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The epidemiologic features of acute encephalitis syndrome in central India

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The epidemiologic features of acute encephalitis syndrome in central India

by

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This work is dedicated to

all those who suffer from encephalitis,

and never know what caused it,

why do they suffer, and how can they prevent it.

Many of them never live to ask these questions,

or are too crippled to even think.

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Introduction

Acute infections are a common cause for seeking healthcare in developing countries, and many such patients present only with fever and without any localizing symptoms or signs. These patients are usually classified as having an acute undifferentiated fever (AUF), which poses a diagnostic as well as therapeutic dilemma. Due to the absence of good diagnostic tests, and evidence-based management algorithms, many patients with AUF are empirically treated for malaria. The non-availability of diagnostic tests is especially a problem in the management of acute encephalitis syndrome (AES), an often fatal subtype of AUF. AES (simply known as brain fever) is a constellation of symptoms and signs, characterized by the presence of fever and the development of altered behavior, with or without seizures or neurological deficits.

To understand the burden and management practices in patients with AUF and its various syndromic subtypes, we first performed a retrospective chart review from electronic medical records of a large teaching hospital in rural central India. The results of this study are presented in Chapter 1. Briefly, we found that in 2006, of 1197 adult patients with AUF, 196 (16.4%) patients had AES, and 42 (21.4%) of them died during their hospital stay. Very few patients underwent diagnostic testing which can help to determine the specific etiology. Despite a negative test for malaria, most patients were empirically treated with anti-malarial medication. Most deaths among patients with AUF were among those with the AES subtype, and improvement in our understanding of this condition and its causes has the potential to help save human lives.

As a next step to understanding epidemiologic features of AES in India, we conducted a systemic literature review, with the aim of understanding what is known about the etiology of AES in India and to identify the research gaps. This systematic review is presented in Chapter 2 of the dissertation. We found that most studies of the epidemiologic features and etiology of AES in India have been done in the setting of an outbreak. These explosive outbreaks mainly affected children, had a high case fatality proportion, and were often attributed to infection with Japanese encephalitis virus (JEV) a mosquito-borne flavivirus. More recently however, many outbreaks caused by Chandipura, Nipah and enteroviruses have been investigated and reported. Certain gaps remain in our understanding of AES in India. First, studies of endemic causes have been few and far between. Second, most such studies have not evaluated multiple potential pathogens, but have rather restricted themselves to testing for Japanese encephalitis virus as the etiology. Third, almost all studies have been limited to children. Hence our understanding of AES in adults remains limited. Last, none of the studies evaluated possible risk-factors for AES by comparing the prevalence of risk factors among cases with the prevalence in the general population.

To fill the existing research gaps, we designed a prospective study of adult-AES in rural central India. Our study consisted of three parts a) Prospective hospital based surveillance to determine incidence, spatial and temporal distribution, and predictors of mortality among adults with AES; b) A case-control study to evaluate environmental exposures and societal risk factors for AES in adults, with cases sampled from the hospital, and controls from the community; and c) Etiologic evaluation of AES cases. To the best of our knowledge, this is the first study of its kind, as there are no other comparable studies of adult-AES in India. The description

of AES cases, their survival experience, and risk factors are presented in chapter three.

AES is usually caused by a diverse group of viruses (flaviviruses, enteroviruses, herpesviruses, paramyxoviruses, etc) but the clinical syndrome may also be seen in some non-viral infections, such as malaria, tuberculosis, bacterial meningitis, and leptospirosis, or even in metabolic encephalopathies. In our etiologic assessment, we found a large proportion of patients to be seropositive for anti-leptospira IgM antibodies. These results were obtained using a commercial enzyme linked immunosorbent assay (ELISA). Although aseptic meningitis is a known presentation in patients with leptospirosis, isolated neurological involvement (termed primary neuroleptospirosis) in the absence of clinically evident hepatic or renal involvement has been described only in few case reports. These findings prompted us to consider neuro-leptospirosis in the as differential diagnosis of AES, and we performed a systematic review to understand if we could rely on ELISA as a diagnostic test for acute leptospirosis. This systematic review is presented in chapter four.

We performed an extensive assay of etiologic diagnostic tests in our AES cases, as well as a battery of research investigations. We collected cerebrospinal fluid (CSF) and acute and convalescent serum samples from most cases, and excluded those with laboratory confirmed non-viral etiologies, such as malaria and, bacterial, tuberculous, and cryptococcal meningitis. The remaining patients were suspected to have viral meningo-encephalitis as a cause for AES. The biological samples from these patients were evaluated using nucleic acid amplification techniques and enzyme linked-immunosorbent assays of spinal fluid in laboratories in India. The results of this etiologic assessment and risk factors for those with known and unknown etiologies for their AES are presented in chapter five of the dissertation.

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Abstract

The epidemiologic features of acute encephalitis syndrome in central India

by

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Acute encephalitis syndrome (AES) is a disease condition characterized be presence of fever, and altered consciousness with or without presence of seizures or a neurological deficit. This definition is broad, and AES may be caused by a wide variety of neurotropic viruses, bacteria, protozoa, fungi or may even be non-infectious in etiology. Most AES is however considered to be due to a viral encephalitis, a condition which is common in many developing countries. The epidemiologic features of AES, especially in adults are still not well defined, and this study is first of its kind from India.

This dissertation consists of three original studies and two systematic literature reviews. We first performed a retrospective chart review of all patients with fever presenting to a single hospital in central India, to determine proportions of patients with AES, and to understand diagnostic and management practices in this condition. We found that AES accounts for about 20% of all acute fevers, has a high mortality, remains frequently undiagnosed, and is mostly empirically treated as malaria. In the subsequent year we designed a descriptive study to understand the temporal and spatial profile, and survival characterictics. We also conducted a case-control study to understand risk factors for AES. We found that most AES occurs in hot and humid months of the year, and proximity to a river or stream may be associated with development of the disease. About half of all patients with AES died within 30 days of disease, and low Glasgow coma score, and need for assisted ventilation significantly predicted hazard for mortality. In our extensive laboratory testing of cerebrospinal fluid and serum samples obtained from 152 AES cases suspected of having viral encephalitis, we found 31 (17%) patients who had a confirmed viral etiology. Enteroviruses were the commonest etiology (9.2% of all AES cases) followed by flaviviruses (4.3% of all AES cases). Based on serology 16 (8.8%) patients had probable leptospirosis. In a third of all cases etiology remained unknown. Low socioeconomic status was the only risk factor significantly associated with AES. These studies have provided novel insights into epidemiology of AES in India, and it is likely that most adult AES is due to water borne enteroviruses rather than vector borne flaviviruses as previously believed.

Chapter 1: The problem of acute undifferentiated fever in central India: The syndromic sub-types, burden of disease and management practices

Abstract

Acute undifferentiated fever (AUF) refers to a febrile illness with no indication of an organ-specific disease. Malaria is one important cause of AUF, while the etiologies of non-malarial acute undifferentiated fevers (NMAUFs) largely remain unknown. The syndromic spectrum of NMAUFs ranges from highly fatal acute encephalitis syndrome (AES) to more benign fever-myalgia syndromes. In developing countries, most NMAUFs are empirically treated with anti-malarial drugs, even in the era of highly specific rapid diagnostic tests (RDTs) for malaria. In order to study the burden of AUF, its syndromic subtypes and drug prescription patterns, we carried out a retrospective review of patients with fever admitted to a rural teaching hospital in the summer (May to October) of 2006 in central India. We categorized patients with NMAUF into different clinical syndromes and determined their demographic profile, their in-hospital course, and the pattern of anti-malarial treatment. The study sample included 1197 adult patients who were investigated for malaria; 1053 (88%) of them had NMAUF and use of further diagnostic tests in this group by clinicians responsible for their care was limited. Despite one or more negative tests for malaria, many patients (39.9 % 95% CI (37.0-43.3)) were treated with anti-malarial drugs. A total of 196 (16.4%) patients had AES, 42 (21.4%) of whom died during hospital stay. These results suggest a need for research in various aspects of AUF, especially to improve diagnostic tests and to help establish evidence-based treatment practices.

Introduction

Acute undifferentiated fever (AUF) refers to a febrile illness with no indication of an organ-specific disease.[1] Non-malarial acute undifferentiated fever (NMAUF) refers to those AUFs, in which diagnosis of malaria has been excluded. Depending on the local epidemiologic spectrum the term acute undifferentiated fever has different connotations.[2] While in the developed world this term often refers to self-limiting viral diseases, in most developing countries malaria and other non-malarial diseases (such as dengue, leptospirosis, enteric fever, and Japanese encephalitis, etc) present as acute undifferentiated fever and are major public health problems.[3, 4, 5, 6] Evaluation of NMAUFs depends on two key steps; first, the identification of distinct clinical subtypes (or syndromes) and second, use of specific laboratory tests to establish a specific etiology.

The first step of a syndromic approach to classification, based on simple and easily elicited clinical signs, can help health workers classify NMAUFs into different categories, such as fever-myalgia[7], fever-arthralgia[7], fever-icterus[8, 9], fever-rash[9], or acute encephalitis syndrome.[7, 9] Each of these syndromes is comprised of a constellation of non-specific signs and symptoms, and can be caused by several diseases which can be prioritized according to public health importance in different areas. While syndromic definitions are used primarily to track emerging infections or bioterrorism threats in various developed countries,[10, 11] they are increasingly being used to determine the burden of various diseases in many resource-poor settings in which diagnostic facilities for etiologic diagnosis of NMAUFs are not avaialble.[8, 9]

Laboratory evaluations of patients with fever in developing countries usually include light microscopy for malaria. According to official estimates in India, although about 100 million

individuals are investigated for malaria by microscopy every year; fewer than 2% of them are slide positive.[12] Thus, most parts of India are classified as a low endemic zone for malaria by the World Health Organization. The annual slide-positivity in all malaria-endemic countries is estimated to be about 5% (6 million confirmed cases among 128 million individuals investigated in 43 countries).[13] Individuals who test negative for malaria could still have malaria (i.e. false-negative microscopy), an organ-specific infection (such as pneumonia, infectious diarrhea, etc), or an acute undifferentiated fever due to a cause other than malaria. In expert hands, malaria microscopy is an accurate tool (sensitivity 99.6, and specificity 100%)[14], but the accuracy of this test can be much lower if microscopists are not well trained (sensitivity 69%, and specificity 62%).[15] Newer histidine-rich protein (HRP-2) based rapid diagnostic tests (RDTs) for falciparum malaria have a high accuracy (sensitivity 92.7% and specificity 99.2%),[16] and hence provide an alternative to microscopy. Because the sensitivity of these tests in the detection of other malaria species is low, as of now RDTs would not be able to replace microscopy. The etiologic diagnosis of NMAUFs is largely based on serologic assays (example for dengue, leptospirosis, Japanese encephalitis, and rickettsiosis) use of which is infrequent in resource-poor countries due to their expense and the need for an advanced laboratory support. Infectious disease serologic tests also have limitations in endemic areas, where multiple pathogens can generate cross-reactive antibodies and where prior infections may be the source of persistent antibodies. [17] More specific polymerase chain reaction based tests for these pathogens are even more expensive, or still under-development, and thus are either infrequently available or used.

The limited diagnostic tests for NMAUF, together with an emphasis on the treatment of malaria, has led health care providers in malaria endemic regions to over-diagnose and over-treat most NMAUFs as malaria.[18] It is estimated that between 30 and 90% of all patients with acute undifferentiated fever are treated with antimalarial drugs, although only 7 to 45% of them have laboratory confirmed malaria.[18, 19, 20] In regions with chloroquine-resistant *falciparum* malaria, expensive artemesinin compounds are increasingly being used as the first line antimalarial agents.[12] The use of anti-malarial drugs in patients with NMAUF continues for treating malaria even in the era of HRP-2 based RDTs and expensive artemesinin-based compounds.[21, 22] Previous research shows that if the diagnosis of malaria is improved and antimalarial drugs are prescribed to only those with a positive diagnostic laboratory test, 60% of the costs of malarial treatment programs can be saved.[23] Over-diagnosis of malaria leads to overestimates of the incidence of malaria, underestimates of the incidence of NMAUFs, leads to distortions the accuracy of data related to malaria resistance, and leads to misallocation of financial and manpower resources.[18] Over-prescription of anti-malarial drugs also has the potential for promoting the development of drug resistance.[24] Such practices were implicated in the emergence of chloroquine resistance[25] and could also lead to resistance to artemesinin derivatives.

In this study, we carried out a retrospective review of electronic-discharge summaries (EDSs) of hospitalized patients (aged >12 years) with fever. We used syndromic classification (Box 1) to categorize all NMAUFs, and determined specific laboratory tests done and the pattern of empirical antimalarial use in patients with each clinical syndrome.

Materials and methods

Setting

The Mahatma Gandhi Institute of Medical Sciences, Sevagram, is a rural medical school and hospital located in a small town in central India. It is a 720-bed teaching institution with

more than 400 000 patient visits and about 8000 patient admissions to the internal medicine wards each year. In year 2006, one-third of all internal medicine discharges carried an infectious disease diagnosis, and one-fourth of all deaths in the hospital were attributed to an infectious disease (Unpublished hospital records). The commonest infectious causes of mortality were septicemia (31%), meningo-encephalitis (18%), tuberculosis (16%), and malaria (15%). In the past about 90% of all malaria cases have been due to *Plasmodium falciparum*, with the remainder due to *Plasmodium vivax.*[26]

Resident physicians, who are supervised by the internal medicine faculty, evaluate all fever patients in the outpatient and emergency departments and admit those who are severely ill to the hospital. All seriously ill patients more than 12 years of age with fever are admitted to the internal medicine wards of the hospital. Three-fourths of all fever related admissions occur in the hot and humid months of June to November (unpublished hospital data), when vector-borne and enteric infections are common. During the study period, the healthcare providers at the MGIMS hospital cared for an exceptionally large number of patients presumed to have had Chikungunya virus infection, a mosquito-transmitted viral disease presenting as an epidemic of fever and severe arthralgia, in several states in India. [27] .

After admission, internal medicine consultants review each patient's history; perform a focused physical examination, and order a complete blood counts, and light microscopy (thin smears) and / or rapid diagnostic tests for malaria for patients with an acute undifferentiated fever. Physicians often treat their patients presumptively with antimalarial medications without waiting for or regardless of the results of malaria microscopy. Additional diagnostic tests (such as chest radiograph, liver and kidney function tests, appropriate bacterial cultures, cerebrospinal fluid examination, etc.) are ordered based on the clinical findings, in-hospital events, and response to initial therapy. IgM ELISA tests for dengue, hepatitis E, and leptospirosis are sometimes done, depending on the ability of patients to pay for the tests, or as determined by the treating physicians. Diagnostic tests for Chikungunya, Japanese encephalitis, hepatitis A and rickettsiosis are never performed as their costs are prohibitive.

Sources of data

In 2005, a hospital information system (HIS) was established in the hospital. The system collects and stores patient related data and supplies that information to health workers on request. A 12-digit unique patient identifier (case record number) is used to track all transactions of a patient admitted to the hospital. This identifier is linked to demographic data, medical history and physical findings; results of all in-hospital investigations and in-hospital and discharge medications. The treating physicians assign a discharge diagnosis to each patient and use the electronic system to prepare an electronic discharge summary (EDS) for all hospitalized patients.

Study design

We used the HIS to electronically retrieve an EDS for all inpatients, aged 12 years and above, who underwent light microscopy or a HRP-2 based RDT for malaria from June to November 2006. We blackened the names and addresses of the patients from EDSs before abstracting the data. A study investigator (RJ) abstracted the data on demographic variables, discharge diagnoses, symptoms and signs, laboratory test results, medication use, length of stay, and in-hospital outcomes and recorded them on standardized forms. We excluded patients who had fever of 14 days or more before hospitalization and those with missing clinical data. We also excluded patients with a definite source of infection identified, such as pneumonia (air-space consolidation on chest radiograph); acute infectious diarrhea (presence of loose stools as a presenting symptom); urinary tract infection (positive urine cultures); smear-positive pulmonary

tuberculosis; and skin or soft tissue infection. The study design was approved by the institutional review boards at MGIMS and the University of California, Berkeley and a request for waiver of consent from the individual patients was granted.

Data analysis

We used abstracted data to classify patients who tested negative for malaria into fever syndromes (fever-myalgia, fever-arthralgia, fever-jaundice and acute encephalitic syndromes) using standardized definitions (See box). Patients with positive anti-dengue IgM antibodies by a rapid test were classified separately in the NMAUF group as having dengue (including dengue fever, dengue shock syndrome, and dengue hemorrhagic fever). We analyzed the pattern of antimalarial use across different syndromic categories. We used bivariate analysis to compare age, sex, symptom duration, hematological findings (hemoglobin, white blood cell and platelet counts) and in-hospital variables in patients with malaria and NMAUFs. We used the t-test for continuous, normally distributed variables and chi-square or Fisher's exact test as appropriate for categorical variables. All tests were two sided, with a *p* value of 0.05 or less considered statistically significant. All statistical analyses were done using Stata statistical software (version 9.0, Stata corp. Lakeway drive TX).

Results

A total of 1671 inpatients were investigated for malaria by commercially available RDT (Parachek-Pf, to detect the HRP2 of *P falciparum,* Orchid Biomedical Laboratories, India; n=1652) and by light microscopy (thin peripheral smear examinations for presence of malarial parasite; n=1314). A total of 1309 patients had both these tests. After excluding 474 (28%) patients who did not meet inclusion criteria from the study (Figure), our analytical sample consisted of 1197 patients (738 men, 61.6%) between 13 and 84 years of age [mean (SD) age 36.6 (17.4) years] who fulfilled the criteria for acute undifferentiated fever. These patients were seen in the hospital from 1 to 14 days [mean (SD) duration 4.7 (3.5) days] after onset of the first symptoms. Malaria was diagnosed in 144 (12%) patients based on light microscopy or RDT; of the patients with malaria, 124 (86%) had *Plasmodium falciparum* infection and the remaining were infected with *Plasmodium vivax.*; the remaining 1053 (88%) patients were negative for malaria and classified as having NMAUF.

A total of 387 (32.3%) patients had fever-arthralgia syndrome, primarily due to a concurrent Chikungunya virus epidemic which took place in the year 2006. Acute encephalitis syndrome (AES) was responsible for the highest mortality among AUF cases (196 cases (16.3%), and 42 deaths (21.4%)). The use of diagnostic tests to detect other infectious causes of the febrile illness in patients with NMAUF was limited. (Table 2) Cerebrospinal fluid (CSF) examination was performed in 90 (46%) of 196 patients with acute encephalitis syndrome, based on CSF cytology, chemistry and negative bacterial cultures, 71 (78.8%) were classified as presumptive viral encephalitis. No specific viral diagnostic tests were performed in these patients. Of remaining 19 patients 12 (13%) were diagnosed as tuberculous meningitis and seven (6%) as pyogenic meningitis.

Of the 176 (16.7%) patients with NMAUF tested for anti-dengue IgM antibodies by a qualitative rapid test, 47 (26.7%) were positive, consistent with dengue as the cause of their illness. Blood cultures were obtained in 240 (22.8%) patients, none of which grew *Salmonella* species. Growth of organisms thought likely to be contaminant (i.e. coagulase negative *Staphylococci* or *Micrococci* species) was reported in 8% of all blood cultures. Very few patients were tested and were positive for leptospira, hepatitis E, or hepatitis B.

A total of 565 (33.8%) patients received anti-malarial drugs, including all 144 patients with malaria, and 421 of 1053 patients (39.9%; 95% CI 37.0 – 43.0) with NMAUF. Of the antimalarial recipients in NMAUF group, 274 (65%) received chloroquine and 144 (34%) received an artemesinin derivative. Of the 144 patients with malaria, 92 (63.8%) received artemesinin derivatives, 44 (30.5%) received quinine and 40 (29.1%) received chloroquine either alone or in combination (Table 1). Compared to patients with NMAUF, patients with malaria were twice as likely to receive an artemesinin derivative (RR 2.46; 95% CI 1.83 – 3.31) Compared to patients with NMAUF, patients with malaria had longer febrile periods, lower hemoglobin levels and platelet counts, and longer in-hospital stays, but lower mortality. (Table 1) The majority of patients with acute encephalitis syndrome (AES) received artemesinin derivatives (70 of 196, 35%). In the remaining syndromic subtypes of NMAUF, chloroquine was used most often.

Of the patients with different syndromic subtypes of NMAUF 20 to 59%, also received empiric antibiotic therapy. The use of empiric antibiotics was highest in the high mortality syndrome of AES. Of the total 196 patients with AES, 117 (59.6%) received antibiotics, and another 34 (17.3%) received both antibiotics and anti-tubercular drugs. Most antibiotic prescriptions were for beta-lactam drugs (example ampicillin, amoxicillin, cephalosporins); a minority of antibiotic prescriptions were for anthracycline derivatives (example Tetracycline, docycycline), fluoroquinolones (example ciprofloxacin), and macrolides (example erythromycin or azithromycin).

Discussion

Our study in a rural teaching hospital in central India shows that 88% of hospitalized adults with acute undifferentiated fever tested for malaria did not have evidence of malaria by light microscopy or by RDT. Despite the availability of the rapid diagnostic test for malaria in the hospital, over-treatment for malaria was common. Forty percent of the patients with a negative test for HRP-2 based RDT received treatment for malaria despite the negative rapid diagnostic test result. AES was responsible for most deaths, while the commonest syndromic subtypes were fever-arthralgia and fever-mylagia syndromes. Our study demonstrates that the syndromic approach to classifying patients is simple and cost-effective and can be used to classify patients with NMAUF. Such an approach could help health workers select cost-effective diagnostic tests for different fever subtypes.[28, 29, 30] A drawback of syndromic classification is that diseases often have a wide clinical spectrum and they can often be classified into more than one category.[31, 32].

Our study has a few limitations. We may have misclassified patients across different categories of NMAUF because we did not collect data prospectively. Because of our focus on hospitalized adults (i.e. the most severely ill patients), our study findings should not be generalized to the acute febrile illness subtypes seen among outpatients or in the community. Our results also cannot be applied to infants and children. In our study, malaria was diagnosed by laboratory testing in 12% of patients, which is higher than the national slide-positivity estimates for India (2%), probably due to a referral bias, better microscopy facilities, and use of rapid diagnostic tests.

The availability in India of point-of-care diagnostics for malaria (such as microscopy or rapid antigen based tests) is limited, particularly in rural areas. A lack of diagnostic facilities and low cost of treatment have led to national guidelines which advocate presumptive treatment of all fever patients for malaria with chloroquine or folate antagonists (sulphadoxine-pyramethamine). As a result, physicians in India, as well as other developing countries often diagnose malaria on

clinical grounds and treat it without obtaining a blood test, despite the lack of accuracy of perception and touch for detecting fever[33] and a lack of accuracy of symptoms and signs to diagnose malaria in adults.[34] The practice of presumptive treatment of malaria continues, even in the era of artemesinin based therapy and in settings (such as the present study) where rapid and sensitive diagnostic tests for malaria are available. The over-emphasis on malaria results in under-diagnosis of NMAUFs,[35] perpetuates irrational medical practices, and leads to worrisome medical, social and economic consequences[18] Our study was not designed to determine the burden of fever patients who receive no diagnostic tests and are presumptively treated with anti-malarial drugs. The majority of such patients are treated in the outpatient settings, and we expect that both the number of such patients and the proportion of them treated with an anti-malarial would be higher than the estimates in the present study.

In a recent study from Tanzania, Reyburn and colleagues, [22] reported that the availability and use of rapid diagnostic tests for malaria did not reduce over treatment of malaria. Of the 1193 patients who received rapid diagnostic tests in their study, only 52% were given a correct prescription. More than half the prescriptions for antimalarial drugs were given to people who had negative test results. Reyburn and colleagues[22] argue, and we agree, that this practice may be due to traditional teaching in medical schools, which makes health workers respond to a perceived increased risk of malaria in hospitalized adults with fever and also due to national guidelines, which overemphasize treatment of malaria. In addition, because of the high prevalence of and the morbidity and mortality from malaria causes, physicians dread failing to treat malaria correctly.[36] Physicians are known to recall their most recent or dramatic clinical experiences and often let these events color their judgments and decision-making. In our hospital too, physicians used the "just in case" defense to justify overuse of antimalarials: "it is better to treat several cases of non-malarial febrile illnesses with an antimalarial drug than to miss one true case."[37] A recent study from Uganda, where malaria is common, suggests that the risk of missing a true case of malaria in the event of a negative diagnostic test is almost negligible : only two malaria cases out of 2359 febrile episodes were missed when febrile children were not given antimalarial treatment when the results of microscopy were negative. [14] Our data also show that had our hospital physicians not prescribed an antimalarial when the rapid diagnostic test was negative (n=421), they would have deprived <1% of malaria cases the benefits of antimalarial treatment (estimated malaria prevalence, 12%; estimated sensitivity and specificity of the rapid diagnostic test, 90% and 96.6% respectively[38]). Clearly, if over-diagnosis of malaria and indiscriminate antimalarial use among hospitalized adults with fever is to be curtailed, physicians need not only avoid these cognitive traps, but must also believe in the diagnostic accuracy of rapid tests (where available) for confirming or ruling out malaria. Such a change could come by having fever treatment algorithms for patients testing negative for malaria, recognizing the importance of non-malarial diagnosis in medical education and practice. National guidelines also need a modification to accommodate different causes of acute undifferentiated fever.

We acknowledge that in resource-restrained settings, neither malaria microscopy nor rapid diagnostic tests for malaria are available or affordable for the vast majority of people suffering from acute undifferentiated fevers, and our arguments do not generalize to these settings. Health workers in such settings argue that insisting on an accurate diagnosis is an ivory tower approach and use this argument to justify empirical treatment of most fevers with antimalarial medications. In order to change this practice, we believe that the availability of rapid diagnostic tests for falciparum malaria should be increased, and more sensitive and specific rapid tests for other malaria species need to be developed. The results of these tests should be acted upon rationally.

Such an investment could have substantial benefits for patient care, reduced ancillary diagnostic testing and shorter hospital stays. In hospital settings, the use of rapid diagnostic tests for other diseases (e.g. influenza) has been shown to result in substantial reductions in inappropriate antibiotic use. [39]As more sensitive, rapid and simple point-of care malaria diagnostic tests become available [40] it is equally important for the heatlh care provider to reserve antimalarial drugs for those who have malaria. The commercially available rapid diagnostic test for malaria and parenteral artemesinin therapy currently cost $2 and $10, respectively, in our hospital; these costs could be substantially lower with their more widespread use.[21] Given the increasing use of artemesinin based therapy for malaria, there is a need to limit the unnecessary use of anti-malarial drugs in patients testing negative for malaria. The cost savings associated with rational use of artemesinin based therapy could help improve the availability of rapid malaria diagnostics.[21]

In our literature review we could not find studies on epidemiologic features of acute undifferentiated fever from India, although a number of recent studies have focused on specific etiologies of NMAUF. The proportion of dengue fever among all fever cases has been estimated to be 14% in a population-based study in rural South India,[41] and 48% in a hospital-based study in urban North India.[42] Leptospirosis, and salmonella infections have been implicated as the cause of one-third[43] and one-tenth[44] of all fever cases in two different studies. Despite NMAUF being common, the studies on its epidemiologic features remain limited. Recently, the public health system in India has initiated a systematic integrated disease surveillance program (IDSP), which aims to compute the burden of infectious diseases, including NMAUFs, in a more comprehensive manner.[45]

In conclusion, our study shows that although most hospitalized adults with acute febrile illnesses in our region do not have malaria, they receive antimalarial therapy. We believe that an over-emphasis on malaria in the national guidelines, the attitudes of treating doctors, and a lack of good quality diagnostic tests for NMAUFs are the main reasons for this practice. The first step in improving diagnostic tests for NMAUFs would be to identify specific etiologies in different clinical-syndromes, so that meaningful diagnostic algorithms are devised. Next, we should develop and deploy rapid antigen based tests for detection of pathogens responsible for NMAUFs, so that the causative organisms can be identified. The diagnosis of NMAUFs can be influenced by antigenic cross-reactivity and possible past or current co-infections with multiple organisms.[5] Because most of these agents are evaluated by serologic tests, testing for multiple organisms in a single battery of tests has limitations.[17] Pan-microbial microarrays are currently being investigated to facilitate identification of causative organisms when multiple etiological possibilities exist.[46] Multiple pathogen detection by nucleic acid amplification techniques is promising and could provide better solutions in the future. The evidence base, when translated into clinical practice, could change the approach to the diagnosis and management of NMAUFs. We suggest that epidemiologists, physicians, microbiologists and funding agencies come together to establish the validity of syndromic classification of NMAUFs and conduct studies that will yield useful answers to the challenges posed by acute febrile illnesses.

Table 1: Clinical presentation and anti-malarial medication use among patients with malaria and non-malarial acute undifferentiated fever syndrome subtypes (n=1197)

Variable	Malaria	Non-malarial acute undifferentiated fever syndrome (NMAUF) (n=1053)						
		All	Dengue	Fever arthralgia	Fever myalgia	Acute encephalitic syndrome	Fever -icterus	Others
N	**144**	**1053**	**47**	**387**	**234**	**196**	**41**	**148**
Percent distribution (95%CI)	12 (10.2-14.0)	87.7 (85.9-88.7)	3.9 (2.8-5.1)	32.3 (29.6-35.0)	19.5 (17.3-21.9)	16.3 (14.3-18,5)	3.4 (2.4-4.6)	12.3 (10.5-14.3)
Age (mean, years (SD))	36.9 (16.4)	36.6 (17.5)	34.5 (16.1)	36.7 (17.6)	33.4 (15.5)	41.8 (19.9)	36.6 (13.7)	35.3 (17.1)
Fever duration* (mean, days (SD))	5.8 (3.6)	4.6 (3.5)	6.2 (3.9)	3.3 (2.8)	4.8 (3.6)	5.2 (3.5)	7.3 (3.8)	5.3 (3.7)
Male Sex N (%)	98 (68.1)	640 (60.7)	26 (55.3)	233 (60.2)	148 (63.2)	126 (64.4)	24 (58.5)	83 (56.0)
Hb * (mean, g/dL (SD))	10.9 (2.9)	12.1 (2.2)	11.9 (2.5)	12.2 (1.9)	12.2 (2.3)	12.1 (2.1)	11.1 (3.3)	11.9 (2.4)
White cell count (mean,$\times 10^3$ /mm^3 (SD))	7.0 (4.7)	7.5 (3.5)	7.3 (3.9)	7.3 (3.3)	7.5 (3.8)	8.4 (3.9)	8.3 (5.2)	7.1 (2.8)
Platelets* (mean,$\times 10^3$ /mm^3 (SD))	164 (123.2)	200 (95.4)	209 (131.1)	201(90.6)	200(92.1)	195.9 (94.0)	199 (94.6)	201(102.9)
Hospital stay* (mean, days (SD))	5.1 (2.8)	4.3 (3.5)	5.8 (6.5)	3.5 (3.1)	3.8 (2.4)	5.8 (3.6)	6.1 (3.9)	4.4 (3.5)
Mortality*	7 (4.8)	55 (5.2)	4 (8.5)	0	0	42 (21.4)	0	9 (6.1)
Any Antimalarial	**144**	**421**	**25**	**103**	**118**	**94**	**20**	**61**

Percent receiving antimalarial in each category (95%CI)	100 (97-100)	39.9 (37.0-43.0)	53.1 (38.0-67.8)	26.6 (22.2-31.3)	50.4 (43.8-57.0)	47.9 (40.7-55.1)	48.7 (32.-64.8)	41.2 (33.1-49.5)
Monotherapy	106 (73.6)	406 (38.5)	24 (51.1)	99 (25.6)	115 (49.1)	93 (47.4)	17 (41.5)	58 (39.2)
Chloroquine (CQ)	19 (13.2)	249 (23.6)	11(23.4)	81 (20.9)	94 (40.2)	16 (8.2)	5 (12.2)	42 (28.4)
Quinine (Q)	25 (17.4)	20 (1.9)	3 (6.4)	2(0.4)	3 (1.3)	7 (3.6)	1(2.4)	4 (2.7)
Artemether (Ar)	62 (43)	137 (13.1)	10 (21.3)	16 (4.1)	18 (7.7)	70 (35.7)	11 (26.8)	12 (8.1)
Combination therapy	38 (26.4)	15 (1.4)	1 (2.1)	4 (1)	3 (1.3)	1 (0.5)	3 (7.3)	3 (2.0)
CQ + Q	4 (2.8)	4 (0.4)	0 (0)	2 (0.5)	1 (0.4)	0 (0)	1 (2.4)	0 (0)
CQ + Sulphonamide	4 (2.8)	4 (0.4)	0 (0)	0 (0)	0 (0)	0 (0)	2 (4.9)	2 (1.3)
Ar + CQ	15 (10.4)	6 (0.5)	0 (0)	2 (0.5)	2 (0.8)	1 (0.5)	0 (0)	1 (0.7)
Ar + Q	15 (10.4)	1 (0.1)	1 (2.1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

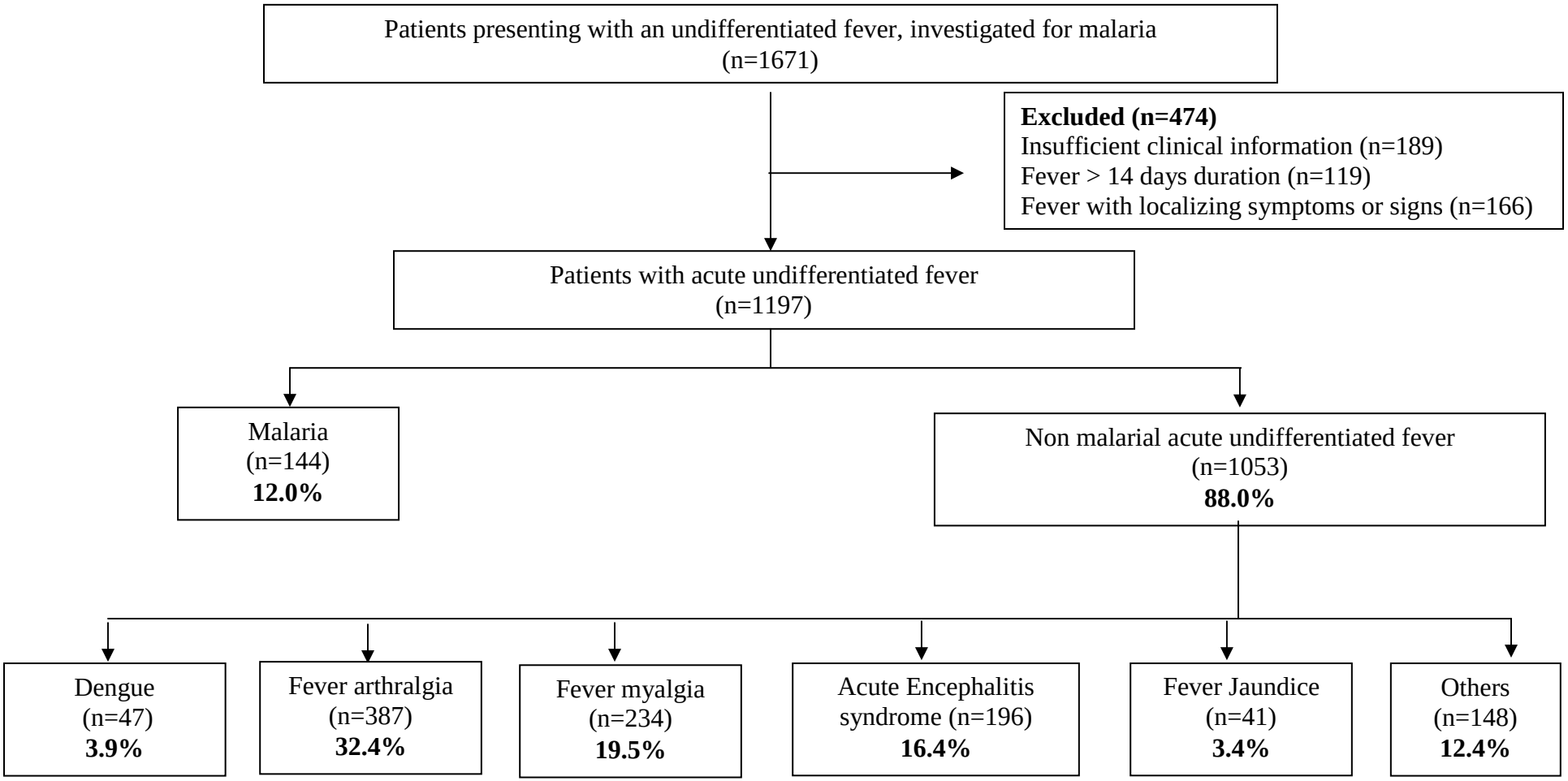
All figures indicate number (%), unless indicated otherwise * For these variables there was a statistical significant difference between the patients with malaria and those with Non-malarial acute undifferentiated fever (NMAUF)

Table 2: Use of diagnostic tests in patients with acute undifferentiated fever (n=1197)

Etiologies of acute undifferentiated fever investigated	Diagnostic test used	Number of positive results / Total number tested	Diagnostic yield (Percent tested positive)
Protozoa			
Plasmodium species	Malaria microscopy	140/1042	13.4
Plasmodium falciparum	HRP-2 based RDT	124/1184	10.4
Viruses			
Dengue virus	Anti-dengue IgM Ab Rapid test	47/176	26.7
Hepatitis B virus	HBsAg	4/ 41	9.7
Hepatitis E virus	Anti-HEV IgM Ab ELISA	2 /11	18.1
Bacteria			
Leptospira	Anti-leptospira IgM Ab ELISA	3 /11	27.2
Tubercular meningitis	CSF cytology and chemistry	12/90	13.3
Bacterial meningitis	CSF cytology and chemistry	7/90	7.7
Salmonella sp.	Positive Blood culture	0/240	0
Gram positive organisms	Positive Blood culture	19/240	7.9

HRP-2 =Histidine rich protein; RDT=Rapid diagnostic test; Ab=Antibodies; ELISA=Enzyme linked immunosorbent assay; CSF=Cerebrospinal fluid; HBsAg=Hepatitis B surface antigen; HEV=Hepatitis E virus.

Figure 1: Study flow chart



(The percentages represent the proportion of each category of the total patients with acute undifferentiated fever, n=1197)

Box: Study definitions

Acute undifferentiated fever (AUF): Fever, without any localized source of infection of 14 days or less in duration. Myalgia, arthralgia, headache, altered sensorium, or jaundice were considered not to have a localizing value.

Localized fever: Fever, with a symptom, sign or an investigation which localized the source of infection to skin or soft tissue, respiratory, gastrointestinal, or genito-urinary systems was defined as a localized fever. Patients detected to have a malignancy or autoimmune disorder, were also classified in this group.

Malaria: Malaria was defined as either a positive peripheral smear by microscopy for *Plasmodium species*, or a positive malarial rapid diagnostic test (RDT) for *plasmodium falciparum*, in presence of a history and clinical features of AUF.

Non-malarial acute undifferentiated fever (NMAUF) All patients with AUF, but negative for malaria were defined as having non-malarial acute undifferentiated fever. This entity was further divided in the following syndromic subtypes.

> ***Fever-arthralgia syndrome***: Presence of fever and tenderness over three or more joint areas.
>
> ***Fever-myalgia syndrome***: Presence of fever, with body ache or headache. Individuals with signs suggestive of raised intracranial tension, or meningitis were excluded from this definition.
>
> ***Acute encephalitis syndrome***: Presence of fever and development of altered behavior, with or without seizures or neurological deficit. Patients with meningo-encephalitis were included in this group.
>
> ***Fever-icterus syndrome***: Presence of fever and jaundice as demonstrated by presence of icterus, or biochemical hyperbilirubinemia. This definition is irrespective of a rise in liver enzymes.
>
> ***Others***: This includes patients with fever and associated symptom not indicated above (including but not limited to vomiting, abdominal pain, skin rash, conjunctival congestion) are classified in this group.

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Chapter 2: Epidemiologic features of acute encephalitis syndrome in India: A systematic review

Introduction

Acute encephalitis syndrome (AES) is defined as the acute onset of fever and a change in mental status (including signs and symptoms such as confusion, disorientation, coma, or inability to talk) and/or new onset of seizures (excluding simple febrile seizures) in a person of any age at any time of year.[1] Prior to the introduction of this term, the condition was known variously as "acute febrile encephalopathy", "viral encephalitis", "infectious encephalitis", "brain fever" etc. The clinical definition of AES was introduced to facilitate surveillance for Japanese encephalitis (JE), a mosquito-borne viral encephalitis. Although this definition is broad and includes illnesses caused by many infectious as well as non-infectious etiologies, most AES is considered to be due to a viral-encephalitis.[2]

JE is considered to be a leading cause of AES in Asia[3, 4] with over 50,000 cases and 10,000 deaths reported each year.[5] Such a high burden of JE has led to adoption of mass vaccination strategies in endemic regions in India[6] (using a live attenuated vaccine shown to provide more than 90% protection).[7] In the absence of readily available diagnostic tests most AES cases and outbreaks in India are ascribed to JE,[8] leading to under-reporting of other potential etiologies. The history of AES in India has paralleled that of JE, when the pathogen was first reported from southern India (Vellore, Tamil Nadu) in 1955.[9] Various subsequent studies have confirmed that most AES in India are due to JE, and it has been considered as the only significant cause of AES in India.[9] However, many recent studies, even in the JE endemic regions, have found alternate novel viral-etiologies for AES outbreaks including enteroviruses,[10]Chandipura virus,[11, 12] and Nipah virus.[13, 14]

Explosive AES outbreaks have a high mortality and hence are a major public health concern in India. The first major AES outbreak was reported from eastern India (Bankura, West Bengal) in 1973.[15, 16] Since that time epidemics of AES have occurred in different parts of India with striking regularity. Although many AES outbreak investigations have been reported in the literature in the past, the surveillance for and investigation into sporadic cases of AES has been limited.[5] Although many AES case-reports and case-series had been reported earlier, [17-20] The first systematic AES surveillance studies were done in Lucknow in Northern India (1957-58)[21] and Vellore in the south (1960-61).[22] Most of the surveillance studies were performed as a part of the Indian Council of Medical Research's JE surveillance studies, focusing mostly on mosquito-borne viruses. The aim of the present study is to systematically review the epidemiologic features of AES in India, both in outbreak and non-outbreak settings, with a focus on viral etiologies.

Methods

Study Definitions

We used the standard definition of acute encephalitis syndrome[1] (fever and altered mental status of less than seven days in duration, with or without seizures or a focal neurological deficit). Studies were classified as "outbreak-investigations" if the occurrence of AES cases was sudden, unexpected, and more than the usual number seen in the same area in same season in previous years. It was expected that these studies would have been performed only after occurrence of the outbreak had been confirmed. Usually these studies would include cases presenting over a period of few days or months. Studies were classified as "surveillance studies" if all consecutive cases presenting with AES from a specified population were planned to be included in the study in a pre-determined manner. Usually these studies included AES cases presenting to a health care facility, over a period of one year or longer.

Various studies used different age cut-points to define the pediatric age group, which varied from 12 to 18 years. Owing to the difficulty in abstracting data using a single cut point of 12 years for pediatric cases in all studies, we used different cut-off definitions, as used by the authors. For the purpose of this review we defined viral diagnostic studies as the investigations conducted on any human sample, including but not limited to serum, cerebrospinal fluid (CSF), throat swab, stool, urine, and tissue aspirates and biopsies. Thus viral diagnostic studies on animal, entomological, or environmental samples were excluded from this review.

Search Strategy

We searched Pubmed, Web of Science, and BIOSIS to identify relevant articles for this review. We used medical subject heading (MeSH) key words "encephalitis" and "India" for the initial search, used study selection criteria to identify the most relevant articles. In addition we hand searched all volumes of the journals "Journal of Communicable Diseases" (Published by the Indian Society of Malaria and Communicable Diseases) and "Indian Journal of Medical Research" (Published by the Indian Council of Medical Research) from year 1973 to date, to identify additional articles. These two journals were specifically chosen as most encephalitis research from India has been published in them. In addition, we looked at the reference lists of original articles, reviews, and book chapters on encephalitis to identify additional articles.

Study selection and data abstraction

The study selection procedures were aimed at identifying original articles about the epidemiologic features of AES in India. A title and abstract review of all the identified articles was performed by an investigator (RJ) who used the following inclusion and exclusion criteria to identify relevant studies:

Inclusion criteria:

1. Original research on human acute encephalitis syndrome cases
2. Cases of AES occurring within the geographical boundaries of India
3. Inclusion of clinical or demographic data describing of human cases

Exclusion criteria:

1. Case-reports, review articles and conference abstracts
2. Secondary laboratory studies on viruses
3. Studies on samples collected from normal human subjects, or human subjects who had symptoms not suggestive of AES.

The full text articles of all the relevant studies were obtained and data were abstracted by an investigator (RJ). The studies were classified as either an outbreak

investigation or a non-outbreak study. The data concerning year, and location of the study, the number of AES cases, the case fatality proportion, and the proportion of pediatric cases in the study were abstracted. Data was also abstracted about type and number of samples collected from cases, viral diagnostic tests performed, and the results of those investigations that helped in determining the etiologies of the cases.

Analysis

Because this review aimed to describe acute encephalitis syndrome cases in India, which had occurred in different populations and at different points of time, a wide heterogeneity in the results was expected. Further, the sites and timing of obtaining human samples, laboratory techniques, and range of viral etiologies investigated are likely to be heterogeneous over time. Owing to these reasons, we did not calculate any pooled estimates for any demographic characteristic or etiologic agents and have provided only a descriptive analysis of the studies.

Results

After electronic searches of databases (Pubmed, Web of science, and Biosis), and hand searches of journals and removal of duplicates, a total of 659 articles were obtained. After title, abstract and full text review 54 articles were found to meet the criteria for the study (Figure 1), 34 being reports of outbreak investigations and the remaining 20 being the reports of the findings of surveillance studies.

Outbreak investigations

The first AES outbreak investigation was from the eastern part of India in the year 1973,[23, 24] and subsequently, a total of 33 studies were reported in as many years. These studies are summarized in table 1. All of these epidemics occurred between May and October, the hot-humid months in India. About half of these epidemics took place in eastern part of India, (seven of them from a single district of Gorakhpur, Uttar Pradesh) (Figure 2) and almost half of these were large (200 or more cases in a single outbreak). The largest of these was an outbreak in Gorakhpur in 1988[25], when more than 4000 AES cases occurred in a four month period.

The case fatality proprotion was reported in 28 studies, and was high (>50%) in eight (28.5%), intermediate (20-50%) in another 16 (57.1%) studies, and low (<20%) in four (14.2%) studies. A total of 19 outbreaks involved primarily children (proportion of pediatric cases ≥80%, 13 outbreaks), or predominantly children (proportion of pediatric cases ≥40%, 6 outbreaks). Only one outbreak (Siliguri, 2001)[13, 14] was exclusively in adults.

The majority of studies (29/34 (85.3%)) were investigated using human samplesto determine etiology, and in half of these either cerebrospinal fluid (CSF) or brain tissue specimens were evaluated for a viral etiology. Uptill 1997 all of the 19 reported AES outbreaks were attributed to JEV infection, and successful viral isolations done in six (31.5%) of them. In the remaining 13 studies attributed to JE, the evidence for JEV infection was serological (heamagglutination inhibition or IgM capture ELISA). In the 13 outbreaks occurring after 1997, viral diagnostic testing was done in 10(76%), and JE was reported as the predominant etiology in only four (30.7%). In an epidemic in Sangli in 1997[26] fewer than 10% sera were positive for IgM antibodies against JEV, and no other etiology was found. The remaining five outbreaks after 1997 were due to Measles (Chandigarh, 1997)[27], Nipah (Siliguri, 2001)[13], Chandipura (Warangal 2003, Vadodra 2004)[11, 28], and enteroviruses (Gorakhpur, 2006)[10].

The last four of the reported epidemics (Siliguri 2001, Warangal 2003, Vadodra 2004, and Gorakhpur 2006) were notable for being attributed to organisms other than JEV. The Siliguri 2001 epidemic which had a high case fatality, in which there wasan association between being a case and exposure to pigs (amplifier host in natural history of JE), was initially considered to be due to JEV. In 2003, after discovery of Nipah virus (a paramyxovirus, with a respiratory-zoonotic route of transmission) as a cause of AES in Malaysia and Bangladesh, the stored samples from the Siliguri outbreak were re-analyzed, and Nipah virus was confirmed as an etiology using RT-PCR, and serology.[13] The Warangal and Vadodra epidemics of 2003 and 2004, respectively were extensively investigated, and the samples from patients were negative for evidence of infection with most conventional AES agents. Chandipura virus (an arthropod borne rhabdovirus) was isolated from few patients, and Chandipur-viral-RNA was detected in 19 of 41 samples analyzed from these two outbreaks.[11, 28] The results of the epidemic in Gorakhpur in 2006 were interesting, as unlike all previous epidemics of AES in the same district, JEV was not isolated from the human samples tested. Instead, this epidemic turned out to be caused by enterovirus-71.[10]

Surveillance studies

All but one of the 20 surveillance studies were prospective, hospital-based evaluations of consecutive AES cases who sought medical attention. (Table 2) These studies were done in the same geographic areas where the AES outbreaks occurred (Figure 3). Although the studies took place over a year or more, yet most incident cases presented to hospitals during the summer and rainy seasons (May to October). Fourteen (70%) of these 20 studies were performed exclusively in pediatric patients; the proportion of patients who were children was more than 40% in two (10%) of the remaining studies that gave a break-down by age. The case fatality proportion was reported in nine (45%) studies and was high (CFR>50%) and intermediate (20-50%) in four studies each, and low (<20%) in one study. In all but two studies human samples were tested for viruses; 14 (70%) of them collected CSF or brain aspirates as well as serum samples.

Of the seven studies conducted before 1974 (before the results from first AES outbreak in 1973 were reported), six studies reported enteroviruses as the main cause of AES based on viral isolation. Most of the enteroviruses were isolated from either stool or rectal swabs from the patients with AES. Of 633 CSF and brain tissue samples analyzed in these six studies, 19(3%) yielded either an enterovirus isolate (i.e. Coxsackie A6, A9, B2, B5, Echovirus 7) or a cytopathogenic agent. Only one study during this period[29] suggested JEV was the cause of most AES cases presenting to hospitals.

A series of JEV epidemics were reported in different parts of country between 1974 and 1985. We did not find any surveillance studies during this period. Between 1985 and 2003, a total of seven studies[30-36] were reported. These were planned with the aim of determining the proportion of AES cases due to JEV infection. The proportion of JEV cases in these studies range from 11 to 60%. These studies either isolated JEV, or demonstrated presence of anti-JEV IgM antibodies in samples from patients with AES.

A total of six studies[12, 37-41] were published after 2003, and performed diagnostic tests for JE as well as one or more other etiologies. Yet, these studies reported only a single predominant etiology of AES, two of them reported JE,[37, 38] and one each reported dengue virus,[41] and Chandipura virus,[12] as sole etiology of AES. One study suggested a

non-viral metabolic etiology for most cases, as all patients were negative for all tested organisms.[39] Only one study[40] from Delhi done in 2004-05 tested for and found multiple etiologies for AES cases. About a third of all patients with AES had enterovirus-71 infection and remaining had either measles, mumps, JEV, dengue, herpes or varicella infection. A total of 28% cases remained undiagnosed.[40]

Discussion

The published literature about the epidemiologic features of AES in India comes largely from outbreak investigations, and surveillance studies, most of which have been conducted in regions that had experienced previous outbreaks. Evidence from these studies shows that JEV is an important cause of AES in India. Other agents have also been shown to cause AES in India in the last decade, and these include dengue virus, measles virus, nipah virus, chandipura virus,and enteroviruses.

The current review has certain limitations. It is based on published journal articles and does not include other potentially valuable sources of information such as technical reports and documents from national and regional disease control organizations. Also it is likely that only studies with positive results are published in peer reviewed journals, and other studies with only negative results are excluded. It is also likely that some AES outbreaks were never investigated. Despite these limitations the current study represents the most comprehensive review of the epidemiologic features of AES in India, as described in studies published literature over the past four decades.

Numerous outbreaks of AES have occurred in India over past three decades, most of which have been attributed to JEV, a flavivirus transmitted to humans in an avian-vertebrate (pig)-arthropod (Culex) life cycle.[9] The evidence of JEV as the etiology in the studies has been based on serology and viral isolation studies, and has been supported by zoonotic and entomologic investigations. However more recent AES outbreaks have been attributed to a vector borne rhabdovirus (Chandipura virus),[11, 28] or water borne enteroviruses.[10] Despite these differences in etiology, the seasonality, pediatric predominance, and high mortality have remained hallmarks of AES caused by both JEV or non-JE viruses.

In contrast to outbreak investigations, AES surveillance studies have produced more variable results. Almost all of these studies were conducted in children, and most of them have looked at only one etiologic agent. Studies done prior to 1974 (when JE epidemics in India had not been reported) had focused on enteroviruses as possible etiologic agents, and used viral isolation as a key diagnostic tool.[21, 42-45] Although in isolated cases enteroviruses were isolated from CSF, isolation of enteroviruses from rectal swabs or stool samples was comparatively more frequent. Most subsequent studies (after 1974, when JE epidemics had become frequent) were conducted in the same regions that had experienced prior AES outbreaks. In regions with prior JE outbreak, about a quarter to one-half of all cases were found to be seropositive for IgM antibodies against JEV.[30, 33, 37] Similarly, in a region with a prior Chandipura virus outbreak, same virus was shown to be responsible for sporadic AES cases.[12] On the other hand a recent surveillance study in a region not known to have past outbreaks reported multiple etiologies as being responsible for AES cases, with enteroviruses and paramyxoviruses being more common.[40]

The diagnostic yield from testing suspected viral encephalitis cases is higher during outbreaks, and early etiologic assessment can provide information that is valuable in guiding their containment. In a region where a past AES outbreak due to a given agent has occurred, majority of endemic AES cases are likely to be due to the same etiology. Based on the reporting of AES cases and outbreak investigations, the National Vector Borne Disease Control Program, an agency of the Government of India has identified certain regions of India as endemic for JEV (See figure 4), [46]and with the support of international health organizations a JEV vaccination program has been initiated in these endemic regions.[47]

Recently, the World Health Organization has proposed definitions and standards for AES surveillance,[48] to improve JE surveillance and disease control programs. Epidemiological data show that the introduction of JE vaccination in endemic regions reduced the overall incidence of AES.[49] A recent systematic review of AES surveillance studies globally[2] reported that in developing countries where the incidence of JE falls as a result of vaccination, the incidence of AES becomes similar to that in the developed world. Thus, it is likely that once the incidence of JE falls either due to periodic fluctuations in the circulation of JE viruses or its vector, AES caused by other neuro-pathogenic etiologic agents will get unmasked, though at a much lower incidence.

To conclude, there are important geographic variations in the epidemiologic features of AES in India. Although most AES cases in India have been considered to be due to JEV, various studies published in the last decade have altered this view. Previously known and newer neuropathogenic viruses have been isolated from AES cases, especially in non-JE endemic regions. Continuous etiology of AES is often difficult during the clinical care of patients, especially in resource poor settings, because the definite viral diagnosis are either available only in research laboratories or prohibitively expensive. As a consequence, periodic surveillance studies of AES can help public health personnel as well as clinicians make informed decisions. There is a paucity of surveillance studies in regions not prone to AES outbreaks, which compromises both clinical and public health decision making. None of the surveillance studies we found included adults, making it difficult to generalize from results about etiologic agents in this age group.

Table1: Outbreak-investigations of acute encephalitis syndrome in India

First author Study year	District, State	AES Cases (Case fatality)	Percent children	Human samples (number)	Viral diagnostic studies performed	Etiologies detected by laboratory tests
Chatterjee 1973[50]	Bankura West Bengal	324 (45.9)	44.7†	None	NA	NA
Banerjee 1973[24]	Bankura West Bengal	NA	NA	Serum (29) Brain tissue (4)	HI for JEV Mouse brain inoculation	31% Sera positive for JEV JEV isolated from one brain tissue
Bhardwaj 1978[51]	Deoria, Uttar Pradesh	78 (NA)	30*	Serum (78)	HI for Gp B Arboviruses Chik / JEV / WNV / DEN2	62% positive for one or more arbovirus. 10% positive for JEV
Mathur 1978[52]	Gorakhpur, Uttar Pradesh	647 (23)	42.5#	Serum (322) CSF (12) Brain tissue (5)	HI for JEV Mouse brain inoculation	JEV isolated in 4 / 5 brain tissue samples. 87% of paired sera sero-positive for JEV
Loach 1978[53]	Champaran, Bihar	NA	NA	Serum (4)	HI for JEV	All JEV positive
Rao 1978[54]	Tamil Nadu	298 (33.2)	84.6†	Serum (70) CSF (29)	Mouse brain inoculation	JEV isolated from 11 cases
Prasad 1978[55]	Kolar Karnataka	71 (25.3)	NA	Serum (33)	HI for JEV	Presumptive / Compatible diagnosis of JEV in 21 (67%) cases
Mathur 1980[56]	Raipur, Madhya Pradesh	33 (54.5)	100*	Serum (10)	HI for Gp B Arboviruses JEV / WNV / DEN2	80% positive for an arbovirus
Rao 1981[57]	Tamil Nadu	607 (24.0)	92.3†	Serum (125) CSF (90) Brain tissue (9)	HI for JEV Mouse brain inoculation	55% of paired sera JEV positive No virus could be isolated
Chaudhury 1982[58]	Goa	35 (37.1)	34.2†	Serum (10) Brain tissue (1)	HI for JEV Mouse brain inoculation	100% seropositive JEV isolated from brain tissue

Mohanrao 1982[59]	Goa	26 (42.3)	38.4†	Serum (14) CSF (7) Brain tissue (2)	HI for JEV Mouse brain inoculation	42.8% presumptive JEV JEV isolated from brain tissue
Chakraborty 1982[60]	Manipur	99 (53.5)	31.3†	Serum (46)	HI for JEV	24% JEV positive
Kar 1982-88[61]	Gorakhpur, Uttar Pradesh	1680 (32.8)	71.7†	Serum (70)	HI for Gp B Arboviruses and JEV	75.7% GpB Arbovirus positive 24.5% JEV positive
Chakraborty 1985[62]	Gorakhpur, Uttar Pradesh	831 (33.3)	64.5†	Serum (8)	HI for Gp B Arboviruses	62% positive for arbovirus group
Angami 1985[63]	Dimapur, Nagaland	50 (60)	56†	Serum (10)	HI for JEV, Gp B Arboviruses, WNV	80% positive for arboviruses 30% positive for WNV
Mukherjee 1985-89[64]	Dimapur Nagaland	220 (14.0)	NA	Serum (37) CSF (1)	JEV IgM ELISA	27% Serum and single CSF sample positive for JEV
Narsimhan 1988[25]	Gorakhpur, Uttar Pradesh	4544 (31.0)	78‡	None	NA	NA
Rathi 1988[65]	Gorakhpur, Uttar Pradesh	875	100†	Serum (670) CSF (25)	IgM ELISA for JEV HI for JEV	JEV IgM CSF 18/25 (72%), JEV IgM Blood 27/53 (51%), HI IgG serum 498/670 (74.3%)
Vajpayee 1989[66]	Rourkela Orissa	254 (40.1)	65.8‡	Serum (4)	HI for JEV	Two JEV positive
Sharma 1990[67]	Haryana	294 (69.7)	NA	Serum (10)	HI for JEV	80% JEV positive
Neogi 1995[68]	Manipur	NA	NA	Serum (16)	JEV IgM ELISA	75% JEV Positive
Thakre 1997[26]	Sangli Maharashtra	52 (3.8)	NA	Serum (52)	JEV IgM ELISA	9.6% JEV Positive
Wairagkar 1997[27]	Chandigarh	51 (52.9)	100*	Serum (11) CSF (17)	JEV, Dengue, WNV IgM ELISA Measles IgM ELISA Cel line isolation	Two specimens confirmed to have measles RNA. Another four specimens showed CPE suggestive of measles, on

					RT-PCR for measles	cell line inoculation IgM anti-measles antibody 17/28 (60%)
Rao 1999[69]	Anantpur, Andhra Pradesh	212 (18.8)	100†	Serum (31)	JEV IgM ELISA	94% JEV Positive
Victor 1999[70]	Dharmpuri Tamil Nadu	3 (NA)	100†	None	NA	NA
Kaur 2000[71]	Assam	152 (42.1)	50.6†	Serum (44)	JEV IgM ELISA	90.9% JEV Positive
Chadha 2001[13]	Siliguri West Bengal	66 (74)	All adults[a]	Serum (17) Urine (6)	Nipah and Measles IgM /IgG Nipah RT-PCR	Nipah antibody 9/17 (52.9) Nipah RNA 5/6 (83.3)
Rao 2003[11]	Warangal, Andhra Pradesh	329 (55.6)	100†	Serum (54) CSF (10) Brain aspirates (55) Brain tissue (1) Throat swab (22)	Serology / PCR for JEV, WNV, Dengue, Paramyxoviruses, Rabies, enteroviruses, influenza, coronaviruses, and mycoplasma Chandipura virus serology/ PCR / cultures (cell lines) Intracerebral mice inoculation	Chandipura virus isolated from 3/22 throat swabs, one brain aspirate, two blood clots. Chandipura virus RNA detected in 4/21 throat swabs, 5 serum samples, one brain aspirate 15/46 patients IgM/IgG positive for chandipura virus antibodies
Gupta 2004[72]	Gorakhpur, Uttar Pradesh	115 (22)	90.4†	None	NA	NA
Chadha 2004[28]	Vadodara Gujarat	26 (78.3)	100‡	Serum (20) CSF (8) Throat swab (14) Urine (10)	JEV, WNV, Dengue IgM ELISA Chandipura virus IgM ELISA RT-PCR for falviviruses (serum),	Chandipura virus isolated in one serum sample 9/20 (45%) samples positive for Chandipura virus RNA on PCR 2 /20 (10%) serum samples

					paramyxoviruses (urine) enteroviruses (serum), and chandipura (serum and CSF) Mouse brain / cell line inoculation (chandipura PCR positive)	positive for enterovirus RNA (echovirus 11, and poliovirus 1) 3/20 sera Chandipura virus IgM positive
Gupta 2004[73]	Bellary Karnataka	73 (1.4)	84*	None	NA	NA
Kumar 2005[74]	Lucknow Uttar Pradesh	278 (37.7)	100	Serum/CSF (223)	JEV IgM ELISA (Xcyton)	JEV IgM positive 77/223 (34%)
Parida 2005[75]	Gorakhpur Uttar Pradesh	326 (23)	NA	Serum (185) CSF (141)	JEV IgM ELISA RT-PCR for JEV Viral isolation in cell lines	JEV isolation 7/326 (2.1%) JEV RNA on PCR 12/326 3.6% JEV IgM positive (50% serum, 30% CSF samples)
Sapkal 2006[10]	Gorakhpur Uttar Pradesh	1912 (21.5)	100[†]	CSF (306) Blood (304) Throat swab (120) Rectal swab (120)	Viral isolation in cell lines Enteroviral RT-PCR	Enteroviral RNA 66/306 (21.5%) CSF samples. Also in 6% rectal swabs, 4% throat swabs, 6% serum samples

HI=Heamagglutination inhibition;

Cut-off age used to define pediatric age group [#] 10 years *12 years; [†]15 years, [‡]18 years

a. This was likely to be a point source epidemic, from a single hospitalized case[14]

Table 2: Surveillance studies of acute encephalitis syndrome reported from India

First author Study year	District, State Study type	AES Cases (CFR)	Percent children	Human samples evaluated (number)	Viral diagnostics performed	Etiologies detected after laboratory tests
Paul 1957-58[21]	Lucknow, Uttar Pradesh Prospective hospital based	27 (NA)	NA	CSF (4) Stool (42)	Intra-cerebral mouse inoculation Cell line inoculation	One cytopathogenic agent (Coxsackie B5) from CSF, and 13 from stool samples
Carey 1960-61[29]	Vellore, Tamil Nadu Prospective hospital based	61 (NA)	NA	CSF and Serum samples	HI for JEV	JEV isolated in 3 cases Presumptive / Compatible JEV diagnosis in another 51 of 61 cases
Nair 1961-67[76]	Delhi Prospective, laboratory based	254 (NA)	100[#]	CSF (254) Stool (254)	Intra-cerebral mouse inoculation	One CSF sample positive for Coxsackie A9. 15 (6%) stool samples positive for an enterovirus Remaining not tested for other pathogens
John TJ 1967-68[45]	Nagpur, Maharashtra Prospective, hospital based	255 (NA)	100*	Serum (146) CSF (172) Rectal Swab (215) Throat swab (217) Urine (120) Others (189)	Cell line inoculation	Enteroviruses (Echovirus 7, Coxsackie B2, and untypable) isolated from eight CSF samples. Overall enterovirus isolated from one of the samples.
Madhavan 1967-68[42]	Pondicherry Prospective, hospital based	26 (NA)	NA	Serum (5) CSF (15)	Cell line inoculation	Enteroviruses (Echovirus 7) isolated

				Rectal swab (1) Stool (1)		from CSF samples of eight cases
Benkappa 1973-74[77]	Bangalore, Karnataka Prospective, hospital based	64 (89.8)	100*	Serum (23) CSF (33) Brain tissue (26) Throat swab (40) Rectal swab (55)	Intracerbral mice inoculation Cell line inoculation	Coxsackie A6 in one CSF sample Eight other enteroviruses in other non-brain/CSF samples
Hardas 1974-75[43]	Nagpur, Maharashtra Prospective, hospital based	90 (NA)	100*	CSF (68) Stool (16) Throat swab (41) Rectal Swab (31)	Cell line inoculation	No agent isolated from CSF. Only three cytopathogenic effects seen. Eight enteroviruses isolated from non-CSF samples
Kumar 1985-88[30]	Lucknow, Uttar Pradesh Prospective, hospital based	740[a] (37)	100*	CSF (394) Brain tissue/ Serum	Intracerebral mice inoculation HI/CFT WNV, Dengue, JEV, Chikungunya virus	JEV positive 92/394 (23.3%) Samples of 14 patients were positive for other viruses.[b]
Chaudhuri 1985-89[31]	Burdwan, West Bengal Prospective, hospital based	762 (25-35)	100*	None	NA	NA
Chattopadhaya 1986-1995[34]	Arunachal Pradesh Retrospective hospital based	162 (62.3)	47.5[†]	None	NA	NA
Devi 1992-93[32]	Cuttack, Orissa Prospective, Hospital based	35 (14)	100*	CSF (35)	JEV IgM ELISA	JEV IgM positive 4/35 (11.4%)
Chatterjee 1996-1999[35]	Burdwan, West-Bengal Prospective hospital based	204 (NA)	NA	Serum (204)	HI for JEV / Dengue / WNV	45/204 (22.0) positive for JEV
Kabilan 1998-99[33]	Madurai, Tamil Nadu Prospective, hospital based	37	100*	Serum (37) CSF (37)	HI and cell IFA for JEV	JEV in 22/37 (59.5%) cases
Kabilan 2002-03[36]	Cuddalore, Tamil Nadu Prospective-Hospital based	58 (NA)	100[†]	Serum (48) CSF (47)	JEV IgM Serum / CSF	JEV Cellular Ag in CSF / toxo-IFA in

					JEV cellular antigen (IFA) JEV RT-PCR	14/47 (32%) JEV-RNA 11/17 (65%) cases JEV IgM CSF in 6/47 (13%) JEV IgM serum in 3/38 (8%)
Kumar 2003-05[41]	Lucknow, Uttar Pradesh Prospective-Hospital based	265 (30.1)	100[†]	Seum (238)	IgM ELISA Dengue HI for JEV / Dengue Dengue PCR positive	Dengue IgM in 52/238 (22%) Dengue RNAin 21 cases JEV HI positive 9/44 (20.4%)
Vashishtha 2003-05[39]	Bijnor, Uttar Pradesh Prospective, hospital based	55 (76.4)	100[†]	Serum / CSF Brain / Liver tissues	Measles and JEV antibody tests (IgM-ELISA)	All samples negative for viral etiology. Liver biopsy suggested hepatic necrosis
Potula 2003[37]	Pondicherry, Tamil Nadu Prospective Hospital based	300 (35.8)	100[‡]	Serum / CSF (212)	JEV cellular antigen (IFA) CSF JEV IgM antibodies CSF micro-neutralization test	184/212 (86.7%) JEV Ag positive; 91/212 (42.9%) JEV IgM positive
Talande 2005-06[12]	Warangal, Andhra Pradesh Prospective, hospital based	90 (54.4)	100[†]	Serum (52)	IgM ELISA for JEV, Chandipura, virus WNV Chandipura virus RT-PCR	Chandipura virus RNA in 20/44 (45.4%) Chandipura IgM in 3/44 (6.8%)
Karamkar 2004-2005[40]	Delhi Prospective, hospital based	157[c] (NA)	100[†]	CSF (57)	CSF IgM antibodies against herpes, measles, mumps, rubella, varicella, JEV,	EV71 20/57 (35.1%) Measles/ Mumps 10/57 (17.5%) JEV / Dengue 6/57 (10.5%)

					Dengue. Microneutralization for EV71 antibodies. Cell line inoculation	Herpes/ VZV 2/57 (3.6%) Others 3/57 (5.4%) Unknown 16/57 (28%)
Roy 2005[38]	Lucknow, Uttar Pradesh Prospective, hospital based	57 (NA)	61.4	Paired serum (13)	HI test for JEV	JEV positive 7/13 (53.8%)

HI=Heamagglutination inhibition;

Cut-off age used to define pediatric age group *12 years; †15 years, ‡18 years

a. Of these 740 cases, in 240 a non-viral diagnosis was established. In another 38 encephalopathy was considered to be related to measles. Of the remaining 462 patients, 394 underwent virology investigations.

b. The other viruses included adenoviruses (5), parainfluenza and influenza (4), polio, coxackie, echovirus (1 each), and untypable (2).

c. Of these 157 cases, 94 were of non-viral etiology and remaining 57 were viral encephalitis suspects. Although CSF samples of all 151 patients were collected, only 57 samples were subsequently evaluated for virology studies.

Figure 1: Literature search strategy

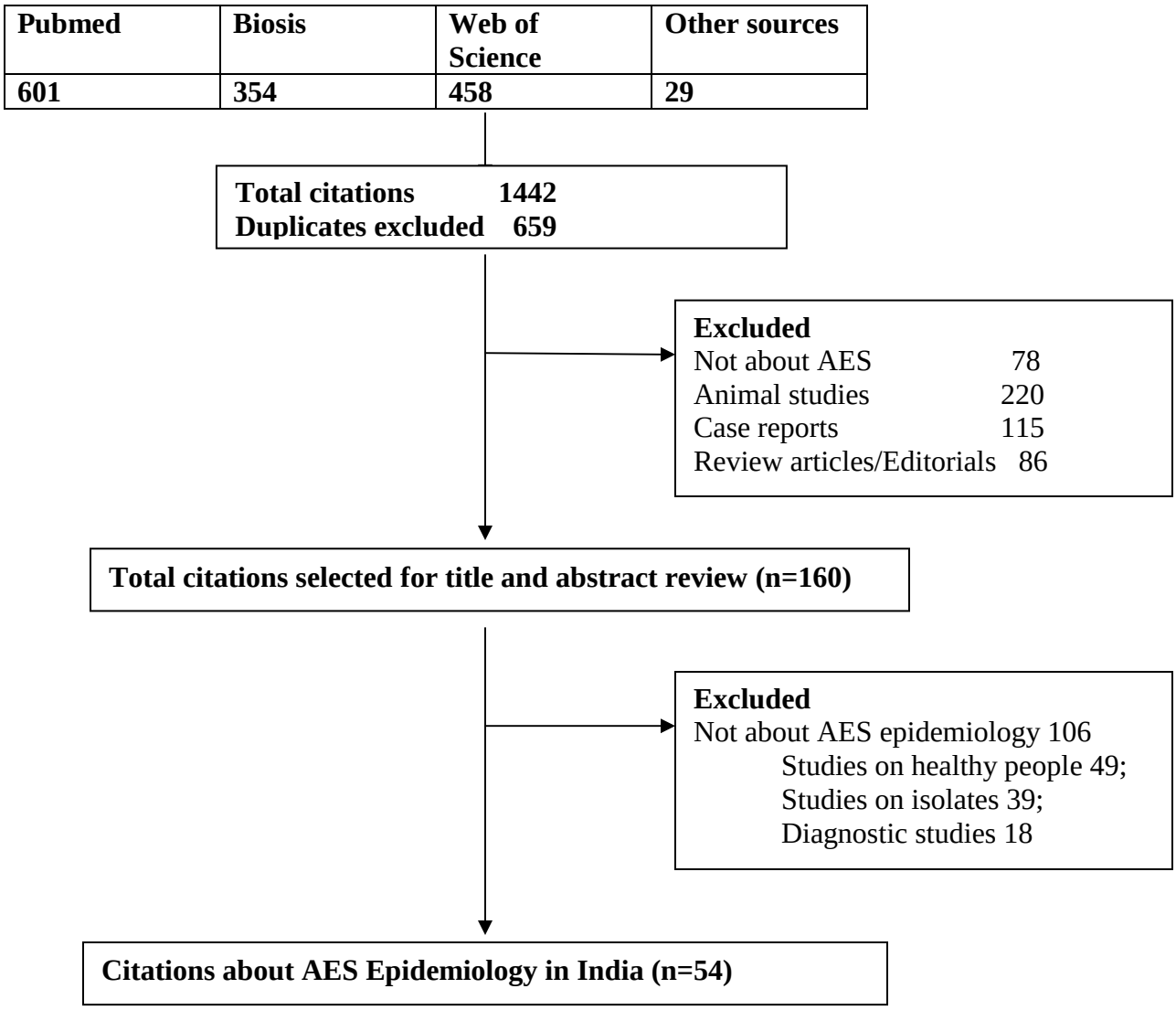
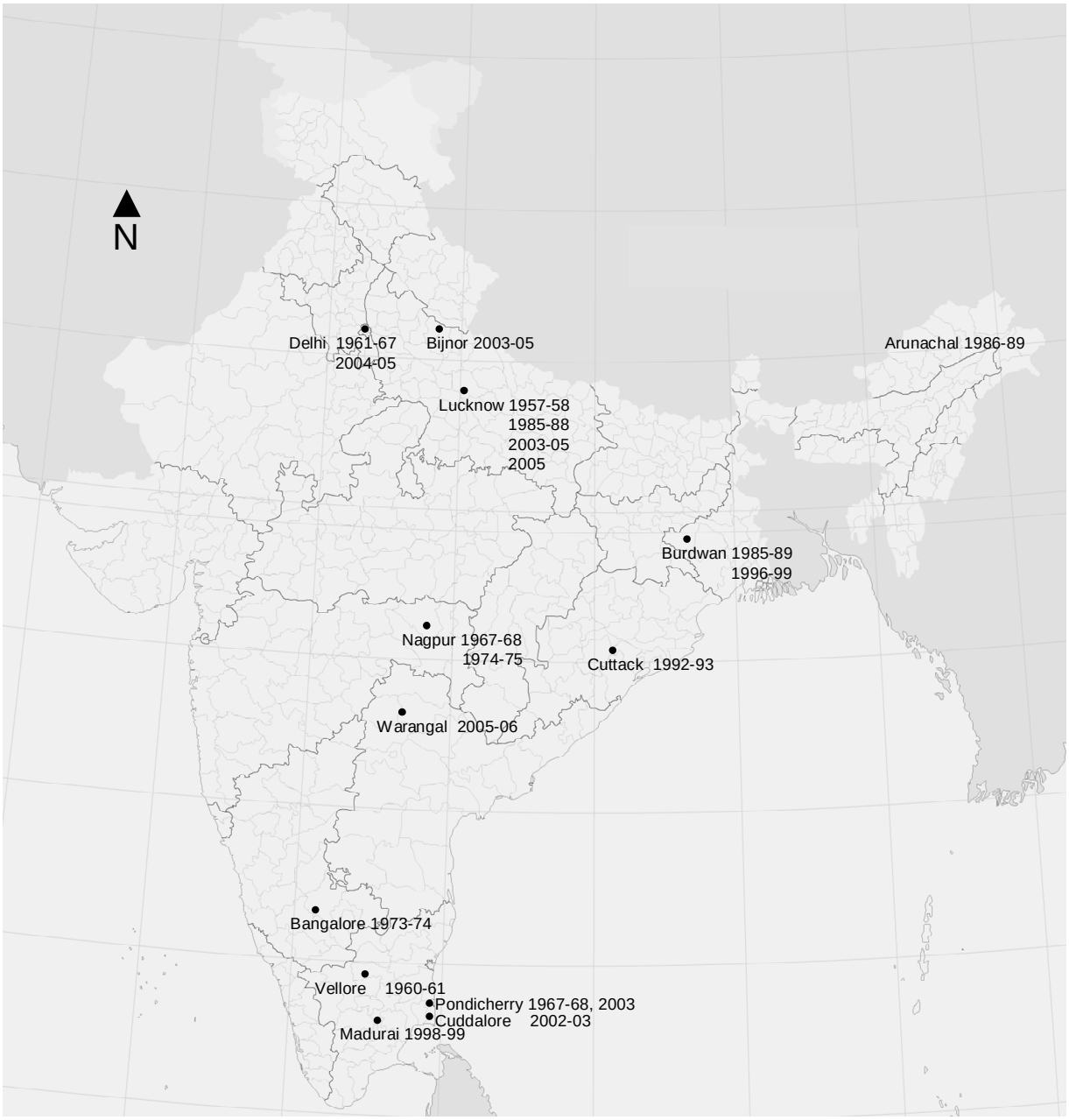


Figure 2: Location of reported acute encephalitis syndrome outbreaks from India (1973-2008)

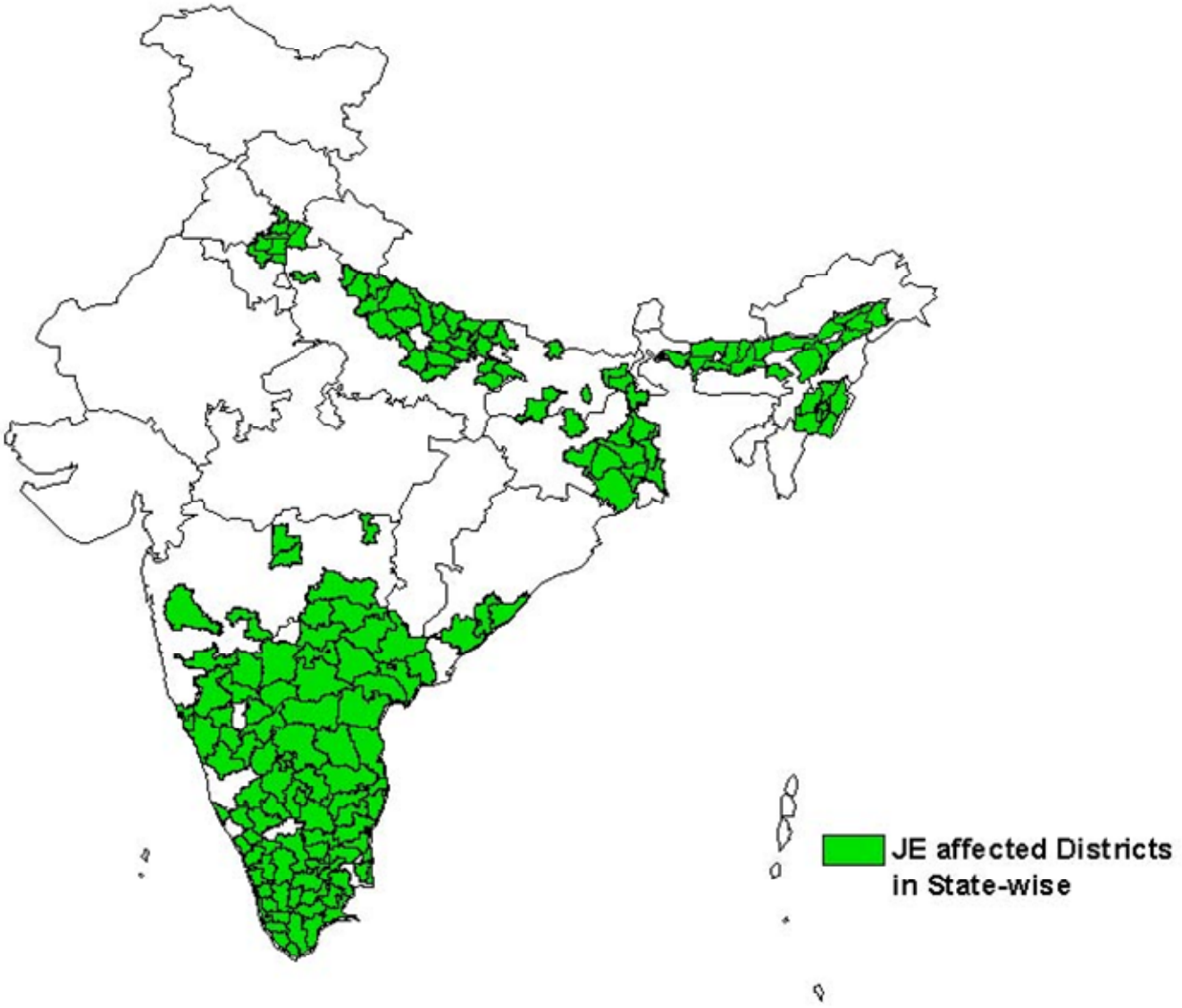
Numbers indicate year of outbreak. Example '73 means 1973.
Underlined years indicate when Japanese encephalitis was not ascribed as the cause of outbreak. These outbreaks were 1997, Chandigarh (Measles); 2001 Siliguri (Nipah); 2003 Warangal & 2004 Vadodra (Chandipura); and 2006 Gorakhpur (Enterovirus)

Figure 3: Surveillance studies of acute encephalitis syndrome in India (1957-2008)



Note: Numbers indicate years when surveillance studies were conducted.

Figure 4: Japanese encephalitis virus endemic districts in India (Source: National vector Borne disease control program, Government of India)



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Chapter 3: The descriptive epidemiologic features of acute encephalitis syndrome in central India

Abstract

Acute encephalitis syndrome (AES) is defined as the acute onset of fever and a change in mental status in a person of any age at any time of year. The present study is a prospective descriptive study of all adult AES cases which presented to a single hospital in Central India. This study aims to determine the incidence, spatial and temporal distribution, predictors of mortality, and environmental / societal risk factors of adult AES in rural central India. All consecutive cases with AES were included in the study, and their time of onset and spatial location of their residence at the time was determined and evaluated. In addition a detailed clinical assessment and follow up was done to determine predictors for mortality and disability. One control was sampled from the same villages giving rise to the case, and environmental and societal risk factors were compared. A total of 183 AES cases were evaluated as part of the study, 64% of which occurred in hot and humid months of the year; the incidence of adult AES in the administrative subdivisions closest to the hospital was 16 per 100,000. Fifty three (36%) of the AES patients died, and having been on assisted ventilation significantly increased hazards of mortality (HR 2.14(95% CI 1.0-4.77)). A high Glasgow coma score (HR 0.76 (95% CI 0.69-0.83)), and a longer duration of hospitalization (HR 0.88 (95% CI 0.83-0.94)) were associated with a lower hazard of dying. As compared to healthy community controls, low socioeconomic status (OR 3.12; 95% CI 1.57 to 6.17)), and household factors that promote vector borne transmission (OR 2.16; 95% CI 1.08 to 4.33)) were significantly associated with the risk of AES. Low socio-economic status, which operates through multiple potential disease transmission pathways, was a major determinant of AES. Poverty not only increases exposure to infectious agents, but also affects the ability of individuals to protect themselves from such exposures.

Introduction

Acute encephalitis syndrome (AES) is defined as the acute onset of fever and a change in mental status (including symptoms such as confusion, disorientation, coma, or inability to talk) and/or new onset of seizures (excluding simple febrile seizures) in a person of any age at any time of year.[1] Although the syndromic definition of AES is broad and potentially includes patients with a metabolic encephalopathy (a non-infectious phenomenon), most AES is due to acute infectious meningo-encephalitis. The reported incidence of AES varies widely by age, geographic location, season, host susceptibility, and efficiency of the health system in detecting it. A recent systematic review of all epidemiological studies estimated the incidence of AES in developed countries to be about 10.5 and 2.2 per 100,000 in children and adults respectively.[2] The incidence of AES in developing countries is estimated to be 6.3 per 100,000 for all ages, but there is a wide variation in the incidence reported from tropical settings, a range from 389 per 100,000 during a 1989 outbreak in India [3] to 0.03 during non-epidemic surveillance in Taiwan in year 1997.[4] Recently it has been suggested that a minimum incidence of 10, 6, and 2 per 100,000 in children, all-age groups and adults respectively is necessary for any AES surveillance program to be called as effective.[2]

Published reports concerning AES in India are largely based on outbreak investigations.[3, 5-7] Most of these outbreaks predominantly affected children and were attributed to Japanese encephalitis virus (JEV) infection. Recently however new viral agents such as Chandipura virus,[8-10] and Nipah virus[11] have however been reported as possible etiologies of AES. Other viruses such as Herpes virus,[12-14] Varicella zoster virus,[15] Dengue virus,[16-18]Enteroviruses, [19, 20] and West Nile Virus,[21-23] also have been reported as etiologies of AES, largely in case-reports or case-series. Although AES occurs in non-outbreak settings and affects adults as well as children, there are no previous published studies from India that describe the epidemiologic features of non-outbreak AES in adults in India. In a previous study of all adults who were admitted with a febrile illness in year 2006, we found that 196(16.4%) of 1197 adults had AES. These patients had a high mortality (21%) and etiologic diagnosis had not been attempted due to a paucity of available diagnostic facilities.[24]

Here we present the results of a prospective descriptive study of all AES cases that presented to a single hospital in Central India. This study answers three specific research questions: a) What is the incidence, and spatial and temporal distribution of acute encephalitic syndrome cases in central India?; b) What are the predictors of mortality and disability in patients with acute encephalitic syndrome in Central India?; and last c) What are the environmental risk factors for AES of presumed viral etiology in rural central India?

Methods

Setting

The Mahatma Gandhi Institute of Medical Sciences (MGIMS) is a teaching hospital located at Sevagram, in district Wardha of Maharashtra state in India. MGIMS is a not-for profit hospital, largely funded by government grants that offers low-cost quality care in the region. It has 720 beds, and of about 400,000 patients who seek care at the hospital annually, more than 40,000 are admitted to the in-patient services; about 10,000 of these inpatients are cared for in the medicine wards. Most patients with AES present during the summer and rainy season (between May and October), a time when the incidence of most acute infectious diseases is also at a peak); they are usually referred to the hospital from primary and secondary care facilities in and around the district, and are admitted in the medicine wards. As part of the standard treatment protocol in the hospital, treating physicians of most patients would perform a lumbar puncture and collect about 3mL of cerebrospinal fluid (CSF) and order to conduct further diagnostic tests. Typically, the tests that are ordered on the CSF sample include a microscopy, biochemistry (CSF sugar and proteins), and bacterial culture.

Patients with AES presenting to the hospital reside either within the district Wardha (which has eight eight administrative subdivisions: Wardha, Seloo, Hinganghat, Samudrapur, Deoli, Arvi, Karanja, and Astha, the farthest of these being about 60 kilometers away from hospital), or in one of the neighboring districts (Yeotmal, Chandrapur, Amravati, Nagpur, Gadchiroli, Nanded, Washim, and Adilabad), which together have about 70 administrative subdivisions, the farthest of these being about 200 kilometers away. The total adult population in these subdivisions is about 14 million (2001 census), spread over 72,000 square kilometer area (between latitudes19 & 22N and longitudes 77 & 80E).

Patients

Initial screening

Between January and October 2007 all consecutive hospital admissions were screened to identify adult patients with: a) fever of 14 days or less, and b) change in mental status (including symptoms such as irritability, somnolence or abnormal behavior, confusion, disorientation, coma, or inability to talk). Fever must have preceded the onset of change in mental status. Other signs and symptoms such as new onset of seizures, and focal neurological dysfunction (including paresis or paralysis, nerve palsies, sensory deficits, abnormal reflexes, generalized convulsions, or abnormal movements) may or may not have been present. Patients were excluded if : a) peripheral blood-smear and/or serology for malaria was positive; b) an alternative explanation for fever (e.g. evidence of a definite localized infection in the form of an abnormal chest X-ray suggestive of pneumonia or tuberculosis; positive acid fast bacilli in respiratory tract secretions; urinary tract infection; or soft-tissue infection with sepsis etc.) was plausible; or c) there was biochemical or clinical evidence of a metabolic encephalopathy (including but not limited to hyponatremia, hepatic dysfunction, hypoglycemia, or alcohol intoxication). All patients who satisfied the eligibility criteria were classified as having AES, and were asked to participate in the study.

Informed consent process

As patients with AES are cognitively compromised, consent was sought from the closest available caregiver of the patient (the order of closeness being spouse, parent, offspring, sibling, friend, other relation or friend). The nature and scope of the study was explained to the caregiver, and consent was obtained for study related procedures which included administration of questionnaire, a home visit to the residence of the patient four weeks after the first interview; and obtaining serum samples (an acute sample at time of hospitalization, and a convalescent sample four weeks later) and CSF samples (an additional 4mL of CSF sample was obtained as part of research protocol, over and above the 3mL collected as part of the standard hospital care). The decision making capacity of the patient was reassessed using a mini mental status examination prior to discharge, and therefater at every follow up visit. When the patient became cognitively competent, a second repeat consent was sought from the patient. In case such consent was denied, the patient was to be withdrawn from the study. All informed consent materials were available in the local language *Marathi* and a written informed consent was obtained. The study design was approved by the institutional review boards of the participating institutes (MGIMS Sevagram, Bhopal Memorial Hospital and Research Center, Bhopal and University of California Berkeley, and San Francisco).

Additional exclusion criteria

Additional patients were excluded from the study, after obtaining the informed consent, if there was a definitive evidence of a non-viral etiology for encephalitis. The additional exclusion criteria were:

a) CSF findings suggestive of bacterial meningitis based on either a positive culture for pathogenic bacteria, The presence of polymorphonuclear white cells in CSF, a CSF glucose < 40mg/dL; or a CSF/blood glucose ratio<0.25.

b) Positive mycobacterial cultures for tubercular meningitis (mycobacterial cultures were done using 1mL of the freshly collected CSF, which was inoculated in BACTEC medium)
c) Presence of a space occupying lesion on brain imaging, if performed.
d) A definite metabolic or known infectious etiology for their illness which could preclude the diagnosis of AES was detected during their hospital stay.
Thus, patients with AES, whose clinical CSF based tests suggested a definite non-viral etiology (such as bacterial, mycobacterial or cryptococcal meningitis) were excluded from further analysis. Remaining patients were classified as having suspected viral encephalitis (VE). The study population consisted of eligible and consenting patients with suspected VE.

Sources of data

Various data sources were used to address the three research questions in this study, which are described as below.

Incidence, and temporal and spatial profile of AES:

We recorded the exact residential address of each case at the time of onset of AES. A study team member visited the residence and physically verified the address about one month after the onset of illness. The number of AES cases for each administrative subdivision over the study period was determined as was the adult population of each sub-division based on census data from the year 2001.

The time of onset of the disease was determined from the date of first sign or symptom in the case, based on information obtained at the time of enrolment in the study. To study the relation between the frequency of cases and local temperature and rainfall, we obtained meteorological data from a meteorology station close to MGIMS.

The coordinates of the village/locality of the case's residence were obtained from the available paper maps (1:25,000 topography sheets of the area) obtained from the Geographical Survey of India. Because the exact location of individual houses could not be located on the paper maps, the approximate geographical center of the village/locality of the case was abstracted, and recorded in decimal digit format. The point data for the location of cases were layered with the hydrology and administrative sub-division vector data using spatial analysis software (ArcGIS).

Survival and disability:

All patients with suspected VE were initially treated in the intensive care ward of the MGIMS hospital; once the symptoms resolved, they were transferred to a general ward before discharge from the hospital. We followed all patients included in the study daily until their discharge from the hospital and after 30 and 180 days of symptom onset after discharge. Mortality was defined as death after in-hospital admission; the time between the symptom onset and the date of death was considered as the survival time. Cognitive disability was measured at day 30 by conducting a mini-mental status examination (MMSE). A MMSE score of less than 25 was considered to be indicative of cognitive impairment.

We collected demographic data i.e. age, gender, socio-economic status score), clinical data on-admission (i.e. duration of symptoms prior to admission,

presence of seizures, Glasgow coma score (GCS), clinical signs of meningitis), and in-hospital clinical data (i.e. requirement for assisted ventilation, gastro-intestinal bleeding, hypotension, days of hospital stay, complete-blood cell count, CSF cell counts and CSF chemistry) and examined their relationship to mortality, and to mortality or disability at day 30. Gender, and presence of seizures, clinical signs of meningitis, requirement for assisted ventilation, gastro-intestinal bleed, and hypotension were collected as binary variables (coded 0,1), while the remaining variables were collected as continuous measures.

Risk-factor assessment

Selection of controls: We designed a case-control study to identify the risk factors for VE. Individuals with suspected VE were defined as cases. All adults living in the same administrative subdivisions as the cases constituted the hypothetical population base from which controls were sampled. An eligible control was an individual 18 years of age or older, who had no past history suggestive of AES, shingles (Herpes zoster) or cold sores (Herpes simplex) in the past, frequency matched by age (within 5 years of the case) and gender. As recall of potential environmental risk factors could be influenced by the length of time between the event or exposure and the interview (e.g. the response to a question on the use of vector control measures could vary if the question is asked in a season of high vs. low vector density), the controls were identified concurrently as the cases. A study team visited the household of each case within four weeks from the onset of first symptoms. During the same visit, a member of the study team selected a house in the village farthest away from the case's house. The selection was done in a manner such that if the case-household was located in the center of the village, the farthest house at the periphery of the village was treated as a control household and vice-versa. An eligible control from such a randomly selected household was approached, and written informed consent was sought. One eligible and consenting control was sought for each case.

In about one-third of all villages none of the eligible controls consented for participation in the study. Usually this was because blood samples were required from controls, and controls were to be tested for HIV. Some refusals were from villages in which a VE case had died.

Risk factor assessment : All cases and controls were administered a standardized questionnaire by a study investigator concerning potential environmental risk factors. The questionnaire had been pilot tested with 30 individuals prior to initiation of the study. All interviews were carried out by either one of the two study personnel throughout the study. To minimise interviewer-bias the interviewers were masked from the study hypothesis, and were instead told that healthy individuals were being included in the study because they might have had subclinical disease. The environmental risk factors were grouped according to mode of transmission of etiologic agents. The three potential modes of transmission that were evaluated were as follows:

1. Factors promoting vector borne transmission
    a. Non-use of personal protection measures

b. Presence of objects noted outside the house, that could be breeding sites for mosquitoes
c. The combination of the above factors

2. Factors promoting zoonotic transmission
    a. Contact with following animals in the following categories at home or work
        i. Cattle and swine (including cows, goats, bulls, buffaloes, pigs etc)
        ii. Dogs and cats
        iii. Poultry and birds (including chicken and other domesticated birds)
3. Factors promoting water borne transmission
    a. Use of household well as the predominant source of drinking water
    b. Open sewage running in front of the house
    c. Pond/stream within 200 meters of the house
    d. A combination of the above factors

In addition measures of poverty were collected including

a. Socioeconomic status score
b. Number of children (age <12 years) in house
c. Overcrowding - Person-density (number of individuals per room)

Socioeconomic status was determined using a score developed for rural India by Tiwari and coworkers. [25] This validated scale consists of seven profiles (house, material possessions, education, occupation, per capita income, possessed land and social profile) which are measured for the household as a unit, with a maximum achievable score of 70 points (See Box 1).

In addition to this questionnaire, a serum sample was collected from all cases and controls to evaluate whether HIV infection was a risk factor for VE. Presence of HIV infection was judged using two sequential rapid-HIV antibody detection tests. A person was classified as positive or negative if both tests were positive and negative, respectively. In case of discordant rapid-test results, confirmation with a conventional ELISA was obtained.

Statistical analysis

The statistical analysis for each of the three research questions was performed as follows:

Incidence, Temporal and spatial profile of AES:

The frequency distribution of all cases per week was calculated as was the distribution of cases of specific etiologies. Clustering by time was determined by comparing the frequency of AES cases in the hot-wet and hot-dry seasons, with the frequency of cases in cold-dry season as a baseline. The relationship between the temporal distribution of cases and meteorological factors was analyzed using temperature and rainfall as continuous explanatory variables and number of cases per week as the outcome. The average temperature for the week was calculated from the mid-points for each day. The rainfall for each seven day period was

summed to obtain a cumulative record for the week. A descriptive analysis of the relationship of the weekly frequency of cases, in relation to high temperature and rainfall (known to be associated with both high vector density and worsening water quality respectively) was performed.

We estimated the cumulative annual incidence of AES per 100,000 adults, for each administrative subdivision. It is possible that patients residing in subdivisions nearer to the hospital are more likely to seek care and be diagnosed producing a higher AES incidence rate. To evaluate the presence of referral bias, we performed a simple linear regression of distance from the subdivision headquarter using AES incidence in the subdivision as the outcome.

The spatial density of AES cases was determined by plotting incidence rates for each sub-division. In addition, we performed a kernel density function from the spatial point-data of AES cases. The number of closely placed points is identified and a density gradient is mapped, to help identify spatial clustering. In addition we examined if proximity of residence to rivers or streams was associated with being an AES case. We obtained a hydrology map of the region, and created buffer zones around all rivers and streams at every one kilometer distance. We determined number of AES cases in each buffer zone, and obtained a frequency distribution of cases by distance from from river or stream. All spatial analysis was done using ArcGIS software (ArcMap version 9.3, ESRI Inc).

Survival and disability:

We compared the demographic, on-admission, and in-hospital characteristics of survivors and non-survivors of VE using the t-test for continuous and chi-square test for binary variables. We considered the time to event for each individual in the study and analyzed the data using 'survival analysis' methods. The Kaplan–Meier product limit estimator was used to estimate survival and for the time-to-event plot. Event-free individuals were right censored on day 30 after symptom onset, as none of them had died after this time. To identify those predictors with the most significant independent influence on prognosis, we used the log rank test for simple comparisons. Crude hazard ratios were computed to assess the strength of association between potential prognostic factors and the outcomes (mortality, and mortality or disability on day 30). We used Cox proportional-hazards regression models for analyses of multiple predictor variables for the study outcomes. These models measured the hazard ratio – the relative effect of a predictive factor on an outcome – by assuming that this relationship is constant over time. Because many of the risk factors were correlated, co-linearity was evaluated by generating correlation matrices and handled by eliminating one of the two collinear variables. A backward stepwise technique was used for model selection. For a variable to exit the model, the p value had to be >0.1. Both the crude and the adjusted hazard ratio estimates were computed along with 95% confidence intervals (CI).

Risk factor analysis:

We calculated frequency distributions of various potential risk factors among VE cases and controls. The risk factors were categorized according to

potential source of transmission. All the risk factor variables were binary and were coded as (0,1), except socio-economic score, which was continuous. Socioeconomic score obtained for the households in our study was skewed towards the left, and according to the classification provided by the authors who created the score, all our households were in low or lower-mid socio-economic status category.[25] Thus for a meaningful interpretation of the score, we converted the scores obtained in our study into tertiles and compared the frequency distribution of patients in the highest vs the lowest tertiles. We performed a univariate analysis of individual risk factors, or their logical combinations, and calculated odds ratios and their 95% confidence intervals to assess the strength of association between risk factors and VE. Age and gender were not analysed in univariate analysis because cases and controls were matched on these variables.

We evaluated different multivariable models, according to the disease transmission hypothesis. Three models evaluated vector , zoonotic, and waterborne transmission as risk factor domains. The risk factors in each domain were separately modeled and adjusted for age and socioeconomic status tertile. This was done to evaluate which transmission risk factor contributed to the development of VE. For the poverty related domain, we adjusted all poverty related variables for age, and key environmental risk factors. We then performed a backward stepwise multivariate logistic regression and included all domain specific variables, age and socioeconomic tertile in the full model. From this full model, variables that did not contribute significantly were dropped one at a time until all those remaining contributed significantly. At each step, the variable with the smallest contribution to the model (largest p value) was dropped. The impact of elimination of each variable on the model was evaluated using the likelihood ratio test. The backward, stepwise process was continued until the best fitting, most parsimonious final model was identified. The fit of the final models was assessed using the Hosmer–Lemeshow goodness-of-fit test. The results of the final models are presented as adjusted odds ratios (OR) with confidence intervals.

Results

A total of 7685 patients were admitted to the medicine wards between January and October 2007, 1689 (21.9%) of whom had an infectious disease diagnosis. Of these patients 183 (10.8%) had signs and symptoms suggestive of AES and were included in the study. Of these 152 (83%) were considered to have suspected viral encephalitis (VE) based on negative results obtained on CSF biochemistry, culture and Cryptococcus antigen testing (Figure 1).

Most VE cases were seen during the hot and wet months between July and October, a period characterized by moderate to high temperatures and heavy precipitation (Table 1, and Figure 2). As compared to the hot and dry season (March to June), the number of AES cases and VE suspects was 2 and 2.5 times higher in the hot and wet monsoon season (July to October), respectively.

Spatial distribution of AES cases, stratified by the season of occurrence, is depicted in figure 3. Most (97 / 183 (53%)) patients with AES resided in sub-divisions of Wardha district (the same district where MGIMS hospital is located). These subdivisions had an annual incidence rate of AES between 10 and 16 per 100,000 adults. The sub-divisions of neighboring districts (from which more than one case presented to the

hospital) had an average annual incidence rate of 4 per 100,000 adults (Figure 4). Because these later sub-divisions were farther from the MGIMS hospital, we attribute this difference in incidence rate to referral bias.

Spatial analysis of point data was performed after excluding the margins (peripheral areas with a low incidence). Kernel density function analysis identified clusters of high case density, and on visual assessment, most of these cluster areas were in proximity to rivers and streams. We created one kilometer buffer zones in the hydrology layer and determined the frequency distribution of cases in each one kilometer layer. Linear regression of the frequency of cases and distance from a river or stream showed that 12 fewer cases were seen for every one kilometer distance farther from the river/stream (Figure 5).

The 152 patients who were suspected to have VE were young (mean age 40.3 , SD (18.3) years), of low socio-economic class (mean SES score 19.4, SD (7.0)), and presented to the hospital after a mean of 5.9 days of symptoms. These patients stayed in hospital for an average 10 days (SD 7.5). This cohort was followed up for death or disability for 180 days after the onset of their symptoms. Five patients were lost to follow up after discharge from hospital and were right censored in the analysis. Of the remaining 147 patients, 53 (36%) died, and another 34 (23.1%) had significant cognitive disability at 30-day of follow up. Thus, only 60 (40.8%) patients were free of death or disability by one month.

All known deaths occurred in the first 30 days after symptom onset; hence survival analysis was restricted to the first 30 days after symptom onset. The product limit survival probabilities (Kaplan Meier survival curve) for 152 patients with suspected VE is depicted in Figure 6. We determined hazards for mortality in all patients with VE by using Cox proportional hazards multivariable regression models. Four variables significantly increased hazard for the outcomes (30-day mortality, and 30-day mortality or disability), namely age, Glasgow coma score (GCS) on admission, duration of hospital stay, and requirement for assisted ventilation (Tables 2 and 3). Higher GCS on admission, and longer duration of hospital stay were associated with a lower hazard for mortality with hazard ratios of 0.76 (95% CI 0.69-0.83), and 0.88 (95% CI 0.83-0.94) respectively. The corresponding hazard ratios for mortality or disability at 30 days were 0.78 (95% 0.72-0.85) and 0.96 (95% CI 0.93-0.99) respectively. Need for assisted ventilation was significantly associated with both outcomes (hazard ratios 2.14(95% CI 1.0-4.77) and 1.92 (95% CI 1.03-3.58) respectively. These hazard ratios imply that the risk of death is reduced by 24% and 12% respectively for every one point elevation in GCS, and for every additional day spent in the hospital. The risk of death was increased by more than two times if the patient required assisted ventilation.

We evaluated whether any environmental or societal risk factors were associated with the risk of VE. Most environmental risk factors were interrelated to each other, and low socio-economic status is a confounder for all different transmission pathways.(Figure 7) None of the environmental risk factors were significant predictors of VE on univariate analysis (Table 4) while two societal risk factors (lowest tertile of socio-economic status score, and household overcrowding defined as a person density of three or more individuals per room in the household) were significant risk factors. To determine the effect of environmental risk factors (for each transmission domain) on VE occurrence, independent of socioeconomic risk factors, we performed a multivariate regression

analysis in which we adjusted for socio-economic tertile. Despite having matched cases and controls for age, we included age in the multivariable models so as to adjust for any residual confounding. Of the vector, zoonotic, and water borne transmission models, only the vector model was statistically significant. The combination of presence near the house of water containing objects which promoted mosquito breeding and non-use of any personal protection measure against mosquitoes was statistically significantly associated with risk of VE (OR 2.16; 95% CI 1.08 to 4.33). We also evaluated the effect of socio-economic status, independent of any environmental transmission risk factor. The lowest tertile of socioeconomic score was a significant risk factor for VE (OR 3.12; 95% CI 1.57 to 6.17) (Table 5).

Only six of 152 (3.9%) VE suspects were HIV positive. Of 100 controls, 2 (2%) were HIV positive. The prevalence of HIV positive individuals in controls in our study was comparable to the known HIV prevalence in our community (1.75%). Being HIV positive was not significantly associated with risk of suspected VE.

Discussion

The current study presents a description of AES in rural central India, and reports a high incidence of AES in adults (upto 16 / 100,000 adult population per year), with almost two-third of all cases occurring in the hot and humid months of the year. About 36% of the patients with AES died within 30-days of onset of the illness. While the requirement for assisted ventilation in the hospital was the only clinical parameter associated with significantly increased hazard of mortality, a high Glasgow coma score and longer duration of hospitalization were associated with a significantly reduced hazard of mortality. Although most AES cases were in individuals living in the administrative subdivisions in proximity to the hospital, this spatial distribution is likely to be due to referral bias. Proximity of the village of residence of a case to a river or stream however is likely to be a spatial risk factor for AES in the region. As compared to healthy community controls low socioeconomic status and factors that promote vector borne disease transmission were significantly associated with the risk of VE.

These findings can help improve our understanding of AES in central India. The high incidence rate that we observed in adults in a non-outbreak setting suggests persistent circulation of the infectious agent causing VE in the community. Most etiologic agents causing VE also cause either a more benign febrile illness or asymptomatic infection among susceptible individuals, but it is unlikely that such infections confer herd immunity and AES persists from year to year. Most AES cases occur in the hot and humid months. This finding, consistent with the findings of previous studies of AES in India, suggests a climate dependent transmission cycle. The hot and humid time of the year is characterized by water-logging, filling up of the perennial rivers and streams, and increased agricultural activity in the rural areas. These conditions have a potential to enhance vector densities (e.g. via increased mosquito breeding), increased contamination of drinking water supplies (as groundwater contaminates wells or perennial rivers wash pathogens downstream), and increased outdoor exposures (leading to increased cutaneous contact with water, and soil). In light of these seasonal conditions, the presence of objects outside the house (such as old tires, pots, or old utensils) where mosquitoes can breed and non-use of any personal protection measures against mosquitos was a significant risk factor for development of VE, even after adjustment for

socioeconomic status. Low socio-economic status could contribute to the risk of AES via multiple potential pathways (figure 7), and hence is a potential confounder of the relationship between environmental exposures (which characterize different transmission domains) and the development of VE. However, low socioeconomic status alone, adjusted for all other exposure variables was also significantly associated with the development of VE. This finding implies that poverty alone was a major determinant of VE.

While routes of transmission, non-human hosts, and host characteristics are well described for most known etiologies of AES, there are only a handful of studies which have evaluated environmental and societal level risk factors for AES. In a recent study of children with Japanese Encephalitis in China, lower family income, lower parental education, poor quality of housing, and residence near periphery of a village were significant risk factors, on univariate analysis, although none of these factors remained significant on multivariate analysis.[26] While a study that evaluated risk factors for St Louis Encephalitis (SLE) in Arkansas in 1991 found low income and low education as significant risk factors for infection with the virus,[27] a study in Laos did not find income as contributing to the risk of infection with vector borne flaviviruses.[28] These two studies evaluated risk of infection (and not that of clinical disease) with specific flaviviruses amongst healthy individuals, as determined by presence of IgM and IgG class of antibodies. The study in Laos was conducted in a single study, and it is likely that both rich as well as poor get similarly infected as they donot live too far away from each other. It is likely that rich, despite having used better personal protection measures still got mosquito bites, which infected them. Authors have called this as a "neighborhood effect". We were expecting a similar "neighborhood effect" in our study as well, as cases and controls in our study lived in close proximity to each other.

Recent studies from Texas and California have found that the presence of hypertension, diabetes mellitus, chronic cardiovascular diseases, and immunosuppression were significant risk factors for West Nile encephalitis, suggesting that individuals with such conditions need to especially themselves from mosquito bites in endemic regions.[29-31] While such studies of AES are rare, yet they can be powerful tools in increasing our understanding of disease.

Our understanding of predictors of survival among patients with VE is largely based on studies done on patients with JE. Significant global differences in mortality and disability due to VE have been observed. Mortality among individuals with VE is extraordinarily high in JE endemic areas in South and South-east Asia ranging from 17 to 50%.[32-34] In the current study 36% of patients with VE died, findings supported by other hospitals in India. In contrast developed countries experience a lower mortality (0 to 5%) among patients with VE, but a high proportion of individuals who survive with significant disability (up to 40%).[35-37] These differences may be due to differences in organism-specific disease severity, but likely also reflects differences in quality of available neuro-intensive care facilities.

A low Glasgow coma score (GCS) has been shown to be a significant predictor of mortality in studies of VE conducted in different age groups and locations.[37-44] Low GCS is also a poor prognostic factor in other central nervous system infections such as tuberculous meningitis, [45] or pneumococcal meningitis.[46] Other reported significant simple clinical predictors of mortality or a poor outcome in such patients include a short

prodromal phase, [44] the presence of seizures,[38] the presence of specific neurological signs (such as disruption of oculocephalic responses, [37] decerebrate posturing[44]), abnormal results of neuroimaging studies and complications such as bronchopneumonia. [42] Other poor prognostic features include investigation results such as CSF pleocytosis, [40] elevated CSF proteins, [47] and hyponatremia. [48] Among patients with JE, fluid management-practices fluid in hospitals (such as sodium restriction) and cardiogenic shock have been associated with adverse outcome. Neither high dose dexamethasone[49] nor interferon-alpha 2a[50] confer any survival or disability benefit.

The epidemiologic features of AES in India have been described both in the context of outbreak investigations and as part of disease surveillance efforts. However, in all of these studies the emphasis was on determining the etiology of AES, rather than on factors associated with an increased or decreased risk of severe squealae. Strength of our study lies in its ability to describe and quantify the risk factors for AES, and the factors associated with an increased hazard of mortality. However our study had certain limitations. First the risk of severe sequlae and hazard of mortality or disability is likely to be etiology-specific. The etiology of most AES cases in developing countries is never determined, due to the lack of availability or use of diagnostic tests. In the absence of such diagnostic tests, an attempt could be made to identify potential risk factors, which might then provide clues about sources and modes transmission. Notable example of such studies include well designed case-control studies that found that contact with live pigs was a risk factor for Nipah virus encephalitis, a finding which led to the discovery of respiratory transmission from pigs to human beings.[51, 52] Another epidemiological study from Bangladesh has recently identified tree-climbing as a risk factor for Nipah encephalitis, and postulated that exposure to fruit-bat secretions was a source of infection.[53]

A second limitation of our study could be due to sampling bias. All AES cases included in this study came from a single hospital, and it is likely that we missed individuals who never sought medical care for their illness or who sought care at another facility. These factors would have led us to underestimate the incidence of AES. However our incidence rate of 16/100,000 (in the sub-divisions of the same district as the hospital) that we found is well above the incidence of 2 per 100,000 for adults[2] that has been suggested as the minimal rate indicative of adequate surveillance for AES. Moreover the hospital where this study was based is one of only two tertiary care referral teaching hospitals in the entire district (population 1.3 million). Those cases that never sought medical attention for AES are likely on average to be poorer than those who sought care. Thus low socioeconomic status as a risk factor for AES might well be a conservative estimate of this relationship. Despite limitation of single hospital based studies, such surveillance systems can help track changes in patterns of AES over a long-term.[54, 55]

Last, the controls in our study were healthy individuals sampled from the same villages as the case. Controls living in the same geography as the case are likely to share many of the environmental and societal risk factors. On the other hand many villages may not have homogenous micro-environment, and important differences may exist within them. We deliberately sampled controls from households in the same village farthest from the case household, so that cases and controls have a greater micro-environmental heterogeneity. This approach is likely to have enhanced the contrast in

environmental risk factors between cases and controls. Our finding that low socioeconomic status was a risk factor for AES demonstrates the diversity that does exist within a single village.

The current study is a descriptive analysis of AES in adults in rural central India, which has a seasonal high incidence of the condition; and individuals who are poor are three times as likely to be affected as compared to those who are economically better off. Poverty not only increases individuals' exposure to infectious agents, but also affects their ability to protect them. Poverty also potentially delays health seeking behavior, leading individuals to present late in course of their disease, and makes families less likely to be able to afford expensive supportive treatments which many patients with AES require. Determining the common etiologies of AES in an area is also important as effective preventive measures can be developed. Chapter five of this dissertation presents results of studies on the biological samples collected from patients with AES and re-evaluation of risk factors for AES due to specific etiologic agents.

Table 1: Temporal profile of acute encephalitis syndrome (AES) cases (n=183) and viral encephalitis (VE) suspects (n=152) by season

	Season		
	Cool and dry (Winter)	**Hot and dry (Pre-monsoon; summer)**	**Hot and wet (Monsoon; Rain)**
Months	**January to February**	**March to June**	**July to October**
Weeks (study period)	8	16	16
Mean daily temperature* (Celsius)	23.17	33.55	28.43
Total rainfall (mm)	0	40.63	103.26
Number of AES cases (% of total)	20 (10.9)	55 (30.0)	108 (59.0)
Number of viral encephalitis suspects (% of total)	15 (9.8)	39 (25.6)	98 (64.4)

* Average temperature on a particular day is calculated as the sum of daily maximum and minimum divided by two. Mean of these daily averages is represented as mean daily temperature.

Table 2: Unadjusted and adjusted hazard ratios for 30-day mortality among patients with AES, who are viral encephalitis suspects (n=152)

Variable	Survived to day 30	Died before day 30	Unadjusted	Adjusted†
	N=99	N=53	Hazard ratio (95% CI)	Hazard ratio (95% CI)
Demographic variables				
Age (yrs)	37.5 (17.1)	45.3 (19.5)	1.01 (1.01-1.03)	1.02 (1.00-1.03)
Male gender*	50 (50.5)*	40 (75.4)*	2.57 (1.37-4.82)	
SES score	19.6 (7.1)	18.8 (6.9)	0.98 (0.94-1.02)	
On-admission variable				
Duration of symptoms (days)	6.4 (5.0)	5.2 (3.4)	0.94 (0.88-1.01)	
Presence of seizures*	23 (23.2)*	11 (20.7)*	0.81 (0.41-1.57)	
GCS on admission	11.2 (2.5)	6.2 (3.7)	0.73 (0.68-0.79)	0.76 (0.69-0.83)
Clinical signs of meningitis*	30 (30.3)*	17 (32.0)*	1.10 (0.62-1.95)	
In-hospital stay and complications				
Hospital stay	11.5 (8.0)	7.1 (5.3)	0.86 (0.80-0.93)	0.88 (0.83-0.94)
Gastro-intestinal bleed*	1 (1.8)*	1 (1.0)*	1.41 (0.19-10.2)	
Hypotension*	0 (0)*	11 (20.7)*	5.90 (2.96-11.76)	
Requirement for assisted ventilation*	5 (5.0)*	28 (52.8)*	7.51 (4.30-13.10)	2.14(1.0-4.77)
Investigations				
Hemoglobin (g/dL)	10.6 (2.3)	10.8 (2.7)	1.04 (0.93-1.18)	
Total leukocyte count (x 10^3 mm3)	7.0 (30.9)	10.9 (4.4)	1.00 (0.98-1.00)	
Platelet count (x 10^6 /mm3)	2.4 (1.3)	2.1 (1.2)	0.99 (0.99-1.00)	
Positive HIV test	2 (2.0)	4 (7.5)	1.99 (0.72-5.55)	
CSF cell count (per mm3)	303 (742)	716 (2485)	1.00 (1.00-1.00)	
CSF sugar (mg/dL)	61.1(20.7)	68.5 (27.8)	1.01(1.00-1.02)	
CSF proteins (g/dL)	114.8 (140.8)	179.5 (201.7)	1.00 (1.00-1.00)	
Obtaining brain imaging*	38 (38.3)*	21 (39.6)*	1.04 (0.60-1.81)	

***** These variables are dichotomous, and the values represent number (percent); Remaining variables are continuous and the values represent means (SD).

† These are adjusted hazard ratios obtained after a multivariable regression using Cox proportional hazards model.

Table 3: Unadjusted and adjusted hazard ratios for 30-day mortality and disability among patients with AES, who are viral encephalitis suspects (n=152)

Variable	No Death or disability by day 30	Death or disability by day 30	Unadjusted	Adjusted
	N=65	N=87	Hazard ratio (95% CI)	Hazard ratio (95% CI)
Demographic variables				
Age (yrs)	33.70 (15.72)	45.17 (18.64)	1.02 (1.01-1.03)	1.02 (1.00-1.03)
Male gender*	37 (56.9)*	53 (60.9)*	1.41 (0.92-2.18)	
SES score	20.01 (7.10)	18.90 (6.97)	0.98 (0.95-1.01)	
On-admission variable				
Duration of symptoms (days)	7.04 (5.54)	5.21 (3.53)	0.94 (0.89-0.99)	
Presence of seizures*	12 (18.4)*	22 (25.2)*	1.06 (0.65-1.02)	
GCS on admission	11.66 (2.35)*	7.86 (3.91)*	0.77 (0.73-0.82)	0.78 (0.72-0.85)
Clinical signs of meningitis*	24 (36.9)*	26 (29.8)*	0.89 (0.56-1.43)	
In-hospital stay and complications				
Hospital stay	9.87 (5.42)	10.11 (8.71)	0.97 (0.94-1.00)	0.96 (0.93-0.99)
Gastro-intestinal bleed*	1 (1.54)*	1 (1.15)*	0.94 (0.13-6.78)	
Hypotension*	0 (0)*	11 (12.6)*	6.03(3.03-12.02)	
Requirement for assisted ventilation*	4 (6.1)*	29 (33.3)*	4.80 (3.00 -7.72)	1.92 (1.03-3.58)
Investigations				
Hemoglobin (g/dL)	10.76 (2.44)	10.75 (2.49)	1.02 (0.93-1.11)	
Total leukocyte count (x 10^3 mm3)	7.63 (3.42)	8.91 (3.76)	1.00 (0.99-1.00)	
Platelet count (x 10^6 /mm3)	2.36 (1.35)	2.23 (1.20)	0.99 (0.99-1.00)	
Positive HIV test	2 (3.0)	4 (4.6)	1.46 (0.53-4.01)	
CSF cell count (per mm3)	390.2 (885.2)	467.4 (1901)	1.00 (0.99-1.00)	
CSF sugar (mg/dL)	58.8 (21.0)	67.42 (24.90)	1.01 (1.00-1.02)	
CSF proteins (g/dL)	122.1 (152.3)	148.74(176.91)	1.00 (1.00-1.00)	
Obtaining brain imaging*	23 (35.3)*	36 (41.3)*	1.12 (0.73-1.72)	

***** These variables are dichotomous, and the values represent number (percent); Remaining variables are continuous and the values represent means (SD).

† These are adjusted hazard ratios obtained after a multivariable regression using Cox proportional hazards model.

Table 4: Univariate analysis of risk factors for AES (VE suspects)

Variable	Cases n=152	Controls n=100	OR (95% CI)
Risk factors promoting vector borne transmission			
Objects promoting vector breeding outside house			
Earthen pots vs.	133 (88)	98 (98)	0.15 (0.01-0.65)
No earthen pots	18 (11.9)	2 (2)	
Old discarded tires vs.	4 (2.6)	4 (4)	0.65 (0.11-3.60)
No discarded tiers	147 (97.3)	96 (96)	
Water drum / water coolers vs.	138 (91.3)	92 (92)	0.92 (0.31-2.51)
No Water drum / coolers	13 (8.6)	8 (8)	
Three or more objects vs.	22 (14.4)	17 (17)	0.82 (0.39-1.76)
Two or less objects	130 (85.5)	83 (83)	
Personal vector protection measures			
Any measure used vs.	59 (38.8)	48 (48)	0.68 (0.39-1.18)
None	93 (61.1)	52 (52)	
Three or more objects + Non-use of vector protection			
Either of two conditions present vs.	109 (71.7)	63 (63)	1.48 (0.83-2.63)
Both conditions absent	43 (28.2)	37 (37)	
Risk factors promoting zoonotic transmission			
Cattle and pigs (includes cow, goat, bull, buffalo, pigs)			
Present in home/occupation vs.	60 (39.4)	51 (51)	0.62 (0.36-1.07)
Absent	92 (60.5)	49 (49)	
Dogs and cats			
Present in home/occupation vs.	11 (7.2)	9(9)	0.78 (0.28-2.24)
Absent	142 (92.7)	91 (91)	
Poultry (includes chicken and domesticated birds)			
Present in home/occupation vs.	12 (7.8)	11 (11)	0.69 (0.26-1.81)
Absent	140 (92.1)	89 (89)	
Risk factors promoting waterborne transmission			
Predominant source of drinking water			

Non-piped water supply vs.	21 (13.9)	12 (12)	1.18 (0.52-2.78)
Piped water supply	130 (86.0)	88 (88)	
Sewage drain in front of house with stagnant water			
Present vs.	114 (75.5)	84 (84)	0.58 (0.28-1.16)
Absent	37 (24.5)	16 (16)	
Pond/stream within 200 meters of house			
Present vs.	133 (88.0)	93 (93)	0.55 (0.18-1.46)
Absent	18 (11.9)	7 (7)	
Sewage drain + Pond / stream near house			
Both present	101 (66.8)	78 (78)	0.56 (0.30-1.05)
One or none present	50 (33.1)	22 (22)	
Risk factors related to poverty			
Socioeconomic status (Tiwari score)*			
Lowest tertile vs.	62 (58.4)	21 (32.8)	2.88 (1.44-5.84)
Highest tertile	44 (41.5)	43 (63.1)	
Overcrowding in household			
Three or more person per room	37 (24.5)	12 (12)	2.38 (1.12-5.30)
Two or less person per room	114 (75.5)	88 (88)	
Number of children (age <12) in household			
Three or more vs.	15 (9.8)	11 (11)	0.88 (0.36-2.23)
Two or less	137 (90.1)	89 (89)	
Immunosuppresion (HIV positivity)			
Positive vs.	6 (3.9)	2(2)	2.01 (0.34 – 20.74)
Negative	146 (96.1)	98(98)	

* Number of cases =106, and number of controls =64 in this analysis. Remaining belong to middle tertile.

Table 5: Multivariate logistic regression models

Model	Variable	OR	95% CI
Vector borne transmission model*	Three or more objects† promoting vector breeding outside household + Non-use of any vector protection measure Both conditions met vs. no condition met	2.16	1.08 to 4.33
Zoonotic transmission model*	Presence of a canine in household vs none	1.45	0.45 to 4.65
Water borne transmission model*	Non-piped water supply vs. Piped water supply at home	2.32	0.90 to 5.68
Poor living condition model‡	Socioeconomic lowest vs highest tertile	3.12	1.57 to 6.17

†Objects include earthen pot, water filled drums, water coolers, old tires etc.

* Models adjusted for age and Socio-economic status

‡ Model adjusted for age, presence of objects promoting vector borne transmission, non-use of personnel protection, presence of canine in household, and non-piped water supply at home.

Box 1A: Socioeconomic status scoring sheet

Part 8: Socio-economic status

House Profile
A1 + A2 score
2
☐☐

A1 Land area

No Land	Up to 1000sq. ft.	1001-5000 sq. ft.	5001-10000 sq. ft.	10001-20000 sq. ft.	>20000 sq ft.
0	2	4	6	8	10

A2 House type (see separate sheet)

Not Applicable	K1	P1	P2	P3	P4
0	2	4	6	8	10

Material possessions Profile
B1 + B2 score
2
☐☐

B1 House hold gadgets (see separate sheet for value)

None	Upto Rs.5,000.00	Rs.5,001-10,000.00	Rs.10,001-50,000.00	Rs.50,001-1,00,000.00	>Rs.1,0
0	2	4	6	8	1

B2 Conveyance used (see separate sheet for value)

None	Upto Rs.5,000.00	Rs.5,001-50,000.00	Rs.50,001-5,00,000.00	Rs.5,00,001-20,00,000.00	>Rs.20,0
0	2	4	6	8	1

Educational Profile

Average score
☐☐

Family Members	0	2	4	6	8	10
1.						
2.						
3.						
4.						
5.						
6.						

0=Illiterate , 2=just literate/religious education/up to 8th , 4=Upto 12th , 6= Upto Graduation/Diploma holders, 8=Upto Post-graduation/Professional degree, 10= Higher studies (Ph.D., MD, MS, D.Litt, MCH, DM etc.)

Occupational Profile
Average score
☐☐

Family Members	0	2	4	6	8	10
1.						
2.						
3.						
4.						
5.						
6.						

See SES sheet for a list of occupations

Economic Profile
Total score
☐☐

E1Average per-capita income per month (all sources)

Up toRs.500.00	Rs.501-2000.00	Rs.2001-5000.00	Rs.5001-10000.00	>Rs.1
2	4	6	8	

Possessed land
Total score
☐☐

F1Possessed Land/House Cost Profile

No Land/house	Up to Rs. 50000	Rs. 50,000 to 5 lakh	Rs. 5 to 20 lakh	Rs. 20-50 lakh	>Rs.50 lakh
0	2	4	6	8	10

Social Profile
G1 + G2 score
2
☐☐

G1- Understanding of social issues :

None	Religious-Cultural	Developmental	Educational	Health promotional	Political
0	2	2	2	2	2

G2- Participation in social activities :

None	Religious-Cultural	Developmental	Educational	Health promotional	Political
0	2	2	2	2	2

Total score : ☐☐

24. SES Category (see SES sheet) : 1 2 3 4 5

Box 1B : Socioeconomic score code interpretation

A1: Land area (as in the questionnaire)

A2: House type

K1=No brick walls/floor kuchha/chhappar/hutments (jhopari)/shanty/khaprail ; **P1**=Plastered or Un-plastered brick walls/floor kuchha or made-up of bricks only/ordinary roof (R.B. roof) or tolly roof or tin roof ; **P2**= Plastered walls/floor cemented or mosaic/ordinary roof (R.B.Roof) ; **P3**= Plastered walls / floor cemented or mosaic/RCC roof ; **P4** = Plastered walls / floor made up of marble or mosaic or tile floorings (excluding toilet & kitchen floorings)/RCC roof or plaster of paris roof design.

B1: House Hold Gadget (Approximate costs):

Radio= Rs 500.00	Colour T.V.= Rs 10,000.00	B/W T.V.= Rs 3,000.00
Tape Recorder = 1,500.00	Basic Phone = Rs.3,000.00	Mobile Phone = Rs 5,000.00
Licensed Arm = Rs 40,000.00	Fan = Rs 1,000.00	Washing Machine = 12,000.00
Refrigerator = Rs 8,000.00	Cooler = Rs 4,000.00	LPG Gas. = 2,000.00
Hand Pump = Rs 4,000.00	Water Pump (Tullu) = Rs 5,000.00	Pumping set= Rs 20,000.00
Generator = Rs 20,000.00	Tubewell=Rs.15,000.00	Milking Animal = Rs10,000.00

B2: Conveyance facilities

Rickshaw = Rs 3,000.00	Cycle = Rs 1,500.00	Moped= Rs 20,000 .00
Scooter= Rs 25,000.00	Motorcycle = Rs 40,000.00	Tempo= Rs 1,25,000.00
Auto= Rs 75,000.00	Jeep= Rs 4,50,000.00	Tractor= Rs 3,50,000.00
Truck= Rs 10,00,000.00	Economic Car (Maruti 800, Fiat etc.)= Rs 2,50,000.00	

C: Educational profile (as in questionnaire)

D: Occupational profile

0 = No gainful employment.

2 = Unskilled Labour (labour, agricultural labour, rickshaw puller).

4 =Class IV employee, skilled worker (tailor, black smith, carpenter, washer-man, potter, barber, driver etc.), hawker, vendor, (goods less than 50,000.00) petty farmer (cultivated land<1 acre), caste occupation.

6 =Class-III employee, primary school teacher, high school teacher, small businessman (having his/her own or rented shop and goods upto Rs. 1,00,000.00), farmer (culivated land 1-10 acres) & private contractor, insurance agents etc.

8 =Class-II employee/junior professionals (experience up-to 5 years),intermediate teacher, principals upto intermediate colleges, farmer (cultivated land upto 10-20 acres), business man (goods upto Rs. 1,00,000.00 - 5,00,000.00), local public leader like corporater, Govt. contractor etc.

10 = Class-I employee/executives/senior professionals (experience more than 5 years), university/degree colleges teachers,principals of degree colleges, professors, farmers (land more than 20 acre), businessman (goods>Rs. 5,00,000.00), leaders (MLA's, MP's etc).

E: Possessed Land (as in questionnaire)

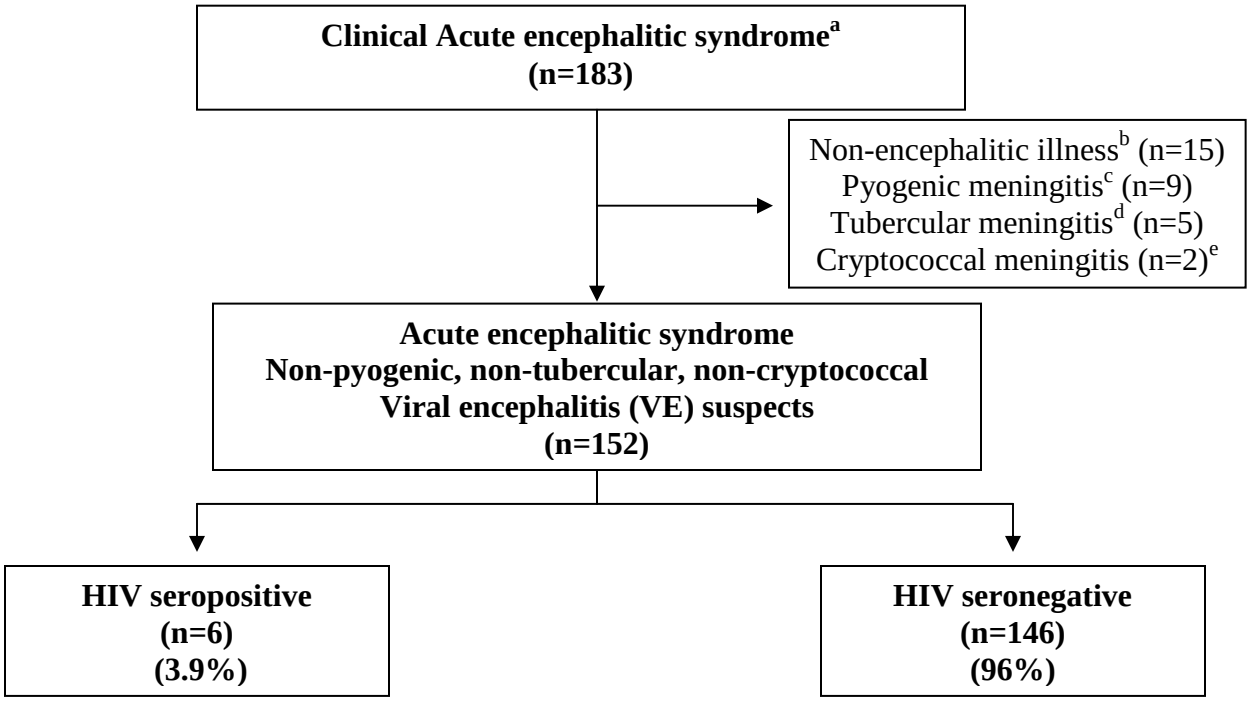
F: Social –Cultural (Mark as applicable for understanding and participation)

Religious-cultural : Understanding & Participation in religious activities as head, priest, worships, rituals, religious preaching, satsang/ participation in marriages, folk meetings, folk activities, parties etc.

Educational: Understanding & Participation in illiteracy removal activities like old age education, orphan education, women education etc.

Developmental: Understanding & Participation in developmental activities i.e.- improving hygiene, sanitation, drinking water, road , school development etc.

Figure 1: Study Flow chart



[a] Acute encephalitic syndrome was defined as presence of fever, which preceded altered sensorium, with or without neurological deficit. All these patients had negative peripheral smears and HRP-2 serology for malaria, had no other primary source of infection, and had a normal chest radiograph. No metabolic abnormality (hypogloycemia, hyponatremia, hyperuricemia or hepatic encephalopathy) was present when these individuals were included in the study.

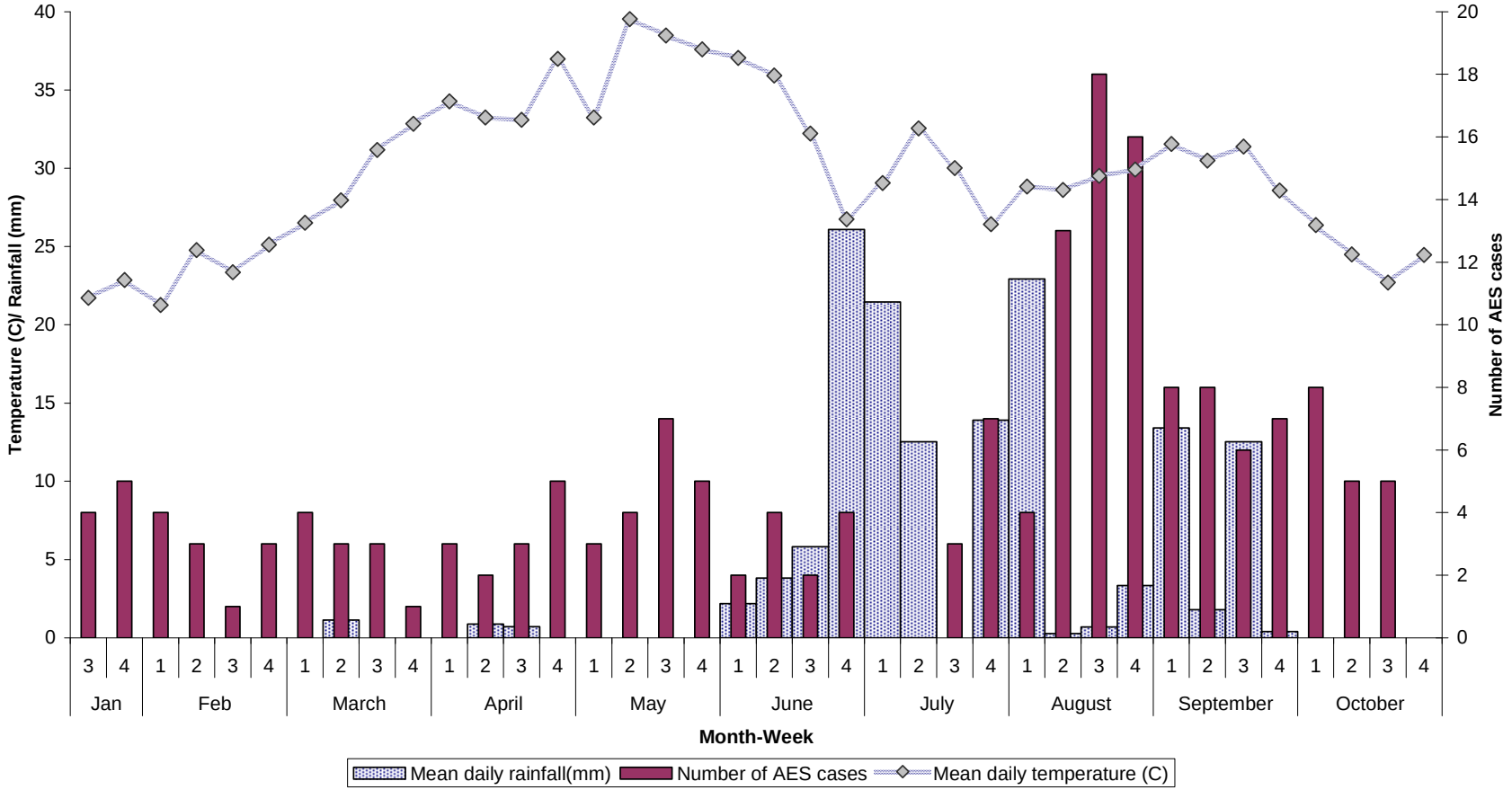
[b] Non-encephalitic illness included individuals who were detected with a non-infectious etiology after inclusion into the study such as intracranial tumor (n=2), venous infarct (n=1), psychiatric illness (n=3), and metabolic abnormalities (n=9).

[c] Pyogenic meningitis was defined as the presence of neutrophils in cerebrospinal fluid sample, CSF/serum glucose ratio <0.25 with or without positive bacterial culture. 4/9 (44%) of individuals with pyogenic meningitis had a positive bacterial culture.

[d] Individuals with a positive cerebrospinal fluid mycobacterial culture on bactec media were defined as having tubercular meningitis.

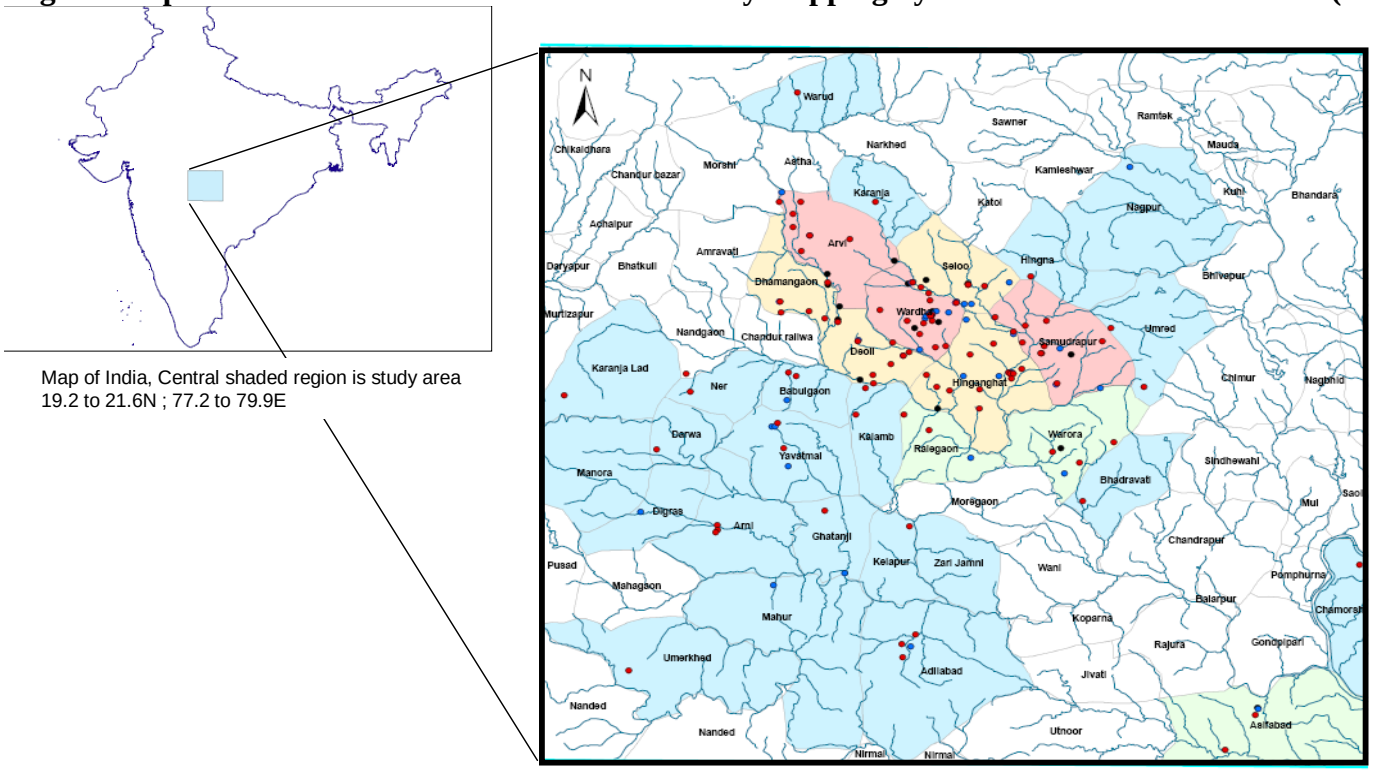
[e] Cryptococcal antigen was tested in HIV positive individuals only.

Figure 2: Temporal profile of all Acute encephalitis syndrome cases (n=183)



Mean daily rainfall for the week has been calculated by dividing cumulative rainfall in the corresponding week by total number of days. Mean temperature for
the week is calculated by dividing the sum total of the daily means (average of maximum and minimum temperature for the day) by the number of days. The first
two weeks for a month correspond to the first fortnight of the month, so the number of days in a week could be either 7 or 8.

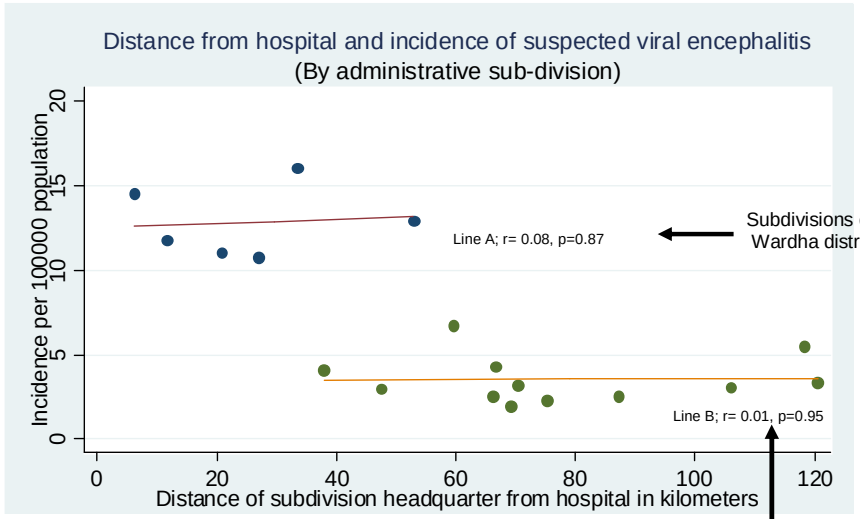
Figure 3: Spatial distribution of AES cases and density mapping by administrative sub-divisions (n=183)



Map of India, Central shaded region is study area
19.2 to 21.6N ; 77.2 to 79.9E

One dot in the map represents each case of AES. The color of dot indicates the season in which the case occurred. The color shades indicate annual incidence rate by each administrative sub-division.

Figure 4: Incidence of AES by sub-division, and distance from referral hospital



Line A represents linear relation between distance of sub-division headquarter from hospital and incidence of AES for the sub-divisions in Wardha district, where the MGIMS hospital is located

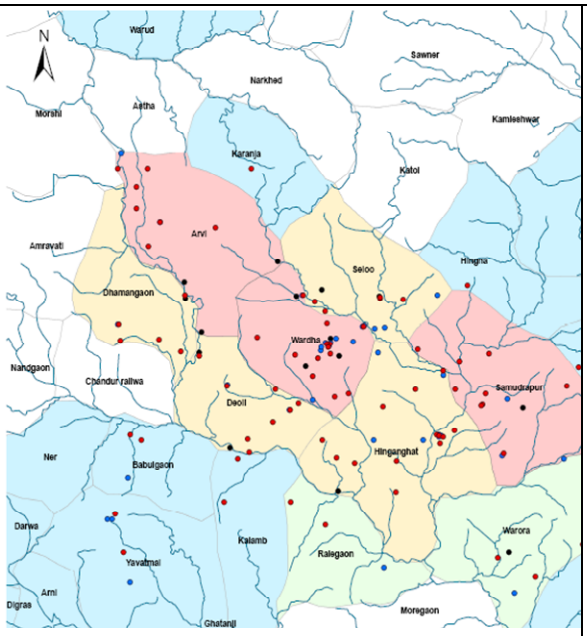
Line B represents linear relation between distance of sub-division headquarter from hospital and incidence of AES for the sub-divisions in districts neighboring Wardha

Sub-divisions from which more than one AES case reported to the hospital are included in this analysis

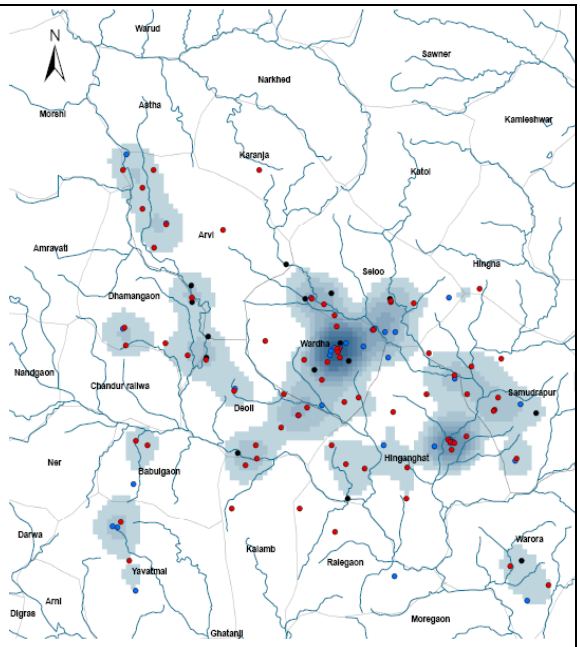
Sub-division	Adult Pop.	Cases	Incidence per 100,000
Samudrapur	80932	13	16.06
Wardha	220176	32	14.53
Arvi	100449	13	12.94
Seloo	93155	11	11.81
Deoli	108650	12	11.04
Hinganghat	148735	16	10.76
Dhamangaon	68948	6	8.70
Asifabad	72801	4	5.49
Warora	116090	5	4.31
Ralegaon	73382	3	4.09
Adilabad	119063	4	3.36
Babulgaon	62407	2	3.20
Arni	97903	3	3.06
Kalamb	67081	2	2.98
Ner	77533	2	2.58
Yavatmal	235177	6	2.55
Ghatanji	87650	2	2.28
Umred	102790	2	1.95

Incidence rate is calculated per 100,000 adults residing in the sub-division, as per government of India 2001 census data.

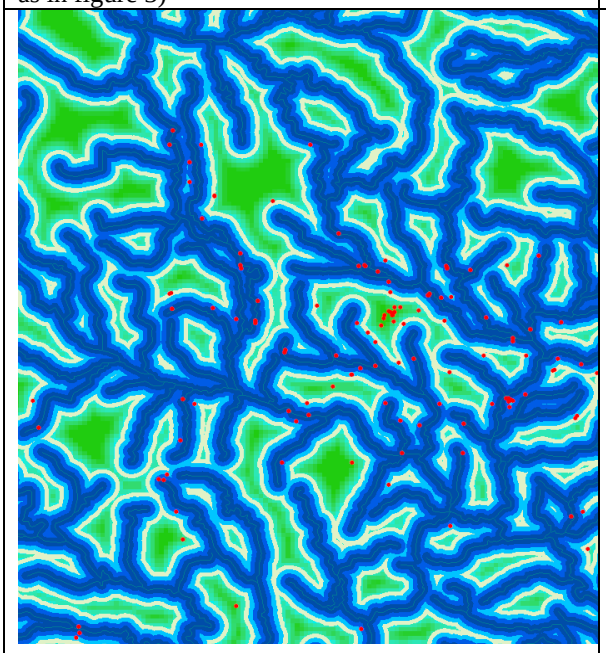
Figure 5: Density mapping of AES cases after restricting the analysis to high incidence areas



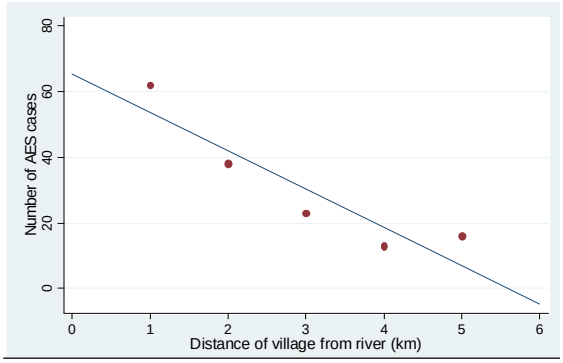
A)Case density by Administrative sub-division. The color coding represents incidence rate (Legend same as in figure 3)



B) Kernel density function shows point locations where the cases were spatially related



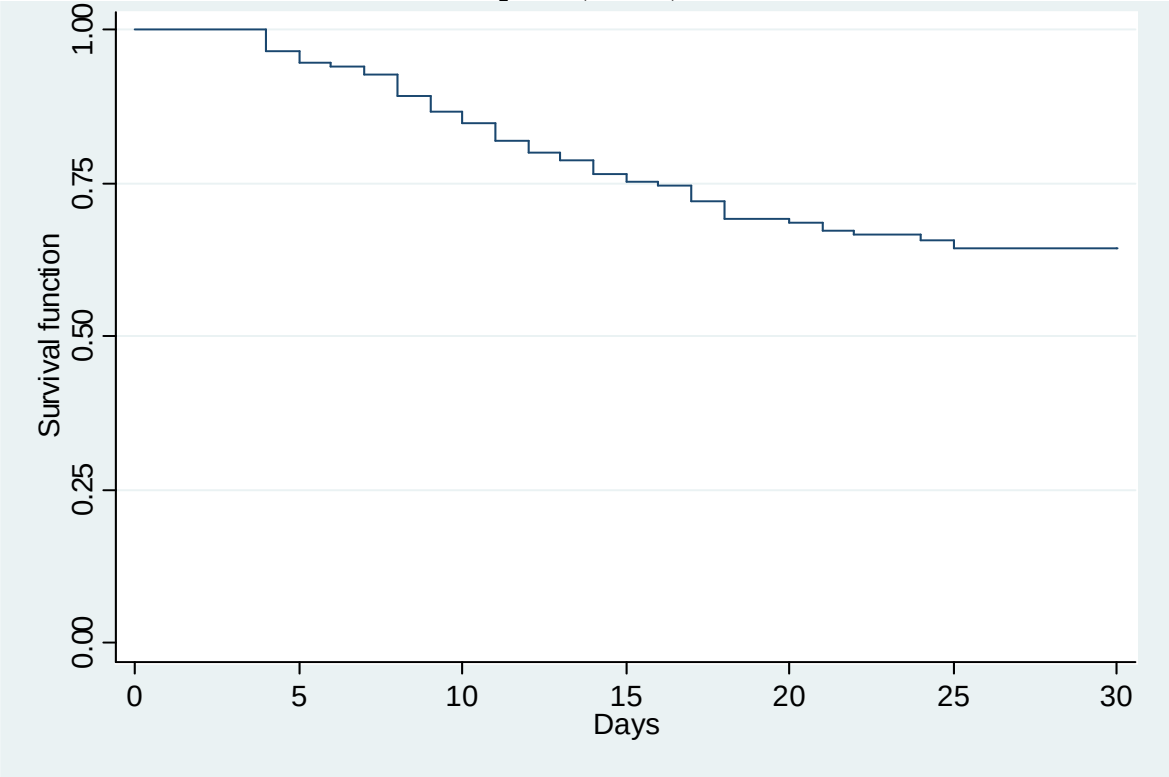
C) One kilometer buffer zones created in hydrology layer (around rivers and streams) and frequency distribution of points in each buffered layer was determined



Distance	Cases	Proportion (of all cases)
Within 1km	62	40.7
1 -2 km	38	25.0
2 -3km	23	15.1
3-4 km	13	8.5
4km or more	16	10.5

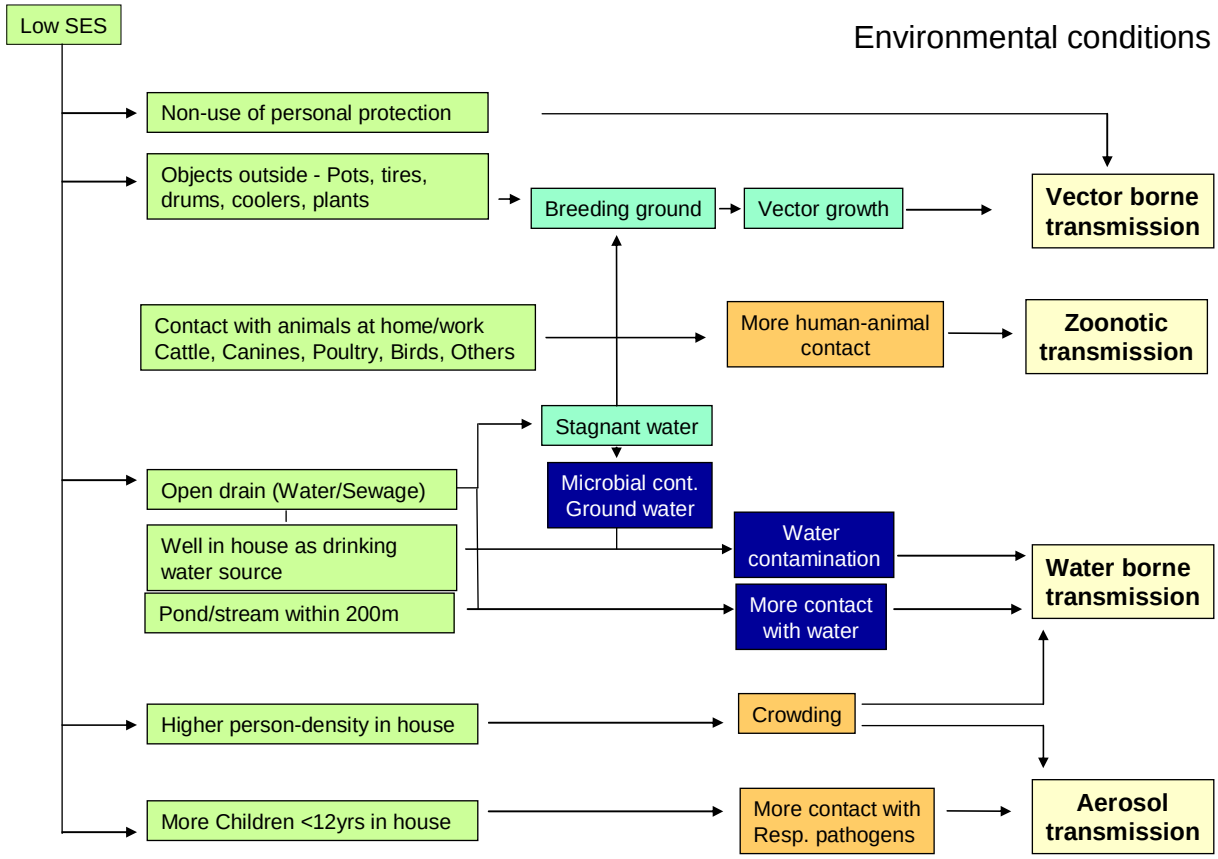
D) Linear regression of Number of AES cases, and distance of village from nearest river / stream;
Cases =65.5 – 11.7*(distance);
There were about 12 fewer cases, for every one kilometer distance away from a river/stream

Figure 6: Kaplan Meier survival curve for the cohort of AES cases, who were VE suspects (n=152)



Product limit estimate of survival function of 152 patients (cumulative at risk period 3529 days)
30-day survival function in patients who are VE suspects is 0.64 (95% CI 0.56-0.71)

Figure 7: Interrelationships between environmental risk factors for AES



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Chapter 4: Accuracy and utility of Enzyme linked immunosorbent assay (ELISA) for diagnosis of acute leptospirosis: A systematic review

Abstract

Leptospirosis is an infection caused by a pathogenic spirochete (Genus *Leptospira*) that grows poorly in culture and has a wide antigenic diversity of its genomospecies and serovars. The microscopic agglutination test (MAT) is a complex serovar specific test, that is the currently accepted reference standard; it is available in only a few reference laboratories worldwide. The enzyme linked immunosorbent assay (ELISA) is an alternate, simpler, genus specific test, that employs broadly reactive antigens to make a diagnosis. We conducted this systematic review to understand the diversity of available ELISA based tests for diagnosis of leptospirosis, and to evaluate their diagnostic accuracy. We used several overlapping strategies to identify 37 full text articles (48 diagnostic studies) for included in this review. We found that compared to MAT as the reference standard, ELISA-based tests aimed at detection of anti-leptospira IgM antibodies have a high sensitivity and specificity. When serum samples were collected in second week of illness (late acute phase), pooled sensitivity and specificity estimates were high ; 92 (95% CI 87 to 95%) and 98 (95% CI 96 to 99%) percent respectively. Thus the pooled positive likelihood ratio of ELISA-based immunoglobulin detection is 57.3 (95% CI 15.65 to 210.34), and the negative likelihood ratio is 0.08 (95% CI 0.05 to 0.14). This review suggests that ELISA-based tests are reasonably accurate for the diagnosis of acute leptospirosis. As there are a wide variety of available ELISA-based tests, however future research is needed to determine the best available tests in geographically diverse regions, so that regional or global recommendation for use can be made.

Introduction

Leptospirosis, an infection caused by a pathogenic spirochete (Genus *Leptospira*), is thought to be the most common zoonosis in the world.[1] Most human infection occurs through the percutaneous route, when either abraded or intact skin comes in contact with water contaminated with the urine of rodents or other animals. Poor sanitation, water-logging, temporary housing, and abundance of rodents are some of the key environmental conditions that promote transmission of *leptospira*. Given these environmental risk factors, residents of urban slums and impoverished rural communities in low and middle income countries are the usual victims of this life-threatening infectious disease.[1]

The diagnosis of leptospirosis is complicated by the antigenic diversity of the causative organism. Genus *leptospira* is further classified into various genomospecies and serovars. Most leptospira organisms pathogenic to humans are categorized under *Leptospira interrogans* genomospecies, which has 24 different serovars.[2] Different pathogenic serovars have been reported to be prevalent in different geographic regions. *Leptospira biflexa* is a major non-pathogenic genomospecies, and its broad immune reactivity has been found to be useful in leptospira diagnostics. The microscopic agglutination test (MAT) is considered as a gold standard for diagnosing leptospirosis. This test aims to identify pathogenic serovars by detecting four fold or greater rises in agglutinating antibodies in paired sera. Performance of this test requires laboratories to

maintain cultures of pathogenic serovars, and to be equipped to perform dark field microscopy. Thus MAT is tedious to perform and is largely limited to a small number of reference laboratories.[3] ELISA is another immunological test used to diagnose various infections; aims to detect broadly reactive genus-specific antibodies of the IgM or IgG class. A Leptospira ELISA is commercially available and it has been proposed that this test may be better than MAT in making a clinical diagnosis during the early acute phase of illness. Although the simplicity of the ELISA and its potential for a wider clinical application make it an attractive alternative, the use of a broadly reactive antigen is still debatable as an appropriate test for diagnosis of leptospirosis in the geographically diverse regions of world.[1] Other types of diagnostic tests which aim at organism or antigen identification (such as nucleic acid amplification, microscopy, and/or culture) have a much lower yield and are virtually never performed to diagnose leptospirosis in clinical settings.

We conducted this systematic review to describe various ELISA-based tests that have been evaluated in diverse geographic regions, and to answer the following specific research questions: a) among patients with leptospirosis, presenting with fever, what is the accuracy of ELISA-based immunodiagnostics (as compared with MAT or culture or microscopy as a reference standard) for the diagnosis of leptospirosis; and b) as compared to MAT, does ELISA help make the diagnosis of leptopsirosis earlier in course of disease.

Methods

We aimed to identify studies from the published literature that had compared diagnostic accuracy of ELISA against MAT, or culture or microscopy as a reference standard. All diagnostic study types, including phase I / II (which evaluate the performance of the ELISA in known reference test positive cases and known negative individuals) and phase III diagnostic studies (which evaluate ELISA among patients suspected to have leptospirosis in a cross-sectional design) were included in the review. To determine if ELISA can diagnose leptospirosis earlier than MAT, we attempted to identify studies which had reported both ELISA and MAT results, stratified by time of sample collection. We used the following definitions in the review process:

1. *Symptomatic human leptospirosis disease* was defined as the presence of clinically evident symptoms (including but not limited to fever, myalgia, jaundice, aseptic meningitis, conjunctival suffusion) in humans, caused as a result of infection with micro-organisms belonging to *Leptospira sp.* This definition excludes sub-clinical or asymptomatic infection with *Leptospira*, and also pure immunological manifestations such as uveitis or iridocyclitis.
2. *Acute leptospirosis* was defined based on symptom duration of 14 days or less. The acute phase of illness was further sub-classified as early acute (less than six days) and late acute (six to 14 days) phase. Symptom duration of 14 days or more (without any upper limit for duration) was defined as the convalescent phase.
3. *Leptospirosis enzyme linked immuno-sorbent assay (ELISA)* was defined as an immunochemical test which detects anti-leptospirosis antibodies in serum samples. When present these antibodies form a complex with specific antigens coated on a solid phase, and their level is estimated from the activity of the enzyme bound to the antigen-antibody complex. This definition includes all ELISA formats (such as the plate, tube or comb) performing either qualitative or quantitative assessment of the

anti-leptospirosis IgG or IgM anti-body levels. The definition excludes rapid diagnostic tests, based on lateral or vertical flow techniques which lack multiple washing steps. Various synonyms of ELISA included in this definition are enzyme immunoassay (EIA), enzyme labeled assay (ELA), and competitive enzyme linked immunoassay (CELIA).

4. *Microscopic agglutination test (MAT)* is defined as a serovar-specific immunological test, wherein a serum sample from the patient is incubated with a battery of live leptospira serovars, The presence of visible agglutination seen on dark field microscopy is considered a positive test.

Search strategy

We used several overlapping approaches to identify all relevant published studies of leptospirosis diagnostic tests. Electronic database searches included PubMed, EMBASE, BIOSIS, LILACS and Web of Science. The strategy for the PubMed search (table 1) is an example of the general approach used to search electronic databases. In addition, published reviews on the topic, hand searches of major infectious disease journals and tropical medicine journals (Journal of Clinical microbiology, Journal of Infectious Diseases, American Journal of Tropical Medicine and International Health, and Transactions of Royal the Society of Tropical Medicine), were used to search for additional articles and reports.

Study selection:

We selected relevant studies without any language restriction and included : a) original studies; b) studies on symptomatic patients (those who had a febrile illness or in whom acute leptospirosis was suspected), and where serum samples were collected at least in the acute phase of the disease, c) studies where ELISA was used as an index test and compared with either MAT, culture, or microscopy as a reference standard. We excluded the following study types a) Case reports, case series, reviews, conference abstracts and letters, b) veterinary, laboratory animal or studies on laboratory isolates; c) studies involving asymptomatic individuals (sero-epidemiological surveys) and studies of individuals who were given an experimental vaccine, and d) studies on samples other than serum (e.g. biopsy samples, saliva, and urine).

Data abstraction

A subset of all the included studies was evaluated by two researchers (RJ and SS), using a standardized data abstraction form and previously agreed upon definitions. Subsequently, data from all of the selected studies were abstracted by a single researcher (RJ) using a standardized data abstraction form. The data collected included information on a) country of study, year of publication, first author, and journal; b) spectrum of patients, study design (cross-sectional / case-control), and time interval between onset of symptoms and sample collection; c) characteristics of the ELISA used including manufacturing (commercial or in-house test), components (antigen source, solid phase, qualitative or quantitative, antibody measured (IgG, IgM or both), and proportion of tested individuals with a positive or a negative test result; d) characteristics of the reference standard (MAT) used, including number of serovars included and; e) numbers of true positives, true negatives, false positives and false negatives. In addition we

applied a 14 point quality assessment scale (QUADAS criteria) and ranked the quality of included studies based on the number of positive responses obtained.[4]

Statistical analysis

We used raw data from each of the included studies to calculate the diagnostic accuracy or test positivity for the ELISA, using MAT / culture / microscopy as the reference standard. The results were cross-tabulated by relevant sub-groups, such as commercial vs. in-house tests, qualitative vs. quantitative testing process, time-periods in the natural history of leptospirosis, and phase I / II (case-control) vs. Phase III (cross-sectional study) designs. The cross-sectional designs, which evaluate the index test and the reference standard on patients suspected of having the disease in a blinded and independent manner, are methodologically superior as compared to phase I / II designs. A substantial degree of variability or heterogeneity is likely even in methodologically similar studies, due to differences in type of antigen used in the ELISA; variations in the reference standard; sampling variability; or differences in the study populations. A major cause of heterogeneity in results is likely to be due to the time interval from the onset of symptoms to the collection of the blood sample for testing. Hence, we performed subgroup (stratified) analyses according to time period (early acute, late acute, convalescent, or unspecified sampling time) and tested for heterogeneity using a chi square test ($p < 0.01$ suggesting significant heterogeneity) for each subgroup. We obtained pooled estimates of sensitivity and specificity in each subgroup to serve as representative summary estimates, using DerSimonian-Laird overall likelihood ratios. All statistical analyses were performed by STATA (version 9, Lakeway drive, TX, USA) and MetaDisc (version 1.4, Madrid Spain).

Results

Of the 6240 articles identified using all search strategies, 57 were selected for a full text review, of which 37 satisfied the criteria required for inclusion in the current systematic review (See figure 1). Nine of the selected 37 articles (24.3%) were in languages other than English (Portuguese-3, Spanish-2, and one each in French, Chinese, Russian, and Turkish). Most of studies (27 of 37 studies; 72.9%) included patients from low and middle income countries, corresponding to how leptospirosis is distributed globally. Some articles evaluated more than one type of ELISA techniques, hence there were a total of 48 studies in these 37 published articles. The quality of diagnostic accuracy studies belonging to three broad categories (phase I/II studies on in-house ELISA tests, phase I/II studies on commercial ELISA tests, and phase III studies) was assessed using a 14 point QUADAS criteria.[4] Overall included studies were of intermediate quality, with a mean score of 6.34 points. The ranges of QUADAS scores for phase I/II studies on in-house ELISA tests and commercial ELISA tests were 3 to 7 points (median 6 points), and 5 to 7 points (median 6 points) respectively. Phase III studies had higher quality scores ranging from 3 to 9 (median 7.5 points).

There was no uniformity in the source of antigen used in different in-house ELISA tests. Both non-pathogenic and various combinations of pathogenic leptospira were used, with different antigen extraction techniques developed in different laboratories. These in-house tests were evaluated in the same laboratories where they were developed, and our literature search did not reveal any external validity study of any given in-house ELISA. One commercial ELISA (Panbio, Australia) has been evaluated in

five different countries (Australia, United Kingdom, Italy, Brazil and Barbados). Four phase I/II studies demonstrated high positivity among known positives (89 to 100%), and high negativity among known negatives (87.5 to 94%) respectively. The cross-sectional Phase III study using this ELISA kit used early acute phase samples, and as compared to MAT, the ELISA had a higher positivity and large number of false positive results. This study showed a lower sensitivity (89%) and specificity (55%) for the ELISA, which may have been due to the use of an imperfect reference standard, especially in early acute phase of illness.

Phase I / II diagnostic studies with in-house ELISA as an index test

A total of 21 studies evaluated in-house ELISA tests to determine whether the ELISA test results differ in people with known leptospirosis (MAT positives) as compared to either healthy controls or those with a non-leptospirosis disease (MAT negatives). All but one of these studies used whole leptospira antigen (LA) prepared from pathogenic species; except one exception [5] which used antigens from non-pathogenic species instead. Of these studies, seven [5-11] used a qualitative dot-ELISA technique, in which binding of anti-leptospira antibodies was seen as a dot-formation on the solid phase and results were read by naked eye. The proportion positive among known MAT positive serum samples ranged from 91.1 to 100%, while proportion negative among known MAT negative sera ranged from 88.9 to 100%.

Another 14 studies [8, 12-22] published in the following years used a quantitative ELISA and measured optical density by spectrophotometer to estimate anti-leptospira antibody levels. Only one of these studies used a recombinant LA (LipL32)[16], while the remaining studies used whole leptospira as a source of antigens. The ELISA tests that aimed to detect IgM class of immunoglobulins (all using whole LA) found 75.7 to 100% positivity among known MAT positives, and 80 to 100% negativity among known MAT negative samples. The LipL32-based test to detect IgG antibodies had a lower positivity of 75% among known positives (Table 2)

Phase I / II diagnostic studies with a commercial ELISA as the index test

Six studies [16, 22-25] have evaluated commercial ELISA kits, manufactured by PanBio (Australia), Biolisa (Germany), and EIE (Brazil). All of these assays were quantitative tests and had 84.9 to 100% positivity among known MAT positives, and 93.1 to 95% negativity among known MAT negatives. (Table 3)

Phase III diagnostic studies with commercial / in-house ELISA as the index test

Eight articles [26-33] (a total of 13 studies) evaluated an ELISA as an index test and MAT as a reference standard, using cross-sectional study design. Four of these studies[28, 30-32] that did not specify the time interval between onset of symptoms and sample collection had similar diagnostic sensitivity (heterogeneity test statistic 1.75, $p=0.62$) but variable specificity (heterogeneity test statistic 51.9, $p<0.01$). This variability in specificity was due to two studies[28, 31] with a small sample sizes and fewer patients in the true negative cell. (Table 4) The pooled sensitivity and specificity estimates were 91% (95% CI 87 to 94%) and 89% (95% CI 85 to 92%) respectively (Pooled LR+ 3.3 (95% CI 0.3 to 30.9), and LR- 0.11 (95% CI 0.07 to 0.17)). The remaining studies aimed to estimate diagnostic accuracy at different times following onset of symptoms (early acute, late acute and convalescent phase) in natural history of leptospirosis. Five studies[26, 27, 29,

[33] that evaluated diagnostic properties in acute phase sera, had a greater heterogeneity in the test results (test for heterogeneity, $p<0.01$); overall sensitivity and specificity estimates were 71% (95% CI 66 to 76%) and 89% (95% CI 85 to 91) percent respectively(Pooled LR+ 4.3 (95% CI 1.8 to 9.9), and LR- 0.30 (95% CI 0.19 to 0.49)). The highest sensitivity and specificity estimates were obtained for late acute samples (collected within 7 to 14 days of symptom onset). The pooled sensitivity and specificity estimates were 92% (95% CI 87 to 95%) and 98% (95% CI 96 to 99%) respectively (Pooled LR+ 57.3 (95% CI 15.65 to 210.34), and LR- 0.08 (95% CI 0.05 to 0.14)). (Table 4, Figure 2). The three studies that used dark filed microscopy [34, 35] or culture[36] as the reference standards, and had lower sensitivity and specificity estimates. (Table 4, Figure 2)

Does ELISA help diagnose leptospirosis earlier in the course of disease than MAT

Fourteen studies[9, 15, 16, 23, 25-28, 33, 37-42] compared ELISA and MAT positivity in samples collected in early acute, late acute and convalescent phase of illness. The early acute phase (within seven days of onset of symptoms) is usual time of first presentation of a febrile patient to a health care provider. The late acute phase and convalescent phase refer to second and fourth week of illness respectively. In early acute phase the proportion of patients who were positive by ELISA was higher than those who were positive by MAT test. The median excess ELISA positivity was 15.5% (range -30.4 to +51.2%). Only two studies reported a higher MAT positivity in this phase. In late acute phase the difference between ELISA and MAT positivity was lower (median excess positivity 7.6%, range -2.7 to +30.9%). Four of the ten studies which had collected samples in late acute phase had higher MAT positivity (Table 5).

As many studies have reported a higher positivity of ELISA in early acute phase as compared to MAT, authors of these studies have contended that ELISA may be better test for early detection of disease. These authors argue that ELISA positive / MAT negative individuals truly have leptospirosis (true positives), rather than representing false positives – especially when early acute phase samples are tested.

Discussion

In the current systematic review, we found that compared to MAT as the reference standard, ELISA-based tests for detecting of anti-leptospira IgM antibodies have a high sensitivity and high specificity. A high diagnostic accuracy was demonstrated both in phase I/II and phase III diagnostic studies. The time interval between the onset of febrile illness and the collection of blood sample influenced the diagnostic accuracy estimates. When serum was collected early (i.e. during first week of illness), the pooled sensitivity and specificity estimates were low (71% (95% CI 66 to 76%) and 89% (95% CI 85 to 91%) respectively). When serum samples were collected after the first week of illness, the pooled sensitivity and specificity estimates were high (92% (95% CI 87 to 95%) and 98% (95% CI 96 to 99%) respectively). Given these estimates of diagnostic accuracy, in the event of a positive test in late acute phase of illness, the likelihood of leptospirosis infection is increased by a factor of 57.3 (95% CI 15.65 to 210.34). In the event of a negative ELISA test, the likelihood of leptospirosis infection is decreased by a factor of 0.08 (95% CI 0.05 to 0.14). The corresponding likelihood ratios in the early acute phase of illness are much lower (LR+ 4.35 (95% CI 1.88 to 9.92), and LR- 0.30

(95% CI 0.19 to 0.49)). Thus the test for leptospirosis ELISA is a reasonable alternative to MAT test, especially in the late acute phase of illness.

Both the MAT and the ELISA tests are based on detection of antibody in human sera, and have certain advantages and disadvantages. MAT is the accepted reference standard but it is quite tedious and resource intensive to perform. It requires that laboratories maintain cultures of pathogenic serovars of leptospira prevelant in the region, so that a standard quantity of viable organisms of each pathogenic serovar can be mixed with different dilutions of paired sera. The resultant agglutination needs to be observed by an experienced microscopist using a dark field microscope. These stringent testing conditions are often not available outside reference laboratories, and hence test has not gained popularity as a routine diagnostic test in patients with a febrile illness. Furthermore, MAT reactivity does not distinguish between IgM and IgG classes of antibodies, and a peculiar phenomenon referred to as a 'paradoxical reaction' is known to occur with MAT, in which a large agglutination reactions with a non-causative serovar may occur in the early acute phase of illness. In addition to these limitations a four-fold or greater rise in titers between acute and convalescent era must be demonstrated for a definite positive MAT result. Because these considerations make MAT less suitable for individual patient diagnosis, the key utility of MAT lies in establishing endemicity of infection with one or more serovars of leptospira in a particular region.[43]

ELISA-based tests were developed so as to provide a simple, yet accurate alternative to MAT for the diagnosis of acute leptospirosis. ELISA can distinguish between IgM and IgG classes of antibodies, which may help to distinguish current infections from those which might have occurred in the remote past. A drawback of the ELISA is that it does not distinguish infection with one serovar from another, and it attempts to diagnose infections caused by all known serovars using a representative antigen set.[2] However, for a clinician needing to take treatment decisions, a broad genus specific diagnosis is adequate as treatment does not differ by genomospecies or serovar.

A key issue in the development of an ELISA test for leptospirosis is to identify a representative antigen, that can help diagnose illness caused by any of the pathogenic circulating leptospira in most regions of the world. Laboratories have struggled with this problem, and have used a variety of whole leptospira antigens in an effort to overcome it. These antigens have been prepared by either sonicating or heating one or more pathogenic or non-pathogenic serovars of leptospira, and impregnating extracted antigens on a solid phase. Results to date suggest that not only different combinations of pathogenic serovars can be used as the source of antigen for such ELISA tests, but so can non-pathogenic *Leptospira biflexa* antigen, which is broadly reactive. As a result antigen derived from *Leptospira biflexa* has been used in commercial ELISA tests. The diagnostic accuracy estimates of commercial and non-commercial ELISA tests are quite similar. Of the two phase III diagnostic studies that evaluated diagnostic accuracy among samples collected during the late acute phase of illness, one study used antigens prepared from pathogenic[33] and other from the non-pathogenic genomospecies [27]. Interestingly the two studies reported similar sensitivity and specificity estimates.

In a number of studies which analyzed samples collected in early acute phase of illness, proportion of positive results was higher with ELISA, as compared to MAT; the median excess positivity being 15.5%. Because many samples are positive by index test (ElISA) and negative by reference standard (MAT), test results of these patients are

analyzed as false positives in diagnostic accuracy analyses. The proportion of true positives is lowered as a result and sensitivity estimates are low. Some authors have suggested that these ELISA positive-MAT negative test result may be due to ELISA performing better than MAT in early acute phase of illness. If this is true, we may be underestimating diagnostic accuracy of ELISA. There is a need for a better reference standard for diagnosis of leptospirosis in early acute phase of illness, and in its absence it will be difficult to assess the diagnostic accuracy of ELISA in early acute phase. On the other hand the high accuracy estimates for ELISA in late acute phase of illness compare with the expected immunological pattern in acute infections, in which IgM antibody levels begin to rise after five to six days, and peak by the second week.

This systematic review has certain strengths: we imposed no language restrictions and used a comprehensive literature search strategy. However there are certain important limitations. First, it is likely that, despite a careful search for published articles, we may have missed some studies not included in electronic databases. Given that most of the included studies had positive results in favor of ELISA, it is likely that some studies with negative results were never published i.e. had a publication bias. Although we have not evaluated the degree of publication bias in our review, it is reasonable to assume that in the event of its occurrence we might have overestimated the diagnostic accuracy of ELISA. Second the included studies were of intermediate and low quality, which could have influenced the overall results in either direction. Due to the small number of diagnostic studies available in the published literature we did not exclude any study based on quality criteria. Most studies, being of "case-control" design (where authors were exploring different ELISA techniques) had lower quality scores simply because these did not represent the right spectrum of patients for a diagnostic study. Also, most studies did not specify if the index test and the reference standard were performed in a blinded and independent manner. Last, there was a wide amount of heterogeneity, even in phase III diagnostic studies, in terms of the prevalence of leptospirosis, the type of antigen used for the ELISA, the number of serovars used in the MAT protocol and the time of collection of serum samples for testing. We tried to minimize heterogeneity by performing a subgroup analysis according to time of sample collection, which is likely to have had the greatest influence on the results. Further subgroup analysis was difficult due to the small number of studies in each group. Despite the residual heterogeneity, we pooled results in each time-dependent subgroup, which may not reflect the true performance of any single ELISA test.

A wide variety of ELISA-based tests have been developed over the past two decades, using different set of antigens. Most of these ELISA based tests have reasonable accuracy, especially in the late acute phase of the illness. However in-house ELISA tests are region and laboratory specific, and none of them have been externally validated. Of the few commercial ELISA tests, only one has been tested in different regions of the world. Large, and high quality diagnostic studies have not been performed using a commercially available ELISA kit. Thus there is a need to bridge the research gap in leptospirosis diagnostics. Future research is needed to determine which are the best available ELISA based tests in geographically diverse regions, so that regional or global recommendation for their use can be made.

Table 1: Sample Pubmed search strategy

Search number	Search string
#1	("Leptospirosis"[Mesh] OR "Weil Disease"[Mesh]) OR ("Leptospira"[Mesh] OR "Leptospira interrogans serovar pomona"[Mesh] OR "Leptospira interrogans serovar icterohaemorrhagiae"[Mesh] OR "Leptospira interrogans serovar hebdomadis"[Mesh] OR "Leptospira Interrogans serovar canicola"[Mesh] OR "Leptospira interrogans serovar australis"[Mesh] OR "Leptospira interrogans"[Mesh])
#2	(((((((("Sensitivity and Specificity"[Mesh] OR "Reproducibility of Results"[Mesh]) OR "Predictive Value of Tests"[Mesh]) OR ("Diagnosis"[Mesh] OR "diagnosis "[Subheading])) OR "Enzyme-Linked Immunosorbent Assay"[Mesh]) OR "Immunoglobulin M"[Mesh]) OR "Agglutination Tests"[Mesh]) OR "Culture Techniques"[Mesh]) OR "Nucleic Acid Amplification Techniques"[Mesh]) OR "Polymerase Chain Reaction"[Mesh]
#3	"Disease Outbreaks"[Mesh] OR "Disease Notification"[Mesh]
#4	(#2 OR #3) AND #1

Table 2: Phase I / II diagnostic studies comparing results of an in-house ELISA as the index test in people with known leptospirosis (MAT positives) as compared to those with non-leptospirosis (MAT negatives)

First Author, year Country (Reference)	*Antigen Source* *Genomospecies serogroup* Extraction method	MAT Number of serovars (Cut-off titer)	No. ELISA positive / No. known leptospirosis (sensitivity; 95% CI)	No. ELISA negative / No. known non-leptospirosis (specificity; 95 % CI)	Quality scoring (points out of 14)
Qualitative ELISA techniques on whole Leptospira antigens to detect all immunoglobulins					
Terpstra, 1980, Aus; NZ; Dutch, Swiss [11]	*L. interrogans Icterohaemorrhagie* Formalin killed	Unsp number (1: 160)	83 / 87 95.4 (88.6-98.7)	201 / 226 88.9 (84.1-92.7)	6
Banfi, 1984, Italy [6]	*L. interrogans Icterohaemorrhagie* Sonicated	Details NA	62 / 62 100 (94.2-100)	536 / 536 100 (99.3-100)	4
Watt. 1988 Philippines [5]	*L. biflexa*	Single serovar *L biflexa* (1:100)	51/55 92.7 (82.4-97.9)	120 /120 100 (96.9-100)	6
Petchclai, 1991 Thailand [8]	*L. interrogans bataviae*	12 serovars (1:100)	60/62 96.7 (88.8-99.6)	40 /40 100 (91.1-100)	5
Ribeiro 1995 Brazil [9]	*L. interrogans icterohaemorrhagie*	20 serovars (1:200)	82/90 91.1 (83.2 -96.0)	152/156 97.4 (93.5-99.2)	5
Da Silva, 1997 Brazil [7]	*L. interrogans canicola, icterohaemorrhagie, hebdomadis, brasiliensis* *L. santarosai cynopterie* Sonicated	22 serovars (1:100)	65/66 98.4 (91.8-99.9)	48/48 100 (92.6-100)	6
Qualitative ELISA techniques on whole Leptospira antigens to detect IgM immunoglobulin					
Tansuphasiri, 2005	*L. interrogans serovars Bratislava, sejore, pyrogenes*	16 seovars (1:100)	95/96 98.9 (94.3-99.9)	232/247 93.9 (90.1-96.5)	7

Thailand [21]	Sonicated				
Quantitative ELISA techniques on whole Leptospira antigens to detect all immunoglobulins					
Mailloux, 1985 France [17]	Whole LA	Details NA	NA[a]	NA[a]	4
Da Silva, 1988 Brazil [15]	*L. biflexa* Sonicated	Unsp number (1: 200)	41/50 82.0 (68.5-91.4)	71/92 77.1 (67.2-85.2)	6
Quantitative ELISA techniques on whole Leptospira antigens to detect IgM immunoglobulins					
Sergeev, 1989 Russia [20]	*L interrogans pomona* Sonicated	Unsp number	231/289 79.9 (74.8-84.3)	242/242 100(97.8-100)	4
Petchclai, 1991 Thailand [8]	*L. interrogans bataviae*	12 serovars (1:100)	62 / 62 100 (94.2-100)	40 /40 100 (91.1-100)	5
Petchclai, 1992 Thailand [19]	*L. interrogans bataviae, pyrogenes, icterohaemorrhagie* Sonicated	12 serovars (1:100)	(bataviae) 101/103 98.0 (93.1-99.7) (pyrogenes) 73/103 70.8 (61.0-79.4) (icterohaemorrhagie) 54/103 52.4 (42.3-62.3)	NA	6
Da Silva, 1992 Brazil [44]	*L. interrogans canicola, icterohaemorrhagie, hebdomadis, brasiliensis L. santarosai cynopterie* Sonicated	Unsp number (1: 200)	26 / 26 100 (86.7-100)	57 /57 100 (93.7-100)	3
Cinco 1992 Italy [13]	*L. interrogans Icterohaemorrhagie (Copenhageni)*	15 serovars (1:100)	78/103 75.7 (66.2-83.6)	117/125 97.5 (92.8-99)	6
Zochowski, 2001	*L. interrogans harjdo* Heat extracted	19 serovars (1:40)	80/83 96.3 (89.7-99.2)	109/117 93.1 (86.9-97.0)	7

UK [22]					
Cespedes, 2002 Peru [12]	*L. interrogans australis, canicola, icterohaemorrhagie L. santarosai cynopterie, grippotyphosa, L. brogpetersenii ballum,* Sonicated	17 serovars (1:100)	39/40 97.5 (86.8-99.9)	79/80 98.7 (93.2-99.9)	7
Nakarin, 2004 Thailand [18]	*L. interrogans bataviae* Sonicated	23 serovars (1:100)	83/85 97.6 (91.7-99.7)	212/220 96.3 (92.9-98.4)	7
Nakarin, 2004 Thailand [18]	*L. interrogans bataviae* Heat extracted	23 serovars (1:100)	70/85 82.3 (72.5-89.7)	176/220 80 (74.0-85.0)	7
Nakarin, 2004 Thailand [18]	*L. interrogans bataviae* Deoxycholate extracted	23 serovars (1:100)	83/85 97.6 (91.7-99.7)	207/220 94.0 (90.1-96.8)	7
Tansuphasiri, 2005 Thailand [21]	*L. interrogans bratislava, sejore, pyrogenes* Sonicated	16 seovars (1:100)	84/96 87.5 (79.1-93.3)	241/247 97.5 (94.7-99.1)	7
Quantitative ELISA techniques on recombinant Leptospira antigens to detect IgG immunoglobulins					
Flannery, 2001 Brazil, US [16]	LipL32 derived from *L. interrogans copenhageni*	Unsp number	75/100 75 (65.3-83.1)	215/236 91.1 (86.7-94.4)	5

ELISA Enzyme linked immunosorbent assay; MAT Microscopic agglutination test; Quality assessment of diagnostic studies done using QUADAS criteria which is a 14 point scale;; Aus Australia; NZ New Zealand; LA Leptospirosis antigen;
a. In this study of all 400 samples, 181 (45.2%) were positive by MAT, and 197 (49.2%) were positive by ELISA

Table 3: Phase I / II diagnostic studies comparing results of a commercial ELISA as the index test in people with known leptospirosis (MAT positives) as compared to those with non-leptospirosis (MAT negatives)

First Author, year Country (Reference)	Commercial IgM ELISA Country of production	MAT Number of serovars (Cut-off titer)	No. ELISA positive / No. known leptospirosis (sensitivity; 95% CI)	No. ELISA negative / No. known non-leptospirosis (specificity; 95 % CI)	Quality scoring (points out of 14)
Winslow 1997 Australia, Fiji [25]	PanBio, Australia	4 serovars (1:50)	41/41 100 (91-100)	217/233 93.1 (89.0-960)	6
Zochowski, 2001 UK [22]	PanBio, Australia	19 serovars (1:40)	75/83 90.3 (81.8-95.7)	110/117 94.0 (88.0-97.5)	7
Vitale, 2004 Italy [24]	PanBio, Australia	19 serovars (1:100)	19/19 100 (82.0-100)	27/29 93.1 (77.2-99.1)	5
McBride, 2007 Brazil [45]	PanBio, Australia	Unsp number	130/146 89.0 (82.8-93.6)	70/80 87.5 (78.2-93.8)	6
Zochowski, 2001 UK [22]	Biolisa, Germany	19 serovars (1:40)	82/82 100 (95.6-100)	97/114 85.0 (77.2-91.0)	7
McBride, 2007 Brazil [45]	EIE Leptospirose, Brazil	Unsp number	124/146 84.9 (78.0-90.3)	76/80 95 (87.6-98.6)	6

ELISA Enzyme linked immunosorbent assay; MAT Microscopic agglutination test; Quality assessment of diagnostic studies done using QUADAS criteria which is a 14 point scale;

* All these ELISA tests use whole leptospirosis antigen

Table 4: Diagnostic accuracy of ELISA tests for leptpspirosis(Cross-sectional studies among leptospirosis suspects)

First Author, year Country (Reference)	ELISA type Antigen source (cut-off value)	Reference standard	Raw data					Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Quality scoring* (points out of 14)
			N	TP	FP	FN	TN					
Any serum sample (not differentaited by time of collection), ELISA vs MAT												
Cursons, 1981 New Zealand [28]	In-house DIG-ELISA *L. interrogans*	MAT	10	8	1	0	1	100 (63-100)	50 (1-99)	89 (67-97)	100 (100)	6
Pappas, 1985 Panama [30]	In-house Dot-ELISA *L. biflexa*	MAT	177	93	14	9	61	91 (84-96)	81 (71-89)	87 (80-91)	87 (79-93)	8
Sencan, 1998 Turkey [31]	In house IgM ELISA *L interrogans*	MAT	24	14	9	1	0	93 (68-100)	0 (0-34)	NA	NA	4
Sharma, 2007 India [32]	In house dot-ELISA *L biflexa*	MAT	495	147	21	16	290	90 (85-94)	93 (90-96)	87 (82-91)	95 (92-97)	4
Early acute samples (collected within 6 days after onset of illness) ELISA vs MAT												
Brandao, 1998 Brazil [26]	In house IgM ELISA *L interrogans*	MAT	108	45	23	1	39	98 (88-100)	63 (50-75)	82 (76-86)	94 (72-99)	7
Cumberland, 1999 Barbados[27]	In-house IgM-ELISA *L. biflexa*	MAT	405	48	15	44	298	52 (42-63)	95 (92-97)	67 (54-77)	92 (80-94)	9
Levett, 2002 Barbados [29]	Commercial ELISA PanBio, Australia	MAT	48	24	9	4	11	86 (67-96)	55 (32-77)	76 (66-84)	70 (46-86)	7
Levett, 2002 Barbados [29]	Commercial ELISA InDx, US	MAT	48	25	9	3	11	89 (72-98)	55 (32-77)	77 (67-84)	76 (50-91)	7
Vanasco, 2007† Argentina [33]	In house IgM ELISA *L interrogans*	MAT	224	79	4	37	104	68 (59-76)	96 (91-99)	91 (88-96)	84 (80-88)	9
Late acute samples (collected between 7 and 14 days after onset of illness) ELISA vs MAT												

Cumberland, 1999 Barbados[27]	In-house IgM-ELISA *L. biflexa*	MAT	296	67	5	8	216	89 (80-95)	98 (95-99)	92 (93-96)	97 (94-99)	9
Vanasco, 2007† Argentina [33]	In house IgM ELISA *L interrogans*	MAT	261	110	1	8	142	93 (87-97)	99 (96-100)	98 (93-99)	96 (92-98)	9
Convalascent samples (collected more than 15 days after onset of illness) ELISA vs MAT												
Cumberland, 1999 Barbados[27]	In-house IgM-ELISA *L. biflexa*	MAT	264	62	12	2	185	97 (89-100)	94 (90-97)	86 (78-91)	99 (96-100)	9
Vanasco, 2007† Argentina [33]	In house IgM ELISA *L interrogans*	MAT	75	26	0	7	42	79 (61-91)	100 (92-100)	100 (94-100)	91 (84-95)	9
All samples, ELISA vs Dark field microscopy (DFM)												
Chandrasheka ran, 2004, India [34]	Commercial ELISA Serion, Germany	DFM	111	41	2	66	2	38 (29-48)	50 (7-93)	NA	NA	7
Sharma, 2008 India [35]	Commercial ELISA Microwell	DFM	276	106	86	30	54	78 (70-85)	39 (30-47)	74 (71-77)	43 (34-53)	9
Early acute samples, ELISA vs Culture												
Camargo 1992 Brazil [36]	In-house IgM ELISA	Cultures	37	4	31	1	1	80 (28-99)	3 (0-16)	NA	NA	3

ELISA Enzyme linked immunosorbent assay; MAT Microscopic agglutination test; DFM Dark field microscopy; N Total number; TP True positives; TN True negatives; FP false positives; FN False negatives; CI Confidence interval. * Quality assessment of diagnostic studies done using QUADAS criteria which is a 14 point scale; †Early acute <10 days, Late acute 10-25 days, convalescent > 25 days after onset of fever.

Table 5: Studies comparing ELISA and MAT positivity in early acute and late acute phases of febrile illness.

First Author, year Country (Reference)	ELISA type Antigen source (cut-off value)	MAT Number of serovars	Early acute phase (0 to 6 days)			Late acute phase (7 to 13 days)		
			ELISA positivity (%)	MAT positivity (%)	Excess ELISA positivity (%)	ELISA positivity (%)	MAT positivity (%)	Excess ELISA positivity (%)
Adler, 1980 New Zealand [37]	In-house IgM-ELISA *L interrogans*	Three	4/8 (50)	1/8 (12.5)	+37.5	8/10 (80)	8/10 (80)	0
Cursons, 1981 New Zealand [28]	In-house DIG-ELISA *L. interrogans*	Two	7/10 (70)	6/10 (60)	+10	4/4 (100)	4/4 (100)	0
Terpstra, 1985 Netherlands [41]	In-house IgM ELISA *L. interrogans*	13	23/33* (69.6)	19/33* (57.7)	+11.9			
Da silva, 1988 Brazil [15]	In-house IgM ELISA *L. biflexa*	Unsp	21/41 (51.2)	0/41 (0)	+51.2	41/41 (100)	41/41 (100)	0
Arimistu, 1994 China, Korea, Italy [38]	In-house IgM ELISA	11 to 19	21/54* (38.8)	20/54* (37.0)	+1.8			
Lin, 1994 China [40]	In house dot-ELISA *L. biflexa*	Unsp	140/228 (61.4)	32/228 (14.0)	+47.4			
Ribeiro, 1995 Brazil [9]	In house dot-ELISA *L. interrogans*	20	24/89† (26.9)	51/89† (57.3)	-30.4			
Winslow, 1997 Australia, Fiji [25]	Commercial IgM ELISA PanBio Australia	4	5/20 (25)	1/20 (5)	+20.0	27/34 (79.4)	20/34 (58.8)	+20.6
Brandao, 1998 Brazil [26]	In house IgM ELISA *L interrogans*	22	36/68 (52.9)	23/68 (33.8)	+19.1	71/86 (82.5)	64/86 (74.4)	+8.1
Cumberland, 1999 Barbados [27]	In-house IgM ELISA *L. biflexa*	22	63/405 (15.5)	32/405 (7.9)	+7.6	72/296 (24.3)	51/296 (17.2)	+7.1
Flannary 2001	rLipL 32 antigen	Unsp	10/28	8/28	+7.2	18/21‡	12/21‡	+28.6

Brazil / US [16]	IgG ELISA		(35.7)	(28.5)		(85.7)	(57.1)	
Croda 2007 Brazil [39]	rLig protein	Unsp	17/21 (80.9)	7/21 (33.3)	+47.6	52/55 (94.5)	35/55 (63.6)	+30.9
McBride, 2007 Brazil [23]	Commercial IgM ELISA EIE, Brazil	12	36/58 (62.0)	11/58 (18.9)	+43.1	33/36 (91.6)	23/36 (63.8)	+27.8
Vanasco, 2007 Argentina [33]	In house IgM ELISA *L interrogans*	13	83/224* (37.0)	116/224* (51.7)	-14.7	111/261 (42.5)	118/261 (45.2)	-2.7

ELISA Enzyme linked immunosorbent assay; MAT Microscopic agglutination test; QUADAS Quality assessment of diagnostic studies; Aus Australia; NZ New Zealand; LA Leptospirosis antigen. DIG Diffusion-in-gel; * Early acute was defined as less than 10 days † Early acute was defined as first sample collected

‡ Late acute phase was defined as 8 to 23 days

Figure 1: Literature search results

Pubmed	Embase	Biosis	Web of Science	Liliacs
3543	1922	1892	804	305

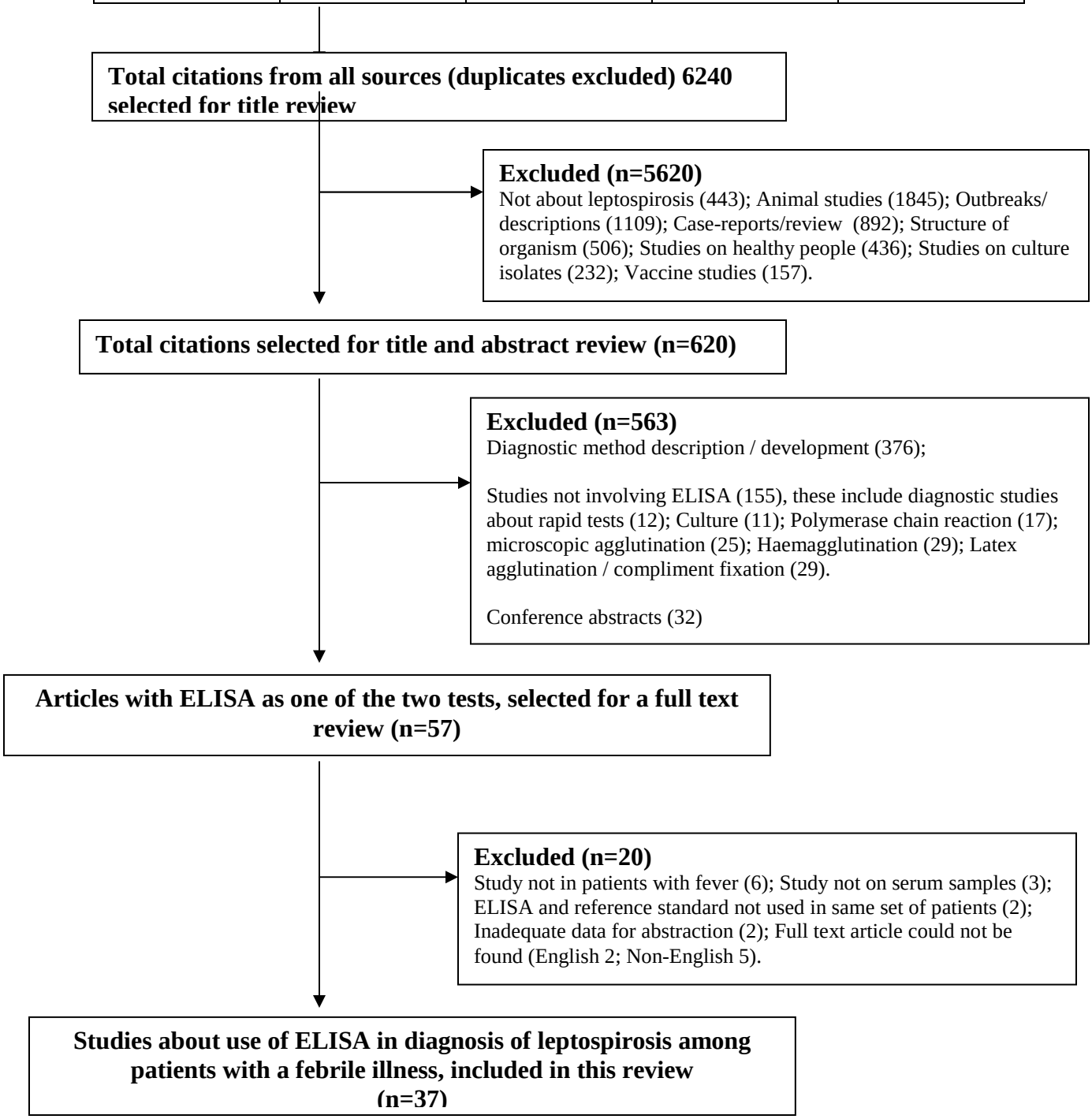
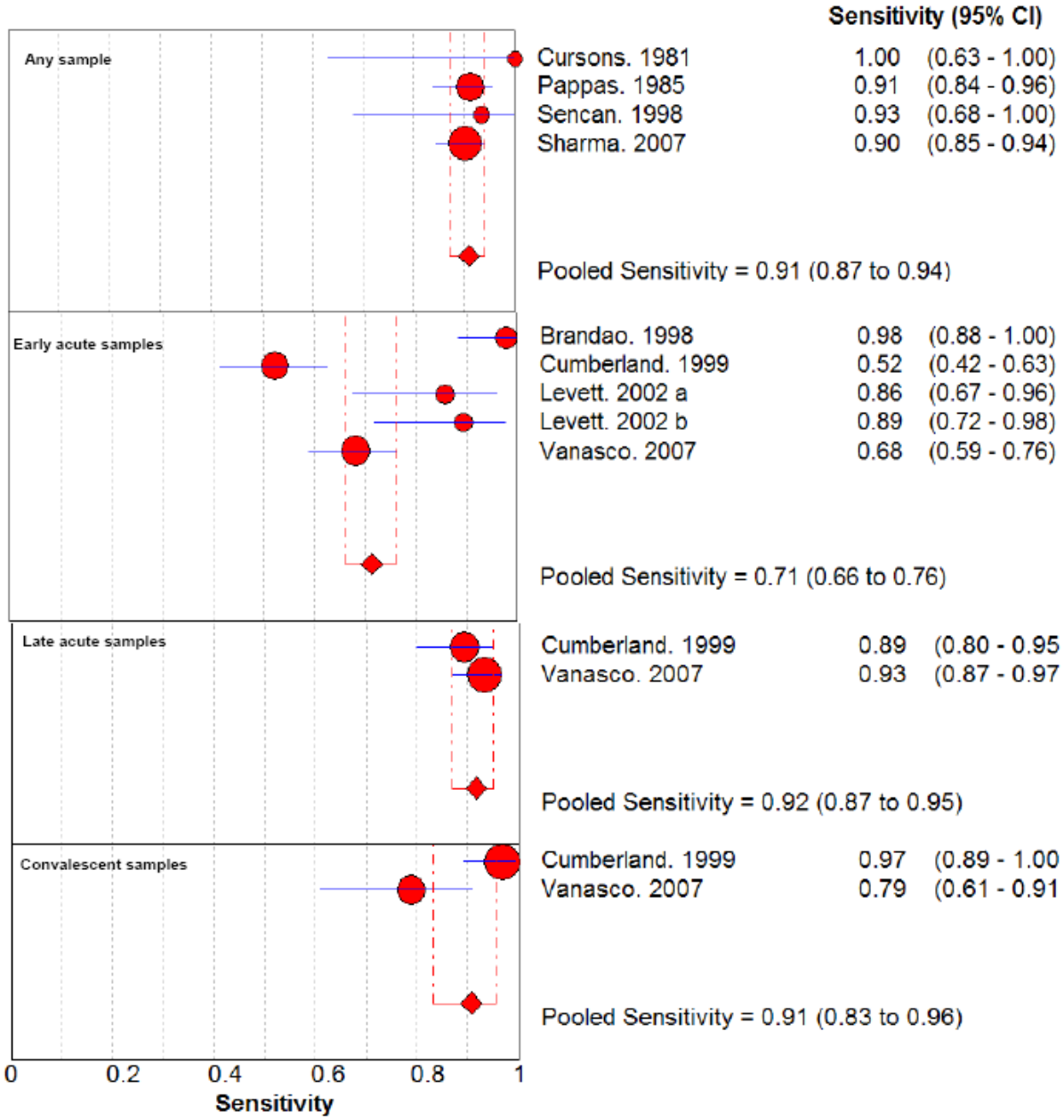


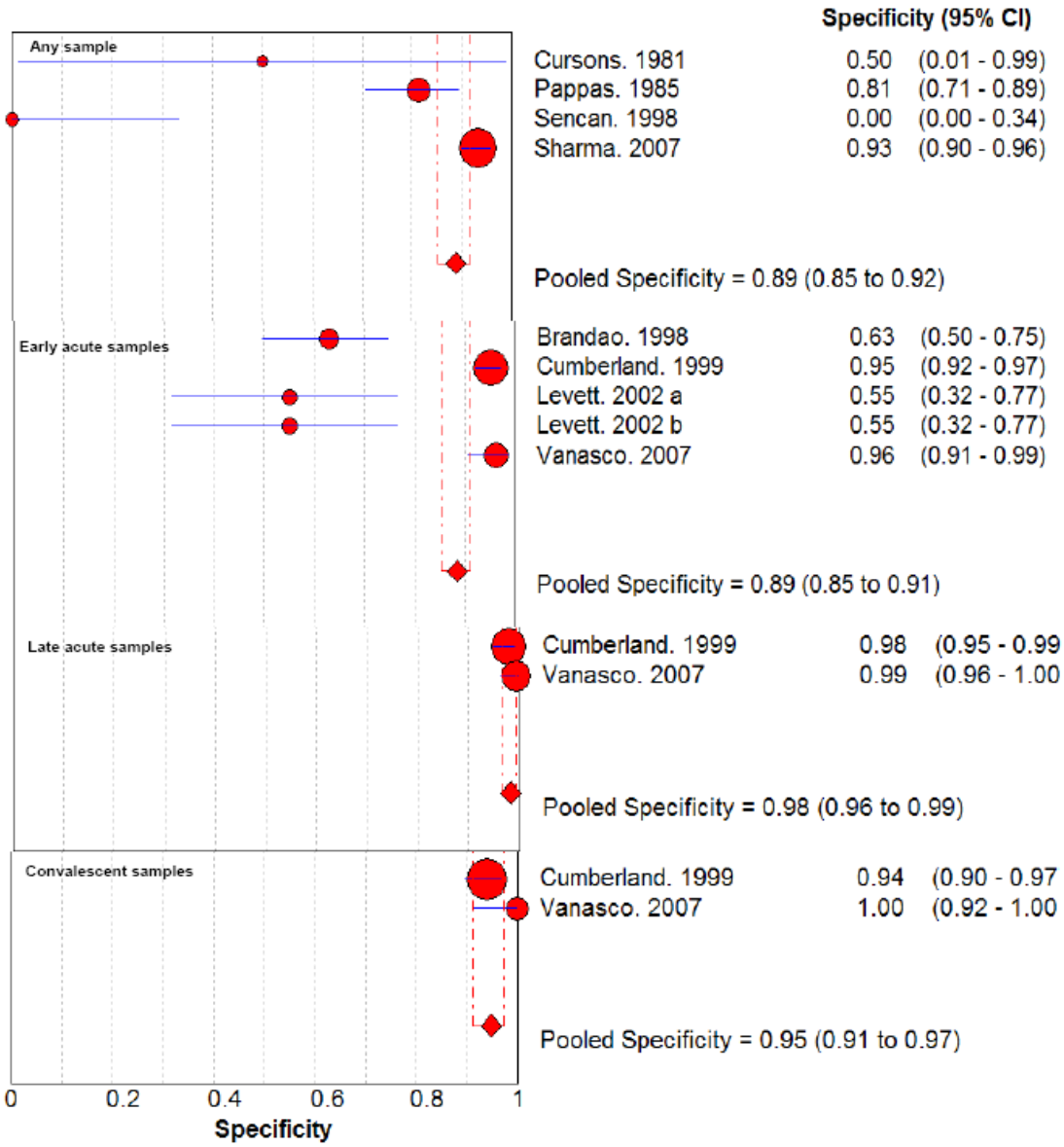
Figure 2: Meta-analysis of ELISA vs MAT in diagnosis of Leptospirosis, stratified by interval between onset of fever and collection of serum samples.

2. A) Test sensitivity



Each circle represents the point estimate for sensitivity for each study. The size of the circle depicts the study size. And the horizontal blue lines represent 95% confidence intervals. The diamond represents the pooled sensitivity and its confidence interval for each sampling category

2.B) Test specificity



Each circle represents the point estimate for specificity for each study. The size of the circle depicts the study size. And the horizontal blue lines represent 95% confidence intervals. The diamond represents the pooled specificity and its confidence interval for each sampling category

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Chapter 5: Etiologic assessment of patients with acute encephalitis syndrome

Abstract

Clinically indistinguishable cases of acute encephalitis syndrome (AES) can be caused by a variety of etiologic agents. Neurotropic viruses including mosquito-borne flaviviruses such as Japanese encephalitis, Dengue, or West-Nile virus cause most AES worldwide, and are considered as the commonest causes of AES in India. Our understanding of the etiology of AES in India is largely based on outbreak investigations or Japanese encephalitis surveillance studies. In the current study, we used multiple viral pathogen testing on cerebrospinal fluid and paired serum samples to determine the etiology of adult AES cases detected through a hospital based AES surveillance. A total of 183 consecutive adults, who met the case definition of AES and were admitted to the hospital between January and October 2007 were included in the study. Of the 183 cases of AES 31 (16.9%) had a confirmed non-viral etiology and the same number (31; 16.9%) had a confirmed viral etiology (17 (9.2%) enterovirus; 8(4.4%) flavivirus; 3 (1.7%) Varicella zoster; 1 (0.5%) herpesvirus; and 2(1.1%) mixed etiology). All viral diagnostic tests were negative in the remaining 121(79.7%) cases. Based on IgM levels in acute and convalescent serum samples, 16 (8.8%) and 48 (26.2%) of them had probable and possible leptospirosis respectively. The remaining 57 (31.1%) cases of AES were classified as being of unknown etiology. Using to community controls as the comparison group, none of the environmental risk factors examined were significantly associated with enteroviral or leptospiral AES. Low socioeconomic status was associated with AES of unknown etiology on univariate analysis. This study is the first description of the etiology of adult-AES in India, and has a potential to provide a framework for future surveillance programs in India.

Introduction

Acute encephalitis syndrome (AES) is a broad syndromic classification encompassing illnesses caused by a variety of etiologic agents. Infection with various neurotropic organisms including viruses, bacteria, mycobacteria, fungi, and protozoa can lead to AES. In addition, certain non-infectious etiologies such as hepatic, renal or hypoxic encephalopathies also have AES like presentation. These etiologies in-turn lead to overlapping pathologic processes such as encephalitis, meningitis and meningo-encephalitis. Because neither the etiologic agents or the pathologic processes are clearly distinguishable from each other, they are classified as AES for the purpose of disease surveillance. Despite a similar clinical presentation, different etiologies have important implications with regards to therapy, prognosis and disease prevention. The high mortality associated with AES, and the inability to treat most viral AES with specific drugs makes it important to develop preventive measures against specific etiologic agents.

The diagnosis of specific etiologies of AES is difficult. First a large number of neurotropic viral agents are known to cause AES. Some of these viruses (such as herpes viruses, enteroviruses, paramyxoviruses and rhabdovirus) lead to sporadic viral encephalitis. Others (such as alphaviruses, bunyaviruses, and flaviviruses) are frequently associated with epidemics of encephalitis.[1-3] Second, there is a wide geographic variation in the incidence of AES caused by these agents. Thus diagnostic tests needed may be

region specific. Third, the technology to detect each of these agents is expensive, and is often not available outside a select group of reference laboratories. Specific diagnostic tests (such as polymerase chain reaction) for many neurotropic viruses are still under development so it is often necessary to rely on the imperfect diagnostic tools. The use of molecular methods to diagnose the etiology of viral encephalitis is costly, and in a developing country such as India, it may not be possible to incorporate such techniques into routine diagnostic testing procedures. However, periodic epidemiological investigations are essential to determine the spectrum of viruses that cause both sporadic and epidemic encephalitis

India has witnessed separated seasonal epidemics of viral encephalitis in the past five years.[4-7] All of these epidemics were initially attributed to Japanese encephalitis virus, but later some of them were determined to be due to new agents such as Nipah virus[8] Chandipura virus.[9, 10] These novel discoveries in the recent years shows that our knowledge about etiologies of AES is still limited. This limitation persists despite advances made in virology in past decades. In a developing country such as India where health care resources are already over burdened, determination of etiological spectrum is important not only to avoid costly empirical treatments, but also to design effective prevention strategies. The purpose of this study is to determine the spectrum of etiological agents causing viral encephalitis in Central India, so that effective planning and implementation of preventive measures can be undertaken.

Methods

Setting, and inclusion and exclusion criteria

This study was conducted at the Mahatma Gandhi Institute of Medical Sciences (MGIMS), Sevagram, India, which is a rural medical school in central India. The methodologies for selecting cases and controls were detailed in Chapter 3. Briefly, all consecutive adult patients with AES admitted to a single hospital were identified; to be included patients had to have new onset of fever and altered behavior for less than five days duration. Patients with malaria and focal infectious processes identified as the source of their fever (pneumonia, acute gastroenteritis, soft-tissue infection with sepsis etc) were excluded. Informed consent was obtained from all patients or their surrogate at the time of enrolment.

During their hospital stay, all patients at MGIMS underwent lumbar puncture and cerebrospinal fluid (CSF) based examinations to determine etiology of AES. Patients were excluded from the study if the CSF based tests suggested the presence of bacterial meningitis (i.e. neutrophils in CSF, CSF/blood sugar ratio 0.25 or less, or positive CSF cultures for a known pathogenic organism); tubercular meningitis (i.e. a positive CSF mycobacterial cultures, or presence of pulmonary tuberculosis); or cryptococcal meningitis (i.e. presence of cryptococcal antigen in CSF, a test performed in HIV-positive individuals only). Patients were also excluded if blood chemistry results or the clinical presentation suggested metabolic derangements (such as hyponatermia, uremia, hypoglycemia, or hepatic dysfunction that could lead to metabolic encephalopathy), or brain imaging suggested an intracranial lesion such as a space occupying lesion or intracranial hemorrhage. All included patients satisfied the CDC criteria for neuroinvasive encephalitis,[11] which are as follows:

> Presence of fever and at least one of the following, (as documented by a physician and in the absence of a more likely clinical explanation)

i. Acutely altered mental status (e.g.disorientation, obtundation, stupor, or coma), or

ii. Other acute signs of central or peripheral neurologic dysfunction (e.g., paresis or paralysis, nerve palsies, sensory deficits, abnormal reflexes, generalized convulsions, or abnormal movements), or

iii. Pleocytosis (increased white blood cell concentration in cerebrospinal fluid [CSF]) associated with illness clinically compatible with meningitis (e.g., headache or stiff neck).

We approached a healthy person from community (i.e. from the same village as the case, but whose house was farthest from the house of the case) to serve as a control for every case. An eligible control was within five years of age as the case and had no history of AES in the past or a febrile illness within past one month. Each control was sampled at the time of the 30-day follow up visit to the case household.

Study variables

We collected demographic (i.e. age, gender, socioeconomic score)and clinical information (i.e. duration of fever, headache, altered behavior, and hospital stay, Glasgow coma scale (on admission), presence of seizures, neck stiffness, hypotension, and need for assisted ventilation, HIV positivity, mortality at day 30, and results from complete blood count and CSF examination) for each included case. In addition, we collected information about environmental risk factors and living conditions (i.e. socioeconomic score , number of individuals per room in household, and number of children below 12 years in house) from all AES cases and community controls. The environmental risk factors included factors that might increase the risk of vector borne infections (i.e.potential vector breeding sites near the households, and personal protection measures used against mosquito bite), waterborne (i.e. type of water supply, a pond or stream near the household, and a sewage drain in vicinity of the house), or zoonotic transmission (i.e. presence of cattle, swine, poultry or canines within house). These variables were previously described in greater detail in chapter 3.

Collection of biological samples

At the time of the initial lumbar puncture (after obtaining informed consent, but before applying all exclusions), we obtained 3mL of additional CSF sample. This sample was divided into three parts of 1.0mL each and was stored at -70°C untill further testing. In addition a serum sample was obtained at the same time as the initial CSF fluid collection and a follow up serum at the time of the 30-day follow up visit. A single serum sample was collected from all controls at the time of enrolment.

Research investigations

A battery of investigations was performed to determine the etiology of AES in *a priori* defined order (CSF RT-PCR, followed by CSF IgM ELISA, followed by serum IgM ELISA) in which test results were to be interpreted. If the CSF RT-PCR was positive for an etiologic agent, it was considered as diagnostic. CSF IgM ELISA results were interpreted for PCR negative cases, and serum IgM ELISA tests were considered as diagnostic only if all CSF-based test results were negative. Patients with AES who had

two or more positive tests using the same testing technique and sample (e.g CSF IgM ELISA positive for dengue as well as for Japanese encephalitis) were classified as having a mixed infection. Patients in whom all test results were negative were classified as having AES of unknown etiology. We used the CDC criteria[11] to classify a case of encephalitis as either a confirmed or a probable case caused by a given etiologic agent based on the following laboratory criteria:

a. Confirmed case
    i. Four-fold or greater change in virus-specific serum antibody titer, or
    ii. Isolation of virus from or demonstration of specific viral antigen or genomic sequences in tissue, blood, CSF, or other body fluid, or
    iii. Virus-specific immunoglobulin M (IgM) antibodies demonstrated in CSF by antibody-capture enzyme immunoassay (EIA), or
    iv. Virus-specific IgM antibodies demonstrated in serum by antibody-capture EIA and confirmed by demonstration of virus-specific serum immunoglobulin G (IgG) antibodies in the same or a later specimen by another serologic assay (e.g., neutralization or hemagglutination inhibition)
b. Probable case
    i. Stable (less than or equal to a two-fold change) but elevated titer of virus-specific serum antibodies, or
    ii. Virus-specific serum IgM antibodies detected by antibody-capture EIA but with no available results of a confirmatory test for virus-specific serum IgG antibodies in the same or a later specimen.

Because a large number of viruses can cause AES, common viral pathogens were classified as first and second line agents based on the known epidemiology of possible agents, in order to optimize the use of limited resources. Japanese encephalitis virus, dengue virus, West Nile virus, enteroviruses, herpesviruses, and Varicella Zoster virus were considered as first line agents. measles and mumps viruses were classified as second line agents.

One aliquot of the CSF sample (volume 1.0mL) was transported to the Defense Research and Development Organization Laboratory at Gwalior, India. Qiagen nucleic acid extraction kits were used to extract DNA and RNA from all samples, using standard techniques. The extracted nucleic acids were tested for first line agents by polymerase chain reaction (PCR). RT-PCR for enteroviruses and herpesviruses using commercial kits (Artus LC-PCR, Germany) was performed at the Virology laboratory at Bhopal Memorial Hospital, Bhopal, India. Enteroviruses comprise of 70 known serotypes, 68 of which infect humans. We used the Artus enterovirus LC RT PCR kit, which amplifies the 114bp region of the enterovirus genome. The analytic sensitivity of this kit is 3.2 copies per microliter. This test is genome specific and does not help identify individual enterovirus serotype. Herpesviruses were detected using the Artus HSV ½ LC PCR kit. This test uses amplification of 148bp region of the Herpes simplex virus genome for detection. The analytic sensitivity of this kit is 1 copy per microliter of CSF. PCR for flaviviruses (Consensus primers YF1, YF3 expected product size 390bp) was performed

at Defense Research and Development Organization laboratory at Gwalior, India. Extracted nucleic acids from a subset of all samples (in patients who had died) were also tested for Chandipura virus (CHPG F2, and CHPG R2, expected product size 200bp), and Nipah viruses (NF1 and NF22 primers, expected product size 1596bp) by conventional PCR.

A second aliquot of the CSF sample (volume 1.0mL) was used to test for IgM antibodies against Japanese encephalitis virus, dengue virus, West Nile virus, and Varicella Zoster virus using commercial IgM capture ELISA kits manufactured by PanBio, Brisbane, Australia. The second line tests for measles and mumps were performed with an IgM ELISA on CSF samples using commercial kits (Serion, Germany). All IgM capture ELISA tests were done in 1:10 CSF dilution and remaining steps as per the manufacturer's guidelines. A third aliquot of the CSF sample (volume 1.0mL) was transported to the DeRisi Laboratory at UCSF, San Francisco for detection of novel viral agents.

During the course of the study, treating physicians ordered anti-leptospirosis IgM antibody testing in some patients with AES, and found the test to be positive. As a result we then tested all of the stored acute and convalescent serum samples by IgM capture ELISA for anti-leptospirosis antibodies. Patients who did not have a confirmed viral diagnosis based on CDC criteria for neuroinvasive encephalitis were classified as having a probable or a possible leptospirosis. Patients who had a two-fold change in serum levels and high IgM levels (above commercially defined cut-off of 11 IU/ml) in either acute or convalescent samples were classified as having probable leptospirosis. Patients with high IgM levels in either the acute or convalescent samples but without a two-fold change, were in turn classified as having possible leptospirosis. The remaining patients were classified as being negative for leptospirosis.

A serum sample was collected from all controls, to look for the evidence of subclinical infection with the causative organisms identified among the cases. The controls with sub-clinical infection were excluded from the case-control analysis for that organism. All control samples were tested for IgG antibodies against enteroviruses (Serion, Germany) and leptospirosis (Serion, Germany) and IgM antobodies against leptospirosis (IgM capture ELISA, PanBio Ltd, Brisbane, Australia). We used this pool of controls and performed the case-control analysis by comparing the risk factor frequency between cases with a specific etiology and the sample of healthy controls, who were seronegative for the specific agent.

Statistical analysis

We performed a descriptive analysis of the demographic and clinical variables and compared their distribution among various etiologic subgroups of AES. These subgroups were: confirmed non-viral, confirmed or probable viral etiology, AES of unknown etiology. We used the chi square test for categorical variables and student's t-test for discrete variables for these comparisons and considered a p value of less than 0.05 as significant. We created three large etiologic subgroups of AES cases: those with an enteroviral disease; those with probable leptospirosis; and those with AES of unknown etiology. Our aim was to determine likely transmission characteristics of AES cases of an unknown etiology. We compared various environmental risk factors (clustered as those related to vector borne, water borne, zoonotic or poor-living conditions as risk groups) between AES cases of unknown etiology, and apparently healthy controls who were

seronegative for leptpspirosis (IgG and IgM antibodies) and enteroviruses (IgG antibodies; n=57 cases, and 57 seronegative controls). We also compared environmental risk factors between enteroviral AES cases and entrovirus IgG seronegative controls (n=17 cases, 68 controls) and between probable leptospiral AES cases and leptospirosis IgG and IgM seronegative controls (n=16 cases and 48 controls).

We calculated odds ratios and their 95% confidence intervals while comparing the distributions of risk-factors between cases and controls. We first performed a univariate analysis of all primary and selected derived variables (such as exposure to three or more objects in the vicinity of household which promoted vector breeding and non-use of personal protection against mosquitoes as a combined vector transmission risk factor; presence of a sewage drain in front of the house and a pond or stream within 200 meters, as a combined water borne transmission risk factor). Socioeconomic status (SES) was collected as a continuous measure, based on type of house, land ownership, object ownership, education and occupation (See Box 1A, B in chapter 3 for details). We converted SES score into tertiles, and compared the distribution of cases and controls in the lowest vs the highest tertile of this score. We defined overcrowding in a household as a the person density in house was more than three or more. Similarly the presence of three or more children in a household were defined as an indicator for overcrowding. Together, low SES tertile, and overcrowding were used to denote poor living conditions.

We performed multivariate logistic regression to determine independent predictors of the risk of AES. We defined transmission models *a priori*, and used variables within a transmission model in the logistic regression analysis. For example, all variables that signify increased risk of vector borne transmission, age, and socioeconomic score were included in the full vector-transmission model. We performed a step wise reduction (using goodness-of-fit test at each reduction step, with a p value of less than 0.05) to identify the most informative variable(s), forcing age and socioeconomic score in each model. We forced age in all models to account for residual confounding, because although cases and controls were age matched, this matching could have been disturbed when seropositive controls were excluded from the analysis. Socio-economic status was included in all models as it is a likely confounder between most exposures and risk of AES. Similar transmission models were evaluated for all case-control analyses. To determine if low socio-economic status was independently associated with risk of AES, we tested a poor living condition model, in which we included the best parameters indentified from each transmission-model and age as variables. Thus we estimated the risk of belonging to lowest tertile of socioeconomic status score as compared to highest tertile, adjusted for age, and factors influencing vector borne, zoonotic and water-borne transmission. The quantum of risk in multivariate analysis was expressed as an adjusted odds-ratio and its 95% confidence intervals. We the considered model to be significant, when the confidence interval of the adjusted odds-ratio did not include one.

Results

A total of 183 adult patients with AES were included in the study between January and October 2007, and 31(16.9%) of them had a confirmed non-viral etiology; the remaining 152 (83.1%) were viral encephalitis suspects. Cases with confirmed non-viral AES had a longer duration of fever and headache; had a higher proportion of individuals with neck stiffness; and had lower CSF glucose levels and higher CSF protein

concentration, and were more likely to be HIV positive as compared to those who were classified as viral encephalitis suspects (Table 1).

We could confirm a viral etiology in 31 (20.3%) of the 152 viral encephalitis suspects and all the viral diagnostic tests were negative in remaining 121 (79.7%). The demographic and clinical characteristics of those with a confirmed viral etiology were similar to those in whom a viral etiology was not identified, except for the presence of neck-stiffness, which was more frequent in those with a confirmed viral etiology. AES cases with a confirmed viral etiology also had a higher mean CSF cell count, a finding which was not statistically significant. (Table 2)

Of the 31 patients with a confirmed viral etiology, 17 (54.8%) were positive by enteroviral RT-PCR, and one (3.2%) by herpesvirus RT-PCR. The remaining 13 patients had anti-viral IgM antibodies in their CSF samples of whom eight (25.8%) had flaviviral encephalitis (four JEV, three Dengue, and one was positive for both), three (9.6%) had varicella-zoster encephalitis, and another two (6.4%) had a mixed CSF serology (positive for both varicella and either Japanese encephalitis and/or dengue). None of the patients had positive CSF serology for West-Nile virus. None of the CSF samples tested for Cahnipura virus and Nipah virus were positive (Figure 1).

We obtained serum anti-leptospira IgM levels in 121 AES cases, and found 16 (13.2%) of them patients to have probable and another 48 (39.6%) patients with possible leptospirosis. (Figure 1, Table 3). We classified remaining 57 (47.1%) patients as being of an unknown etiology. Patients with probable and possible leptospiral AES had significantly lower mortality (0% and 27.6% respectively) as compared to those with AES of an unknown etiology (49.1%). None of the patients with probable leptospirosis required assisted ventilation, as compared to 29.8% patients in unknown AES subgroup. (Table 3) There were no significant differences in the clinical and demographic characteristics between those with enteroviral or flaviviral AES, and those in whom etiology remained unknown. (Table 4)

We performed anti-leptospira IgM testing in 100 serum samples obtained from healthy controls as well, and found 25 of them to be above the commercially defined cut-off. A total of 15 controls had positive IgG antibodies against leptopsira. (Table 5) These findings make it difficult to interpret the results of a single positive IgM test, as in endemic areas anti-leptospira antibodies are known to persist for long periods of time. Because the probable leptospirosis subgroup has a more strict definition, we compared environmental risk factors of this subgroup with seronegative community controls. A large proportion of controls were seropositive for IgG antibodies against enteroviruses, suggesting past manifest or sub-clinical infections in the population. A total of 78 controls were seronegative for enteroviruses, and 59 each for leptospiroris and both enteroviruses and leptospirosis respectively. From this pool of seronegative controls we randomly selected 68, 48, and 57 controls and compared their environmental characteristics with 17, 16, and 57 patients with enteroviral, probable leptospiral and AES of unknown etiology respectively. (Table 5)

As compared to community controls, none of the environmental risk factors examined were significantly associated with enteroviral or leptospiral AES cases. On univariate analysis cases with AES of unknown etiology had a 2.6 times higher risk of being in the lowest tertile of socioeconomic score as compared to seronegative community controls (Table 6). This risk was not statistically significant in a multivariate

model, that included variables associated with increased vector-borne, water borne or zoonotic transmission. In multivariate disease-transmission models, enteroviral AES cases had a significantly higher risk of being in the lowest tertile of socioeconomic score. None of the parameters in other models conferred a significantly increased risk. (Table 7)

Discussion

In this study of consecutive adult patients with AES who presented to a single hospital in rural central India 17% patients had a confirmed non-viral etiology, and in a similar proportion a confirmed viral etiology could be identified. Enteroviruses were the commonest etiology of viral AES followed by flaviviruses (9.2% and 4.3% of all AES cases respectively). An interesting finding was that a large proportion of cases were seropositive for leptospirosis, some of whom may have had primary neuro-leptospirosis. Key strengths of this study include its sampling all consecutive patients; the obtaining of paired sera and CSF samples from all patients, and use of advanced viral diagnostic tests for a battery of neurotropic agents. This study, which combines expertise from epidemiology and virology is the first description of the etiology of adult-AES cases from India in absence of an outbreak. This study has the potential to serve as a model for future AES surveillance in India. This study had a number of interesting and new findings. We have discussed these findings and their limitations in the following sub-sections.

Clinical and demographic features

The patients with AES and a confirmed non-viral etiology were expected to have a different clinical profile. All confirmed bacterial, tubercular, and cryptococcal meningitis cases were in this group, and these conditions which are more common in immuno-compromised individuals. These conditions are also known to produce higher CSF proteins, have lower CSF glucose levels, and meningeal signs. Interestingly we found that pre-hospital symptom duration of patients with a confirmed non-viral etiology was longer as compared to patients who were viral encephalitis suspects. Our data show that the AES patients who were suspected to have viral encephalitis had a more catastrophic course. They on an average had alteration in conscious after a brief duration of fever. In contrast those with a confirmed non-viral etiology had a longer febrile course before they developed altered consciousness. The duration of altered behavior before presenting to the hospital was similar in both groups, it suggests a similar health seeking pattern when this symptom is present.

Individuals with a confirmed viral etiology had more severe meningeal inflammation as evidenced by more cells in the CSF and a higher proportion of those with neck stiffness. This finding has also has been previously reported. Patients with higher viral loads are known to have greater meningeal inflammation, and hence more cells in their CSF samples. Such patients are more likely to have positive results on viral diagnostic tests. Other clinical and demographic features were similar in patients with encephalitis of confirmed viral etiology and those of unknown etiology, which suggests a a viral etiology in even those patients in whom viral diagnostic tests were negative.

Enteroviruses as a key viral etiology of AES

The proportions of AES patients with confirmed viral, enteroviral and unknown etiology in this study are similar to what has been reported elsewhere. [12-13] Yield of viral

diagnostic tests in patients with aseptic meningitis and encephalitis has invariably been low. Despite using a wide array of diagnostic tests, at least a third of all cases remain of unknown etiology in most studies.[12] In a recent large study from the state of California (the California Encephalitis Project), a total of 1571 patients with encephalitis were evaluated over a seven year period, and an infectious etiology could be identified in only 15% of them; 73 cases (4.6%) had enteroviral encephalitis.[13] Another study from California reported that ~ 10% of all samples received from AES cases as a result of West Nile Virus surveillance were positive for enteroviruses on PCR.[14] In another study from Finland, which included 144 consecutive patients over a four year period, about 34% of all cases remained undiagnosed, despite extensive use of PCR based methods.[12] In this study 26% of all patients had enteroviral disease and 17% had herpesvirus as an etiology.[12] Recently two other studies from India have reported enteroviruses as a predominant causative agent in AES cases. In an outbreak investigation of 306 patients from northern India, evidence of enteroviral infection was seen in 66 (21%) of all patients.[15] In a hospital-based study of children from Delhi in north India, 20 (13%) of all 151 AES cases were reported to have an evidence of enteroviral infection.[2] Enteroviruses are a diverse group of about 70 viruses and are responsible for many respiratory and gastrointestinal illnesses. Enteroviral encephalitis is however a rare complication of enteroviral infection.[13] In our study all cases diagnosed as having enterovirus had viral RNA was detected by RT-PCR in CSF samples. This makes diagnosis of enteroviral encephalitis in our study patients more secure than in studies in which the virus was shown to be present in stool samples or in respiratory secretions.

The reported proportion encephalitis cases we have attributed to enteroviral encephalitis may be an underestimate for two principal reasons. First, enterovirus is present in the CSF only briefly and in later stages of encephalitis translocates to brain parenchyma.[13] Given this fact, we may have failed to detect many enteroviral encephalitis cases, and some patients with encephalitis of unknown etiology may have had enteroviral encephalitis. Interestingly, the clinical features and proportion of cases who died was similar in those with enteroviral encephalitis, and those in whom no etiology could be found. Second our study was limited to adults, and enteroviral encephalitis (particularly infection with EV-71 subtype) has largely been reported in neonates and children.[2, 15] Many published reports of enteroviral encephalitis descriptions come from outbreaks that have primarily affected neonates and children.[16, 17] Studies which have included patients of all age-groups (such as the California Encephalitis Project) less than half of all confirmed cases have been in adult age-group.[13] In general the etiologic agents that are widely prevalent in the community will produce many apparent and in-apparent infections, conferring some element of immunity to adults. In such circumstances neonates and children have a higher risk of being affected, especially in outbreaks. It is likely that the burden of enteroviral encephalitis is higher in pediatric population, and our study in adults likely underestimates the community burden of enteroviral encephalitis.

Flaviviral encephalitis

Despite the fact that the region in which this study was conducted is situated in a Japanese encephalitis belt, only eight of our AES cases were due to flavivirus encephalitis. We used two diagnostic tools (CSF PCR (using universal primer) and CSF IgM serology) to look for the presence of three flaviviruses (JEV, dengue virus, and

West-Nile virus). Most AES outbreaks in India have been attributed to JEV, and given the high mortality, the need for assisted ventilation in many patients, seasonal distribution of AES cases in our population, and the infrequent use of personal protection measures against mosquito-bite in the community, we had expected a higher proportion of our cases to be due to flavivirus. Although the finding that flavivirus encephalitis is less common than enterovirus encephalitis is surprising, similar results have also been reported in a recent study[15] conducted in the same area (Eastern Uttar Pradesh, India) where JEV epidemics used to occur annually. [18-21] In that study the shift in etiologic agent was attributed to the population now being largely immune to JEV as a result of recent JEV vaccination. The same logic, however cannot be applied to our findings as no such immunization campaigns have been launched in this area till date. While it is likely that flaviviral encephalitis is a more common cause of outbreaks in this area, other endemic neuroinvasive viruses (such as enteroviruses, herpesvirus, or varicella) may account for more sporadic cases.

Positive leptospirosis serology in AES cases

Another interesting finding in our study was the high proportion of cases of AES in which we found that IgM seropositivity against leptospirosis.One-third of all seropositive patients had a two-fold or a greater change in IgM antibody levels between their acute and convalescent serum samples (probable-leptospirosis). The remaining two-thirds were seropositive without a demonstrable two-fold change (possible-leptospirosis). None of the patients who died could have been classified as probable leptospirosis, because paired sera were not available for them. Still, mortality in possible-leptospirosis group was significantly lower (27%), as compared to mortality among those with a confirmed viral etiology (45%) or those with encephalitis of unknown etiology (49%). Those with probable or possible leptospirosis also had a lower need for assisted ventilation, and had higher Glasgow coma scores on admission. These features suggest that patients in these groups differed in important respects. Lower mortality in the possible / probable leptospirosis group could either be due to lower virulence of the organism, or due to leptospira being highly susceptible to commonly prescribed antibiotics. Most patients with AES in our hospital received empiric antibiotics (usually beta-lactams) as documented in Chapter 1 of this dissertation, and this treatment may have led to a lower mortality among AES cases with serological evidence of leptospirosis.

However caution is appropriate when interpreting these findings, as none of the patients in this study had clinical features typically associated with leptospirosis. The patients did not have jaundice, or renal failure; in fact presence of these features was an exclusion criteria and intended to exclude any case potentially attributable to encephalopathy. In addition none of these patients had acute respiratory distress syndrome, which has been reported in severe cases of leptospirosis and hantavirus infection. Although aseptic meningitis is known to occur with leptospirosis, isolated neurological involvement as a presentation of leptospirosis has rarely been reported as "primary neuro-leptospirosis".[22] Previous reports suggest that primary neuro-leptospirosis may present as aseptic meningitis, encephalitis, intracranial bleeding, or cerebellitis.[22] Because leptospira are sensitive to commonly used antibiotics such as penicillin and other beta-lactams, primary neuroleptospirosis has a low mortality. Primary neuro-leptospirosis has been reported as either isolated case-reports[23, 24] or a case-series of 31 cases who

presented to a single neurological center over five year period.[25] In another study from Brazil, of a little over 100 CSF samples from patients with aseptic meningitis, 38% were positive for leptospirosis by PCR and 8% were positive by IgM-ELISA.[26] The authors of this study were cautious in interpretation of their findings, but had suggested that neuro-leptospirosis should be considered as a diagnosis when no other etiology is evident.[26] In a narrative review of the condition, it was argued that neuro-leptospirosis is often overlooked as a possible diagnosis because bacterial infections are not considered to be a cause of aseptic meningitis.[27] Unlike bacterial meningitis, leptospiral meningitis is described as having lymphocytic pleocytosis, a mildly elevated CSF protein level and normal sugar level in CSF – features which are usually seen with viral meningitis and encephalitis. Therefore it seems likely that some AES cases are due to leptospirosis, and that this diagnosis is frequently not considered.

We used a commercial serum IgM capture ELISA to detect anti-leptospira antibodies in our study patients. There are wide geographic and laboratory variations in the results obtained with available anti-leptospira ELISA-based tests. The traditional test used to diagnose leptospirosis (microscopic agglutination test or MAT) is difficult to perform and not available outside a few reference laboratories worldwide. Commercially available ELISA based tests can be performed easily. We performed a systemic review and a meta-analysis of the available ELISA based tests for the diagnosis of leptospirosis to determine if these tests would be useful in our setting. The results of this review are presented in Chapter 4. Briefly, we found that compared to the MAT as a reference standard, ELISA-based tests aimed for detection of anti-leptospira IgM antibodies have a high sensitivity and specificity. When serum samples were collected in the second week of illness (i.e. late acute phase), the pooled sensitivity and specificity estimates were 92% (95% CI 87 to 95%) and 98% (95% CI 96 to 99%) percent respectively. Although most phase III diagnostic studies had used in-house ELISA based assay, the commercial ELISA most frequently used in phase I/ II studies is manufactured by PanBio, Australia. We used the same IgM capture ELISA in our study, and believe the test to be reasonably sensitive. However we found a relatively high background prevalence of IgM positivity in the population giving rise to , making it difficult to interpret the positive test results in our “possible leptospirosis” group. As it is not practical to classify AES cases as neuroleptospirosis based on a single positive test result, we used a more stringent definition to classify individuals as having probable-leptospirosis.

Approaches to identify etiology of unknown AES

About one-third of all AES cases in our study were of undetermined etiology. To determine the likely etiology in these “unknown AES” cases, we used three principle strategies. First we compared the distribution of the clinical and demographic features of these patients with those patients with a known viral etiology. Second, we compared these unknown AES cases with cases classified as possible and probable leptospirosis There two strategies suggested, that in terms of survival characteristics, AES cases were more similar to those in whom a viral etiology could be confirmed. As a fourth strategy we conducted a case-control study as another strategy to identify potential transmission characteristics (or risk factors) for unknown AES disease. Low SES is a likely confounder in each transmission model, as an alternate transmission pathway can operate between low SES and AES. Thus, we adjusted for SES in each transmission model, but

none of the transmission characteristic was a significant risk factor for AES of unknown etiology.

Could low socio-economic status in itself lead to AES, thru one or more pathways? The relationship between SES and risk of viral infectious is likely to be complex (See chapter 3). And evaluation of low SES itself as an independent risk for causation of AES is equally complex. It is likely that one or more variables we adjusted for in multivariate analysis (e.g. lack of personal protection against mosquito-bite, non-piped water supply, or exposure to cattle /swine / poultry) were intermediaries between low SES as an exposure and AES as an outcome. One plausible explanation for the result of our study is that low SES is a likely confounder to the operating causal pathway.

The results of the case-control study demonstrate that most exposures we studied were equally common in cases and controls. About one-quarter of controls had serological evidence of prior infection with enteroviruses and leptospirosis. In absence of a contrast between cases and controls, either in exposures or the outcomes it is difficult to determine specific risk factors. Moreover, the exposures we examined in our study were broad, self-reported environmental variables which are susceptible to measurement and misclassification bias. A relatively small sample size, potential selection bias (controls were from same village as the case), and information bias (interviewers being un-blinded to case-control status) are other likely limitations of our study. Future studies will benefit from using more focused and objective measures of exposure, specifc to feco-oral or water-borne transmission.

The current study has generated several novel hypotheses. First, most AES is likely to be due to enteroviruses (water borne), rather than due to flaviviruses (vector borne). Second, individuals of low-SES are at higher risk for AES. Last, some cases of AES in adults may be caused by easily treatable leptospira. Subsequent studies need to focus on subtype of enterovirus involved; confirmation of occurrence of neuro-leptospirosis; and on how AES can be prevented, especially in those of a low-SES. We have demonstrated that it is possible to establish a hospital-based surveillance for AES, and have established the etiology of many cases. We believe that this information will be valuable in planning future research designed to test the various hypotheses this study has generated.

Table 1: Characteristics of patients defined as viral encephalitis suspects and those with conformed non-viral acute encephalitis syndrome (n=183)

Variable	AES viral encephalitis suspect N=152	AES with confirmed non-viral etiology N=31	P value
Age (years)	40.2 (18.3)	37.8 (18.3)	0.49
Male gender n(%)	90 (59%)	17 (54%)	0.65
Socioeconomic score	19.38 (7.02)	18.70 (7.28)	0.62
Fever duration (days)	8.9 (7.2)	18.9 (29.4)	<0.01
Headache duration (days)	4.8 (5.2)	11.6 (25.5)	<0.01
Altered behavior duration (days)	1.4 (2.2)	0.8 (1.6)	0.26
Seizures n(%)	34 (22.4)	5 (19.1)	0.72
Glasgow coma scale (on admission)	9.4 (3.8)	10.2 (3.2)	0.38
Neck stiffness n(%)	47 (30.9)	15 (60)	<0.01
Hypotension n (%)	11 (7.2)	2 (8)	0.89
Need for assisted ventilation n(%)	33 (21.7)	4(16.0)	0.51
Hospital stay (days)	10.0 (7.5)	9.2 (8.0)	0.61
Hb g/dL	10.7 (2.4)	11.0 (2.8)	0.52
Total Leukocyte count (x 10^3 /mm3)	8.35 (3.5)	4.78 (2.5)	0.61
Platelet count (x 10^6 /mm3)	2.29 (1.26)	2.27 (1.32)	0.94
CSF cell count (per mm3)	432 (1519)	921 (1935)	0.14
CSF sugar (mg/dl)	63.7 (23.6)	52.5 (38.1)	0.03
CSF proteins (mg/dL)	137.3 (166.8)	246.9 (297.4)	<0.01
HIV positivity n(%)	6 (3.9)	4 (12.9)	0.04
Mortality n(%)	53 (36.0)	10 (58.8)	0.06

Table 2: Characteristics of patients with suspected and confirmed viral encephalitis as a cause of acute encephalitis syndrome (n=152)

Variable	AES viral encephalitis suspect (viral etiology not confirmed) N=121	AES with confirmed viral encephalitis N=31	P value
Age (years)	39.9 (17.9)	41.2 (19.5)	0.72
Male gender n(%)	76 (62.3)	14 (46.6)	0.11
Socioeconomic score	19.4 (7.0)	18.8 (7.3)	0.65
Fever duration (days)	9.0 (7.8)	8.4 (3.7)	0.67
Headache duration (days)	4.7 (5.5)	5.3 (4.1)	0.59
Altered behavior duration (days)	1.3 (2.2)	1.9 (2.3)	0.15
Seizures n(%)	29 (23.6)	5 (16.6)	0.35
Glasgow coma scale (on admission)	9.5 (3.6)	9.0 (4.3)	0.52
Neck stiffness n(%)	31 (25.6)	16 (51.1)	0.01
Hypotension n (%)	8 (6.6)	3 (9.6)	0.55
Need for assisted ventilation n(%)	27 (22.3)	6 (19.3)	0.72
Hospital stay (days)	9.7 (7.1)	11 (8.4)	0.41
Hb g/dL	10.8 (2.3)	10.2 (2.7)	0.21
Total Leukocyte count (x 10^3 /mm3)	8.1 (3.9)	9.3 (4.4)	0.87
Platelet count (x 10^6 /mm3)	2.2 (1.1)	2.5 (1.8)	0.24
CSF cell count (per mm3)	317 (770)	904 (360)	0.07
CSF sugar (mg/dl)	64.8 (23.7)	59.2 (22.9)	0.24
CSF proteins (mg/dL)	135.5 (175.6)	144.7 (126.9)	0.78
HIV positivity n(%)	5 (4.1)	1 (3.2)	0.81
Mortality n(%)	39 (33.6)	14 (45.1)	0.23

Table 3: Subgroup analysis of those in whom viral etiology not confirmed, but had possible or probable leptospirosis based on serology (n=121)

Variable	AES viral encephalitis suspect (viral etiology not confirmed) All (n=121)	Probable leptospirosis (n=16) **A**	Possible leptospirosis (n=48) **B**	Unknown AES (Negative for any viral agents, or leptospirosis) (n=57) **C**	P value A vs C	P value B vs C
Age (years)	39.9 (17.9)	41.3 (18.2)	39.5 (17.1)	40.0 (18.9)	0.80	0.87
Male gender n(%)	76 (62.3)	6 (37.5)	29 (60.4)	40 (70.1)	0.01	0.29
Socioeconomic score	19.4 (7.0)	22.0 (7.3)	18.1 (6.5)	19.9 (7.1)	0.29	0.19
Fever duration (days)	9.0 (7.8)	8.8 (5.0)	8.4 (4.4)	9.5 (10.4)	0.79	0.47
Headache duration (days)	4.7 (5.5)	4.5 (4.8)	5.7 (5.2)	3.9 (5.9)	0.74	0.09
Altered behavior duration (days)	1.3 (2.2)	0.62 (0.95)	1.4 (2.7)	1.3 (2.1)	0.21	0.79
Seizures n(%)	29 (23.6)	4 (25)	14 (29.1)	11 (19.3)	0.61	0.23
Glasgow coma scale (on admission)	9.5 (3.6)	11.2 (2.2)	9.7 (3.3)	8.9 (4.12)	0.03	0.30
Neck stiffness n(%)	31 (25.6)	2 (12.5)	11 (22.9)	18 (31.5)	0.13	0.32
Hypotension n (%)	8 (6.6)	0	2 (4.1)	6 (10.5)	0.17	0.22
Need for assisted ventilation n(%)	27 (22.3)	0	10 (20.8)	17 (29.8)	0.01	0.29
Hospital stay (days)	9.7 (7.1)	9.1 (5.0)	9.8 (7.0)	9.8 (7.9)	0.72	0.97
Hb g/dL	10.8 (2.3)	10.9 (2.4)	10.7 (2.5)	11.0 (2.1)	0.60	0.87
Total Leukocyte count (x 10^3 /mm3)	8.1 (3.9)	10.3 (4.7)	9.2 (30.7)	9.5 (40.9)	0.37	0.96
Platelet count (x 10^6 /mm3)	2.2 (1.1)	2.2 (0.9)	2.2 (1.2)	2.2 (1.08)	0.95	0.83
CSF cell count (per mm3)	317 (770)	107 (160)	250 (476)	444 (1042)	0.20	0.25
CSF sugar (mg/dl)	64.8 (23.7)	57.6 (25.6)	68.3 (21.6)	63.9 (25)	0.37	0.34
CSF proteins (mg/dL)	135.5 (175.6)	104.3 (157.3)	106.5 (141.0)	159.7 (192.4)	0.29	0.11
HIV positivity n(%)	5 (4.1)	1 (6.2)	0	4 (7.0)	0.91	0.06
Mortality n(%)	39 (33.6)	0	13 (27.6)	26 (49.1)	<0.01	0.02

Table 4: Subgroup analysis of patients with enteroviral (n=17) and flaviviral encephalitis (n=8) as compared to patients with AES of unknown etiology (negative for any tested pathogen) (n=57)

Variable	Enteroviral encephalitis (n=16)	Flaviviral encephalitis (n=8)	Unknown AES (n=57)	P value Enteroviral vs Unknown	P value Flaviviral vs Unknown
Age (years)	45.2 (20.7)	44.2 (18.4)	40.0 (18.9)	0.33	0.55
Male gender n(%)	9 (52.9)	5 (62.5)	40 (70.1)	0.18	0.66
Socioeconomic score	18.8 (7.6)	21.0 (7.5)	19.9 (7.1)	0.58	0.69
Fever duration (days)	7.4 (3.0)	8.8 (3.7)	9.5 (10.4)	0.40	0.85
Headache duration (days)	4.2 (3.3)	6.5 (6.1)	3.9 (5.9)	0.82	0.26
Altered behavior duration (days)	1.7 (2.0)	2.8(3.2)	1.3 (2.1)	0.44	0.07
Seizures n(%)	4 (23.6)	0	11 (19.3)	0.70	0.17
Glasgow coma scale (on admission)	9.0 (4.7)	8.3 (3.6)	8.9 (4.12)	0.94	0.69
Neck stiffness n(%)	9 (52.9)	3 (37.5)	18 (31.5)	0.10	0.73
Hypotension n (%)	2 (11.7)	1 (12.5)	6 (10.5)	0.88	0.86
Need for assisted ventilation n(%)	3 (17.6)	2 (25)	17 (29.8)	0.32	0.77
Hospital stay (days)	12.7 (10.7)	8.1 (4.2)	9.8 (7.9)	0.23	0.54
Hb g/dL	11 (2.5)	10.3 (2.7)	11.0 (2.1)	0.95	0.48
Total Leukocyte count (x 10^3 /mm3)	14.1 (8.2)	3.2 (8.1)	9.5 (40.9)	0.37	0.21
Platelet count (x 10^6 /mm3)	2.5 (1.2)	2.8 (2.7)	2.2 (1.08)	0.31	0.23
CSF cell count (per mm3)	389 (689)	2226 (5570)	444 (1042)	0.85	0.03
CSF sugar (mg/dl)	57.82 (25.5)	53.6 (13.8)	63.9 (25)	0.37	0.25
CSF proteins (mg/dL)	86.5 (56.5)	266.8 (226.7)	159.7 (192.4)	0.12	0.15
HIV positivity n(%)	1 (5.8)	0	4 (7.0)	0.87	0.43
Mortality n(%)	8 (47)	4 (50)	26 (49.1)	0.88	0.96

Table 5: Tests performed on serum samples of potential controls, so as to identify controls without evidence of past infection with specific etiologic agents (n=100)

Test	Result	No of negative controls sampled	Cases against which these controls compared	Case-control ratio
Anti-enteroviral IgG antibodies				
Positive / Equivocal	22			
Negative	78	68	Enteroviral RT-PCR positive (n=17)	1:4
Anti-leptospirosis antibodies (IgG & IgM)				
Both positive	7			
IgG positive / IgM negative	11			
IgM positive / IgG negative	23			
Both negative	59	48	Probable Leptospirosis (2-fold change in leptospirosis IgM levels) (n=16)	1:3
Anti-leptospirosis antibodies (IgG & IgM) and Anti-enteroviral antibodies (IgG)				
Either test positive	41			
Negative for all	59	57	Negative for enteroviruses, flaviviruses, herpesviruses and leptospirosis (n=57)	1: 1

Table 6: Univariate analysis of risk factors for encephalitis of unknown etiology, due to enteroviruses and probable leptospiral encephalitis

	Encephalitis of unknown etiology			Enteroviral encephalitis			Probable leptospiral encephalitis		
Variable	Case (n=57)	Control (n=57)	OR (95% CI)	Case (n=17)	Control (n=68)	OR (95% CI)	Case (n=16)	Control (n=48)	OR (95% CI)
Risk factors for vector borne transmission									
Presence of objects promoting vector breeding outside the house									
Earthen pots vs.	48	55	0.2	12	66	0.09	14	46	0.30
No earthen pots	9	2	(0.01-1.01)	4	2	(0.01-0.74)	2	2	(0.02-4.65)
Old discarded tires vs.	1	2	0.49	0	3	0	0	2	0
No discarded tiers	56	55	(0.01-9.74)	16	65	(0-5.59)	16	46	(0-5.93)
Water drum / water coolers vs.	50	51	0.84 (0.21-3.15)	15	66	0.45 (0.02-28.5)	16	47	-
No Water drum / coolers	7	6		1	2		0	1	
Three or more objects vs.	9	7	1.33 (0.4-4.58)	2	12	0.62 (0.06-3.30)	1	10	0.25 (0.01-2.10)
Two or less objects	48	50		15	56		15	38	
Use of personal protection against vector-bite									
Any measure used vs.	27	30	0.93	5	29	0.56	7	24	0.77
None	30	29	(0.41-2.07)	12	39	(0.13-1.96)	9	24	(0.20-2.79)
Three or more objects promoting vector breeding and non-use of personal protection measure against vector-bite									
Either of two conditions present vs.	7	5	1.35 (0.31-6.07)	1	7	0.44 (0.01-4.16)	1	8	0.30 (0.01-2.97)
Both conditions absent	28	27		11	34		9	22	
Factors promoting zoonotic transmission									
Cattle/ swine (Cow, bull, goat, pig etc)									
Present in home/occupation vs.	18	30	0.41 (0.18-0.95)	8	35	0.83 (0.24-2.78)	6	24	0.6 (0.15-2.18)
Absent	39	27		9	33		10	24	
Dogs and cats									

Present in home/occupation vs.	3	5	0.57	2	4	2.13	1	5	0.57
Absent	54	52	(0.08-3.15)	15	64	(0.17-16.38)	15	43	(0.01-5.79)
Poultry (Chicken, birds etc)									
Present in home/occupation vs.	3	8	0.34	3	10	1.24	3	6	1.61
Absent	54	49	(0.05-1.54)	14	58	(0.19-5.73)	13	42	(0.22-8.88)
Factors promoting water borne transmission									
Water supply									
Non-piped water supply vs.	14	8	1.99	2	7	1.24	1	8	0.33
Piped water supply	43	49	(0.69-6.01)	14	61	(0.11-7.54)	15	40	(0.01-2.90)
Sewage drain outside house									
Present vs.	43	19	0.50	12	60	0.40	12	42	0.42
Absent	14	8	(0.16-1.43)	4	8	(0.08-2.13)	4	6	(0.08-2.44)
A water pond/stream within 200 meters of house									
Present vs.	49	55	0.22	12	65	0.13	15	45	1.0
Absent	8	2	(0.02-1.20)	4	3	(0.01-0.96)	1	3	(0.07-55.9)
Sewage drain and Pond/stream near house									
Both present	37	0	0	9	57	-	11	39	-
One or none present	2	47	(0-1.57)	1	0	-	0	0	
Risk factors pertaining to poor living conditions									
Socioeconomic score									
Lowest tertile vs.	29	15	2.64	9	16	3.93	8	13	1.69
Highest tertile	19	26	(1.03-6.84)	4	28	(0.89-19.9)	8	22	(0.43-6.59)
Overcrowding at home									
Three or more person per room	11	5	2.48	5	9	2.97	6	8	3.0
Two or less person per room	46	52	(0.72-9.75)	11	59	(0.64-12.22)	10	40	(0.67-12.50)3

Number of children at home									
Three or more vs.	6	5	1.22	1	6	0.68	3	4	2.53
Two or less	51	52	(0.29-5.39)	15	62	(0.01-6.40)	13	44	(0.32-16.92)
Immunosupression									
HIV seopositivity									
Positive vs.	4	2	2.07	1	0	-	1	0	-
Negative	53	55	(0.28-23.69)	16	68	-	15	48	

Table 7: Multivariate logistic regression for risk factors for encephalitis of unknown etiology

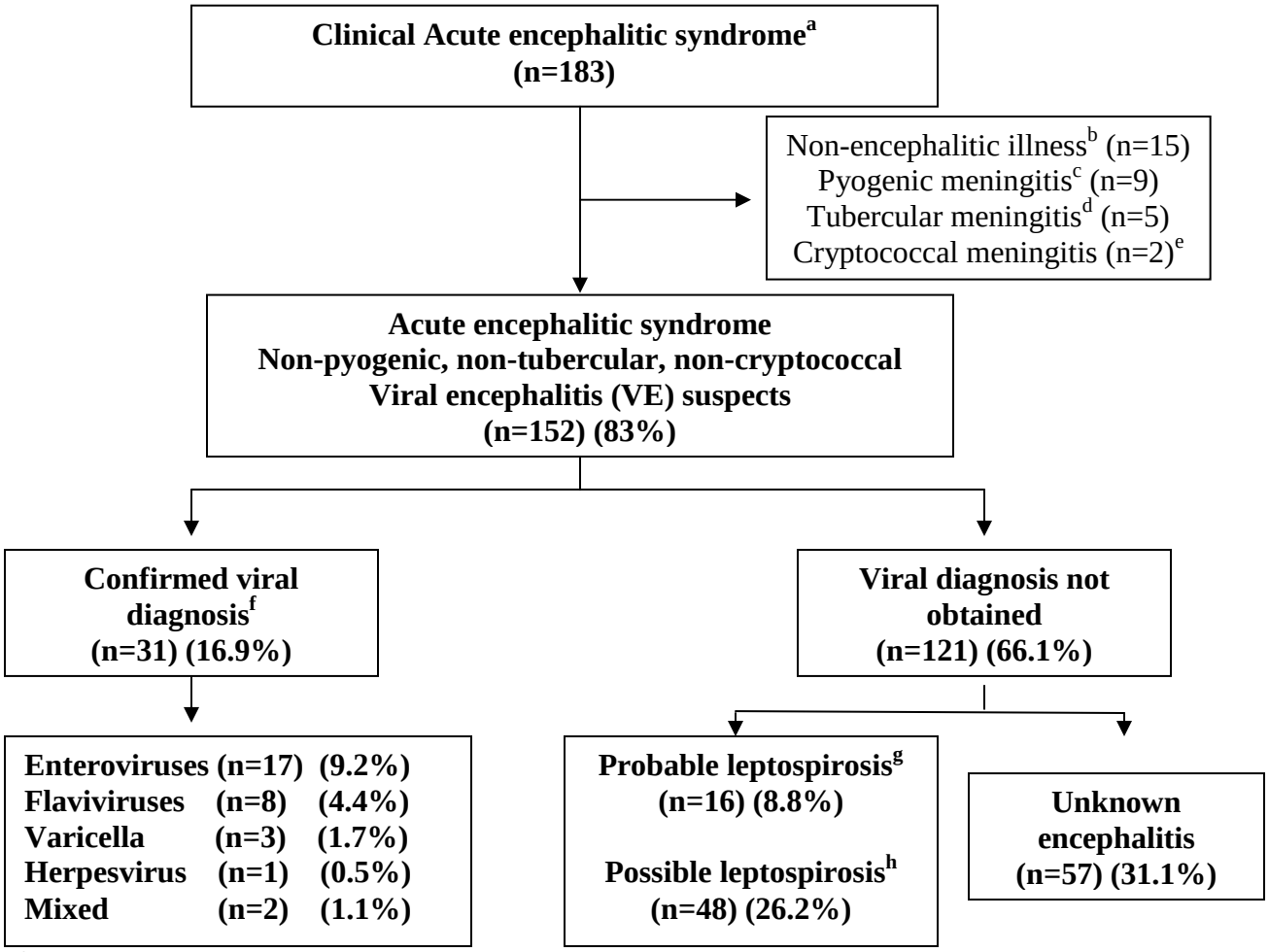
Model	Variable	Unknown encephalitis OR (95% CI)	Enteroviral encephalitis OR (95% CI)	Leptospiral encephalitis OR (95% CI)
Vector borne transmission model*	Three or more objects† promoting vector breeding outside household + Non-use of any vector protection measure Both conditions met vs. no condition met	1.76 (0.46-6.7)	0.63 (0.06-6.17)	0.42 (0.03-5.78)
Zoonotic transmission model*	Presence of cattle in household vs none	0.35 (0.12-0.98)	3.06 (0.22-41.7)	0.54 (0.86-1.85)
Water borne transmission model*	Non-piped water supply vs. Piped water supply at home	0.43 (0.15-2.1)	0.94 (0.16-5.42)	3.4 (0.38-30.5)
Poor living condition model‡	Socioeconomic lowest vs highest tertile	1.8 (0.47-7.5)	5.80 (1.15-29.2)	1.81 (0.47-7.26)

†Objects include earthen pot, water filled drums, water coolers, old tires etc.

* Models adjusted for age and Socio-economic status

‡ Model adjusted for age, presence of objects promoting vector borne transmission, presence of cattle/poultry in household, and non-piped water supply at home.

Figure 1: Study Flow chart



[a] Acute encephalitic syndrome was defined as presence of fever, which preceded altered sensorium, with or without neurological deficit. All these patients had negative peripheral smears and HRP-2 serology for malaria, had no other primary source of infection, and had a normal chest radiograph. No metabolic abnormality (hypogloycemia, hyponatremia, hyperuricemia or hepatic encephalopathy) was present when these individuals were included in the study.

[b] Non-encephalitic illness included individuals who were detected with a non-infectious etiology after inclusion into the study such as intracranial tumor (n=2), venous infarct (n=1), and psychiatric illness (n=3), metabolic abnormalities (n=9).

[c] Pyogenic meningitis was defined as presence of neutrophils in cerebrospinal fluid sample, CSF/serum glucose ratio <0.25 with or without positive bacterial culture. 4/9 (44%) of all individuals with pyogenic meningitis had a positive bacterial culture.

[d] Individuals with a positive cerebrospinal fluid mycobacterial culture on bactec media were defined as having tubercular meningitis.

[e] Cryptococcal antigen was tested in HIV positive individuals only.

[f] Viral diagnosis was confirmed either by PCR or by demonstrating IgM antibodies against a specific virus in CSF sample, as defined by CDC criteria for a neuroinvasive encephalitis.

[g] Probable leptospirosis was defined as a case where a two-fold change in serum anti-leptospira IgM antibodies was seen in acute and convalescent samples, and one of these two samples had IgM levels above the commercially determined cut-off level (11 IU/mL or above). [h] Possible leptospirosis was defined when one or both acute and convalescent samples serum samples had IgM levels above commercially defined cut-off, but without a two-fold change in levels.

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Conclusion

Introduction

Acute encephalitis syndrome (AES) is a broad clinical diagnosis that refers to inflammation of brain matter, usually caused by an infectious etiology.[1] In its most dramatic form, AES epidemics have an explosive onset, are often highly seasonal and result in exceptionally high mortality over a short period of time.[2] In addition to agents that produce epidemics of AES, many other infectious agents that cause AES are endemic in certain parts of the world and are responsible for sporadic cases throughout the year. Even when a given etiologic agents is endemic in the community, its transmission is often seasonal and depends on various environmental characteristics, such as vector densities, water-contamination, and meteorological conditions.[3] AES surveillance studies, that identify and evaluate cases throughout the year can help us understand the true burden of AES and improve diagnostic and therapeutic decision-making for individual patients. While outbreak investigations have helped to improve our understanding of AES-epidemics in India,[4] only a small handful of AES-surveillance studies have been conducted in the region.[5-7] These studies have focused on AES in children and have largely been restricted to cases of AES caused by Japanese encephalitis virus.

The overall goals of this dissertation were to determine the burden of AES in adults in central India, and to describe its etiologies and risk factors. We set-out to achieve these goals by means of three original studies and two supporting systematic literature reviews, which have been presented in detail in the previous five chapters. Briefly, the two systematic literature reviews summarized what is known about epidemiologic features of AES in India, and about ELISA-based tests for the diagnosis of leptospirosis (a potential cause of some adult AES cases). Information from these systematic reviews was used in the design and conduct of the three original studies, which comprised of i) A retrospective chart review of all hospitalized adults with acute undifferentiated fever, to determine the proportion of those with AES, and existing diagnostic and management practices; ii) Prospective hospital-based surveillance for AES to describe the temporal and spatial characteristics, survival patterns, and risk factors of AES in adults; and iii) A diagnostic assessment of adult AES cases, with a focus on viral etiologies. The conclusions from these three original-studies are detailed below:

Project #1 A retrospective chart review of patients with acute undifferentiated fever

This study was designed to characterize the burden of AES as a syndromic subtype of AUF, and the current use of diagnostic procedures and management practices in patients with acute undifferentiated fever (AUF).[8] We carried out a retrospective review of electronic-discharge summaries of hospitalized patients (aged >12 years) admitted with fever to a large teaching hospital in central India in the year 2006. We abstracted data from over 1600 discharge summaries, and identified 1197 patients with AUF, who were classified into syndromic categories based on clinical presentation. Of these 1197 patients 196 (16.4%) had AES. Patients with this syndromic subtype experienced a high mortality with a total of 42 (21.4%) patients with AES dying during their hospital stay. The only specific diagnostic test available and used by clinicians was light microscopy or a histidine rich protein based rapid diagnostic test for malaria; despite the availability and use of these tests 40% of patients with a negative malaria test were

treated with anti-malarial drugs. Despite the fact that every sixth patient with AES died, and two-thirds of all AUF mortality was attributable to AES, almost no specific diagnostic tests were available for this syndromic subtype. Cerebrospinal fluid cytology, biochemistry, and bacterial cultures were the only 7.7% of all patients with AES had results compatible with bacterial meningitis.

This study showed that in central India, most adult patients with AES remain of undetermined etiology, and are often treated with empirical therapies unlikely to be useful. A lack of good quality diagnostic tests is the main reason for this practice; a first step to changing current practice would be to identify specific etiologic agents in different clinical-syndromes of AUF, so that meaningful diagnostic algorithms can be devised. The subsequent projects we carried out were a logical step in this direction.

Project #2 Prospective hospital-based surveillance for patients with acute encephalitis syndrome

Because surveillance studies of endemic AES in adults and studies of risk factors for AES in India have not been reported previously, the second study was designed to answer three specific research questions: a) What are the incidence, spatial and temporal distributions of AES cases in Central India?; b) What are the predictors of mortality and disability in patients with AES in Central India?; and last c) What are the environmental, and socio-economic risk factors for AES of presumed viral etiology in rural central India?

We established an surveillance for AES in a teaching hospital in central India. This hospital is one of the two tertiary care hospitals in the district, in which facilities for mechanical ventilation are available; hence most cases from the district and from subdivisions of the neighboring districts are referred to this hospital by their primary care physicians. All consecutive adult patients with AES who presented to the hospital were reported to the investigators, who recorded the time of onset; the geographic location of case houses; the symptoms and signs on presentation; in-hospital course; the results of investigations performed as part of the care of patients; patients were followed up on day 0 and 180 after onset of their symptoms. For every case, we also sampled a control from the same village as the case, frequency matched by age and gender. We also administered a standardized pilot tested questionnaire to all cases and controls to collect infromation about potential environmental and societal risk factors for AES. In addition, both cases and controls were tested for HIV to evaluate its role as a risk factor for AES.

We found the incidence of AES among adults to be high (between 10 and 16 / 100,000 adult population per year from the subdivisions in the same district as the hospital); Of 183 AES cases that presented during the study period, 64% had onset during the hot and humid months of the year. Spatial analysis of point data was performed in high incidence areas, and clusters with high kernel density were found to be located in proximity to rivers and streams. The incidence of AES cases diminished with increasing distance from a such that there were 12 fewer cases seen for every one additional kilometer distance farther from the river/stream. A total of 53 patients (36%) died, and another 34 (22.3%) had significant residual cognitive disability at 30-day of follow up. In Cox proportional hazards multivariable regression models, four variables were associatd with a significantly increased hazard for mortality, and mortality or disability by day 30: age; Glasgow coma score (GCS) on admission; duration of hospital stay; and requirement

for assisted ventilation. Of the 183 cases, 152 (83%) had AES of a presumed viral origin. We compared socio-economic and environmental risk factors between AES cases with suspected viral-encephalitis and apparently healthy community controls. Low socioeconomic status (OR 3.12; 95% CI 1.57 to 6.17), and factors likely to promote vector borne disease transmission (OR 2.16; 95% CI 1.08 to 4.33) were significantly associated with the risk of AES of presumed viral origin. Only six (3.9%) cases and two (2%) of controls were HIV positive and this was not a significant risk factor for AES, so the study had a limited power to detect the relationship between AES and HIV infection.

In this study of adult AES in rural central India, we found a high incidence of AES (10-16/100,000), as compared to the recently suggested minimum surveillance standard for AES in adults (2/100,000).[2] Thus, hospital based surveillance is feasible and useful for studying AES epidemiology. We also found that AES was more common during the hot-humid months and that residence near a river/stream increased the risk of AES. We found that individuals with a low socioeconomic status were three times more likely to develop AES, as compared to those who are economically better off. Potential pathways whereby those of low socioeconomic status are put at a higher risk of AES include more common exposure to infectious agents that cause AES, and reduced ability of individuals to protect them. Poverty may also potentially delay health seeking behavior, causing individuals to present late in the course of disease, and making families less able to afford the expensive supportive treatments that many patients with AES need.

Project #3 Etiology of AES of suspected viral etiology in central India

Our systematic review of the epidemiologic features of AES in India had revealed no prior study that tested for multiple etiologic agents among adults with AES in a non-outbreak surveillance setting. Multiple pathogen testing in AES cases is important because in a country like India, a number of non-viral conditions such as cerebral malaria, and tubercular or bacterial meningitis are possible etiologies of AES.[2] These conditions can be reliably diagnosed by examination of peripheral blood smear, and cerebrospinal fluid (CSF) cultures, and biochemical findings, leaving the remaining cases of AES as viral-encephalitis suspects. A large diverse group of neurotropic viruses (e.g. flaviviruses, enteroviruses, herpesviruses, paramyxoviruses etc) are known to cause AES. Nucleic acid amplification and serology-based diagnostic tests on CSF and sera have been developed for many of these agents, but their availability in routine clinical practice in India is limited due to their high cost. Based on what is known about the prevalence of these agents, we included common viral agents in our priority list of potential etiologies. We used the CDC definition for a confirmed case of viral encephalitis when interpreting the results of various diagnostic tests.[9] We also considered neuro-leptospirosis [10]as a potential etiology, as many patients with AES were seropositive for IgM antibodies against leptospira. Our systematic review of the enzyme linked immunosorbent assay (ELISA) for detection of anti-leptospira antibodies suggested that we could rely on this test for the diagnosis of acute leptospirosis (pooled sensitivity and specificity estimates being 92% (95% CI 87 to 95%) and 98% (95% CI 96 to 99%) percent respectively when test is performed in late acute phase of illness). Thus, in this study we evaluated multiple potential etiologies among AES cases suspected to have viral encephalitis.

In our extensive laboratory testing of CSF and serum samples obtained from 152 AES cases suspected of having viral encephalitis, we found 31 (17%) patients who had a confirmed viral etiology. Enteroviruses were the commonest etiology (9.2% of all AES

cases) followed by flaviviruses (4.3% of all AES cases). We examined serum anti-leptospira IgM levels in AES cases, and found 16 (8.8%) patients to have probable leptospirosis. The remaining 57 (31.1%) AES cases were of an unknown etiology. None of the environmental risk factors we examined were significantly associated with enteroviral or leptospiral AES cases when they were compared to community controls. On univariate analysis cases with AES of unknown etiology had a 2.6 times higher risk of being in the lowest tertile of socioeconomic score as compared to seronegative community controls, but this difference was not significant on multivariate analysis. Thus, we did not find any environmental or socio-economic risk factor to be associated with specific etiologic subtypes of AES.

This study has generated several novel hypotheses. First, contrary to what has been previously reported enteroviral-AES was twice as common as flaviviral-AES. Thus most AES cases of AES in central India are likely to be water borne, rather than vector borne. In India current public-health focus for control of seasonal febrile illnesses is on vector-control, an approach that may need re-examination. Second, enteroviruses are an important cause of AES in adults, and should be the subject of further study. Third, individuals of low-SES are at higher risk for AES. Last some adults with AES may have leptospirosis, which is relatively easily treatable. Subsequent studies need to focus on subtypes of enterovirus causing AES; confirmation of the role of neuro-leptospirosis in AES; and a focus on how AES can be prevented, especially in those of a low-SES. We have demonstrated that it is possible to establish a hospital based surveillance for AES, and have narrowed the range of known etiologic agents responsible for this life-threatening condition. We believe that this information will be valuable in planning future work, and in developing and testing various hypotheses this study has generated.

Overall the three projects in this dissertation have provided novel insights into AES in India. Despite a high burden of mortality from AES in adults, published studiesof AES have been infrequent. Both our retrospective chart review and a prospective surveillance results suggest that about one-third of all adults with AES die within a month of onset of their illness. The seasonal predilection of this syndrome suggests a limited duration of transmission of the etiological agent, facilitated by hot and humid environmental conditions. These environmental conditions are supportive of both vector borne (more vector-breeding sites and high vector densities) and water-borne transmission (seasonal streams, contaminated water supply etc) in a typical tropical country setting. Low socioeconomic status increases individual's exposure to these conditions and also was a significant risk factor in our analyses. In our etiologic assessment, we found enteroviruses to be responsible for most AES cases of which a viral etiology could be identified. . This suggests a greater contribution of contaminated water supply and poor sanitation to the risk of AES. Vector borne flaviviruses were less common cause of AES in our population in absence of an outbreak.

Of all cases with AES, we could confirm an etiology in only one-third (half of these being viral, and half non-viral). Another one-third were negative all any tested etiologic agents, and in remaining one-third positive serologic tests suggested a presumptive diagnosis of neuro-leptospirosis. Thus, despite and extensive battery of diagnostic tests being used most AES cases remain of unknown etiologies. This offers both an opportunity for further discovery, as well as humility about limitations of

currently available diagnostic tests. The current work provides a framework for expanded AES surveillance, and also a basis for future viral discovery.

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