"DETECTION OF ENTEROVIRUSES IN DENGUE NEGATIVE PATIENTS WITH SUSPECTED ENTEROVIRUS ENCEPHALITIS EV'sE OR MENINGO ENCEPHALITIS AND APPLICATION OF REAL TIME REVERSE TRANSCRIPTASE PCR ASSAY FOR THE DIAGNOSIS OF ENTEROVIRUSES"

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BONAFIDE CERTIFICATE

This is to certify that the dissertation entitled ""**"DETECTION OF ENTEROVIRUSES IN DENGUE NEGATIVE PATIENTS WITH SUSPECTED ENTEROVIRUS ENCEPHALITIS EV'sE OR MENINGO ENCEPHALITIS AND APPLICATION OF REAL TIME REVERSE TRANSCRIPTASE PCR ASSAY FOR THE DIAGNOSIS OF ENTEROVIRUSES"** submitted by **Dr.A.SANGEETHA** to the Tamilnadu Dr. M.G.R Medical University, Chennai, in partial fulfillment of the requirement for the award of M.D. Degree Branch – IV (Microbiology) is a bonafide research work carried out by her under direct supervision & guidance.

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This is to certify that the Dissertation **"DETECTION OF ENTEROVIRUSES IN DENGUE NEGATIVE PATIENTS WITH SUSPECTED ENTEROVIRUS ENCEPHALITIS EV'sE OR MENINGO ENCEPHALITIS AND APPLICATION OF REAL TIME REVERSE TRANSCRIPTASE PCR ASSAY FOR THE DIAGNOSIS OF ENTEROVIRUSES"** presented here in by **DR.A.SANGEETHA** is an original work done in the Department of Microbiology, Tirunelveli Medical College Hospital, Tirunelveli for the award of Degree of M.D. (Branch IV) Microbiology under my guidance and supervision during the academic period of 2017 -2020.

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DECLARATION

I solemnly declare that the dissertation titled **"DETECTION OF ENTEROVIRUSES IN DENGUE NEGATIVE PATIENTS WITH SUSPECTED ENTEROVIRUS ENCEPHALITIS EV'sE OR MENINGO ENCEPHALITIS AND APPLICATION OF REAL TIME REVERSE TRANSCRIPTASE PCR ASSAY FOR THE DIAGNOSIS OF ENTEROVIRUSES"** is done by me at Tirunelveli Medical College hospital, Tirunelveli. I also declare that this bonafide work or a part of this work was not submitted by me or any others for any award, degree, or diploma to any other University, Board, either in or abroad.

The dissertation is submitted to The Tamilnadu Dr. M.G.R.Medical University towards the partial fulfilment of requirements for the award of M.D. Degree (Branch IV) in Microbiology.

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PROTOCOL TITLE: DETECTION OF ENTRO VIRUSES IN DENGUE NEGATIVE PATIENTS WITH SUSPECTED EV-SE OR MENINGIO-ENCEPHALITIS AND APPLICATION OF REAL TIME REVERSE TRANSCRIPTASE PCR ASSAY FOR THE DIAGNOSIS OF ENTEROVIRUSES
PRINCIPAL INVESTIGATOR: Dr.A.SANGEETHA, MBBS.,
DESIGNATION OF PRINCIPAL INVESTIGATOR: PG STUDENT
DEPARTMENT & INSTITUTION: TIRUNELVELI MEDICAL COLLEGE, TIRUNELVELI

Dear Dr.A.SANGEETHA, MBBS.,, The Tirunelveli Medical College Institutional Ethics Committee (TIREC) reviewed and discussed your application during The IEC meeting held on 15.12.2017.

THE FOLLOWING DOCUMENTS WERE REVIEWED AND APPROVED

1.	TIREC Application Form	☐
2.	Study Protocol	☐
3.	Department Research Committee Approval	☐
4.	Patient Information Document and Consent Form in English and Vernacular Language	☐
5.	Investigator's Brochure	☐
6.	Proposed Methods for Patient Accrual Proposed	☐
7.	Curriculum Vitae of The Principal Investigator	☐
8.	Insurance /Compensation Policy	☐
9.	Investigator's Agreement with Sponsor	☐
10.	Investigator's Undertaking	☐
11.	DCGI/DGFT approval	☐
12.	Clinical Trial Agreement (CTA)	☐
13.	Memorandum of Understanding (MOU)/Material Transfer Agreement (MTA)	☐
14.	Clinical Trials Registry-India (CTRI) Registration	☐

THE PROTOCOL IS APPROVED IN ITS PRESENTED FORM ON THE FOLLOWING CONDITIONS

1. The approval is valid for a period of 2 year/s or duration of project whichever is later
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3. A written request should be submitted 3weeks before for renewal / extension of The validity
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CERTIFICATE – II

This is to certify that this dissertation work titled **"DETECTION OF ENTEROVIRUSES IN DENGUE NEGATIVE PATIENTS WITH SUSPECTED ENTEROVIRUS ENCEPHALITIS EV'sE OR MENINGO ENCEPHALITIS AND APPLICATION OF REAL TIME REVERSE TRANSCRIPTASE PCR ASSAY FOR THE DIAGNOSIS OF ENTEROVIRUSES"** of the candidate **Dr.SANGEETHA** with registration Number **201714305** for the award of **M.D.** Degree in the branch of **MICROBIOLOGY (IV)** . I personally verified the urkund.com website for the purpose of plagiarism Check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows **2 percentage** of plagiarism in the dissertation.

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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3374469/
https://fdocuments.net/amp/document/treatment-of-human-enterovirus-infections.html

Instances where selected sources appear:

5

CONTENTS

ABBREVIATIONS

AFI Acute febrile illness

AFP Acute flaccid paralysis

AHC Acute Hemorrhagic Conjunctivitis

Bp Base pair

CDC Centre for disease control and Prevention

cDNA Complementary deoxyribonucleic acid

CNS Central nervous system

Cont Control

CO2 Carbondioxide

CPE Cytopathic effect

CSF Cerebrospinal fluid

CV Coxsackievirus

DMSO Dimethyl sulfoxide

dNTP Deoxy-nucleotide triphosphate

DTT Dithiothreitol

ECV Echovirus

ELISA Enzyme Linked Immunosorbent Assay

EMEM Eagle's minimal essential growth medium

EV Enterovirus

FBS Fetal bovine serum

FCS Fetal calf serum

GI Gastrointestinal infections

HEp-2	Human epithelial carcinoma cell line
HEV	Human enterovirus
HRV	Human rhinovirus
HSV	Herpes simplex virus
ISVC	Integrated shell vial culture
IC	Intracereberal
IgG	Immunoglobulin G
Kb	Kilobases
MEM	Minimal essential medium
mg	Milligram
ml	Milliliter
MEGA	Molecular evolutionary genetic analysis
MRI	Magnetic resonance imaging
MOI	Multiplicity of Infection
NaCl	Sodium chloride
NPEV	Non polio enterovirus
ORF	Open reading frame

p1, p2, p3 The three positions of a codon

PABP1 Poly (A) binding protein 1

PBS Phosphate-buffered saline

PCR Polymerase chain reaction

PEG	Polyethylene glycol
PFU	Plaque forming unit
PV	Poliovirus
Rct	Reproductive capacities at different temperature

RD	Human rhabdomyosarcoma cell line
RNA	Ribonucleic acid
RT-	PCR Reverse transcription - polymerase chain reaction
RT	Reverse transcriptase
RTI	Respiratory tract infection
SD	Standard Deviation
SEAR	South East Asia Region
SVDV	Swine vesicular disease virus
TBS	Tris buffered Saline
TCID	Tissue culture infective dose
TE	Tris EDTA buffer
µg	Microgram
µM	Micromole
Tm	Melting temperature
UTR	Untranslated region
URIs	Urinary respiratory infections
UV	Ultraviolet
VP	Viral protein
WHO	World Health Organization
WPV	Wild Polio Virus
W/V	Weight/volume

INTRODUCTION

The family Picornaviridae comprises many viruses which include the Enteroviruses under the genus Enterovirus. All the viruses in this family include positive-sense RNA that is single stranded , a wide range of human and mammalian diseases are being associated with them.The faeco-oral route is the main mode of the transmission of infection after which the virus stay and replicate in the intestinal tract and hence the name 'Enterovirus'.A wide range of clinical manifestations are being associated with the Enteroviruses that include- acute respiratory illness,Hand foot and mouth disease(HFMD),aseptic meningitis, acute haemorrhagic conjunctivitis ,myocarditis, neonatal multiorgan failure, meningoencephalitis and acute flaccid paralysis(1).Complete recovery of the patient is being observed following majority of the enteroviral infections but occasionally sever manifestations including Meningitis, Myocarditis, Encephalitis,Neonatal sepsis and Polio occur(2) that results in fatality.

In endemic areas , the prevalence of encephalitis caused by Enterovirus according to a study by Shikha Jain et al is 22% and the incidence is 3%.

The History of poliovirus(PV) and that of Enteroviruses(EVs) is similar. The World Health Organisation has taken much efforts that in most of the countries in World, the Polio viral infections are under contol and so the Clinical features ,their complications , Diagnosis and treatment of the Non-Polio Enteroviruses have started gaining attention(3).

The Previous categorization of the Enteroviruses demarcated them into Polioviruses, Coxsackieviruses, Echoviruses and other Enteroviruses.in the recent times, Enteroviruses are divided into four Human Enterovirus (HEV) species, denominated as Human Enterovirus (HEV)-A,B,C,D, depending on similarity within the RNA coding region of the protein VP1,that is in the capsid and has the major neutralization 2 target (4).In 1947, When the poliomyelitis outbreak occurred ,Coxsackei viruses were isolated in Coxsackie,Newyork

which were obtained by inoculating into suckling mice and the pathogenicity that resulted in mice discriminated these viruses from PV. In the very next year,aseptic meningitis cases were seen from where isolation of the first coxsackievirus (CV) group B was done. In newborn mice,Coxsackie virus group A resulted in Flaccid limb paralysis with myositis but the Coxsackie group B viruses in the same resulted in Spastic paralysis with generalized infection involving Muscle,Brain,Heart,pancreas and Brown fat. Echoviruses were isolated from stool samples of people who were asymptomatic for the first time inn 1951. The name Echoviruses was given to them as they were the isolated from enteric regions, cytopathogenic effects in tissue culture, isolated from humans, and orphans (i.e., not associated with a known clinical disease).

The infections that are caused by the EVs could be mistaken often as other viral infections or bacterial infections and may prompt to treatment, diagnostic test and procedures that may not be necessary(2). Though the clear identification of viral serotype is not made,bacterial infection should be excluded that the patient should not undergo unnecessary diagnostic tests and getting administered with unnecessary antibiotics. In recent times, Molecular methods, mainly Polymerase chain reaction exactly identifies the Enteroviruses in the clinical sample and many new antiviral drugs are being developed. Hence Prognosis have become better as the Entero viral infections are diagnosed and treated at an earlier stage that might become severe later (3). Enterovirus 71 is a single serotype of Entero virus, has caused outbreaks of Hand Foot and Mouth disease often, posing a major public health problem (5). Some Enterovirus serotypes casue infection and results in disease within a community, every year .and few of these serotypes produce illness many times than other serotypes. Though specific tissues are preferred by some of the Enteroviruses, thesekind of tissue tropisms are not very unique neither to the virus nor to the tissue that has been infected (3).

The Enterovirus ,when isolated through cell culture technique,is considered as the gold standard method for the diagnosis of infection with Enterovirus. As the molecular methods are being in progression ,the infection of Enterovirus greatly relied upon amplifying the most conserved region of the nucleic acid which is the 5` translated region and such advanced molecular techniques include Polymerase chain reaction (6). More than isolating the virus and demonstrating them in cell culture,these kind of assays remain more sensitive.From a clinician's view, if it is identified that the infection is being caused by some virus that belong to the genus Enterovirus,its enough. But, for the sake ofepidemiological analysis, typing of the virus by molecular method has to be done, especially by sequencing of various variable regions of the genome of the virus partially(6).

Public health has got significant impact by these Enteroviruses as they can cause major outbreaks in a communit. Repititive infections of known pathogenic strains and the evolution of new fresh strains should be forecasted (7). So a continuous surveillance of many strains of Enteroviruses, that are in circulation is imperative. Rapid molecular diagnostic methods that are new should be admitted to determine the enteroviral diseases and to establish the strains causing the disease.

Although there are many antiviral drugs developed against many viral diseases, no approved antivirals are there for the management of infections acquired by Enteroviruses. Increased morbidity and henceforth the serious sequelae after such infections due to enteroviral diseases occur which put forth, there is a crucial demand for the making out new antiviral drugs that are active against Enteroviruses.

This study is aimed at detecting the Enteroviruses associated with meningitis, encephalitis and meningoencephalitis . In South India, as the published literatures about the enteroviral infections that are prevalent is very limited, this study will summate information to the knowledge that we have now about these diseases.

AIMS AND OBJECTIVES

1. To detect the Enterovirus with real time Reverse transcriptase PCR clinically suspected among meningitis and meningoencephalitis patients.
2. To provide definite diagnosis, improve patients management and reduce hospital stay with high speed,accuracy, sensitivity, specificity and quick turn around time of PCR
3. To reduce unwanted use of antibiotic in viral meningitis and meningoencephalitis

REVIEW OF LITERATURE

3.1.Classification

Enterovirus in the family *Picornaviridae,* is a small spherical,positive sense,single stranded ,non-envelope RNA viruses which cause a range of diseases in humanss.Derived its names :*Entero*: from Greek *enteron* meaning 'intestine'.these virions are spherical about 22-30nm in diameter,having naked RNA genome surrounded by a protein shell capsid with 60 identical building units the capsomeres,each capsomere contains 1 copy of four structural proteins VP1-VP4. The 8-stranded anti parallel β-barrel structure folding pattern is similar in polypeptides VP1-VP3(10) copy of each structural protein forms a protomer ,and 5 of them form pentamer .an icasahedral capsid has 12 pentamers.VP1-VP3 forms the protein shell while VP4 lies on the inner surface. Being non enveloped they are not sensitive to organic solvent.

Order:Picornavirales

Family:Picornaviridiae

Genus:Enterovirus

The six genera that make up the Picornaviridae are:

- . the Enteroviruses
- the Rhinoviruses
- the Aphthoviruses, which cause foot-and-mouth disease of cattle
- the Cardioviruses of mice, which include Mengo virus and Encephalomyocarditis virus (EMCV) as well as Theiler’s virus

- the Hepatoviruses, which include hepatitis A viruses(described in Hepatitis A and E)
- the Parechoviruses,(11) which encompass the viruses previously designated echovirus 22 and echovirus 23

Enterovirus serotypes. Genera are defined on the basis of physical and chemical properties likevirion density, acid sensitivity(6).serologically there were 4 groups of EVs depending on the disease pattern they cause in infected experimental animals,their CPE seen in tissue culture

Group[a]	Major disease
Polioviruses 1–3	Paralytic poliomyelitis, Aseptic meningitis
Coxsackie viruses A1–22, 24	Aseptic meningitis, Herpangina, Conjunctivitis (A24)
Coxsackie viruses B1–6	Aseptic meningitis,Fatal neonatal disease Pleurodynia , Myo- or pericarditis
Echoviruses 1–9, 11–21, 24–27, 29–34	Aseptic meningitis Rashes
Enteroviruses 68–71	Conjunctivitis (enterovirus 70),Polio-like illness (enterovirus 71)

The earlier taxonomy of Enteroviruses includes

poliovirus (**PV**) serotypes 1 -3,

coxsackievirus (**CV)** group **A**Serotypes 1 – 24(no type 15,18,23)

coxsackievirus(**CV**)group **B** serotypes 1 - 6;

echoviruses **(ECV**)Serotypes 1 -33(no 7, 9, 10, 11,22,23,28 or34) , and 29 (Pallansc et al. 2006)(12).

After **1970**, new enterovirus serotypes have been assigned with numbers and not sub classified as enterovirus or echovirus to the above groups, but ,they have classified as enterovirus (EV) serotypes 68 to 71, 73, 74 to78, and 89 to 91. Human Enteroviruses are subdivided based on biological property and molecular characteristics of the viruses. Based on sequence analysis Genus Enterovirus are divided among Human Enterovirus A-D species. Species differs from each other by more than 40% nucleotide sequence divergence into capsid regions(Knowles et al. 2011). Crystal structures of many enteroviruses have been resolved

Enterovirus A: coxsackie virus (CV)-A7, CV-A16, enterovirus (EV)-A71

Enterovirus B: CV-A9, CV-B3, E-1, E-7, E-11, E-12, swine vesicular disease virus (SVDV)-1

Enterovirus C: PV-1, PV-2, PV-3, CV-A21, CV-A24

Enterovirus D: EV-D68

Enterovirus E: EV-E

Rhinovirus A: RV-A1, RV-A2, RV-A16

Rhinovirus B: RV-B14

Rhinovirus C: RV-C15.

Species demarcation criteria([12])

Members of a species of the genus *Enterovirus*:

1. They may share more than 70% amino acid identity in the polyprotein, and aa identity in the non-structural proteins 2C + 3CD[2]
2. They may share more than 60% amino acid in P1 region.
3. They are host specific and have specific tissue trophism.
4. Their genome (G+C) composition may vary by no more than 2.5%,
5. They all share a common genome organization.

- **Hepatitis A virus** (genus *Hepatovirus*)- previously classified as enterovirus 72 is now assigned into a separate genus.
- Echovirus-8 is now reassigned as Reovirus.
- Echovirus-20, 23 is now assigned to new genus Parechovirus.

The host of Picarnovirus varies among each species and each strain.

3.2. Epidemiology

EVs infections are encountered everywhere in nature with pleomorphic clinical presentations. The Enterovirus infection and disease patterns have found to be associated with demographic areas.(13) Humans are the only known reservoir for members of the human enterovirus group.Virus is generally shed forlonger periods in feces (1 month or more) than from theoropharynx, and fecal contamination is the usual sourceof infection although enterovirus 70, the agent of acutehemorrhagic conjunctivitis, has been found almost exclusively in conjunctival and throat specimens.

INFLUENCE OF CLIMATE

In temperate climates enteroviruses are more prevalent in the summer and autumn[3](Moore 1982)(14). (i.e. when warmth and humidity are at their peak). In

tropical climates EVs tend to circulate all the year round or are associatedwith the rainy season.[2](Melnick 1996)(15), whereas in temperate SPREAD WITHIN FAMILIES(Kogonet al. 1969, Lagercrantz et al. 1973)(16-17)Young children are the usual reservoir of EV infection. The secondary attack rates in susceptible family members are reported as 92 % for Polioviruses,76 % for Coxsackie viruses and 43%t for Echoviruses. The highspread of Polioviruses and Coxsackie viruses may be related to their longer periods of excretion . [4]Gear et al. 1973, Helfand et al. 1994(18-19) described in their study that Coxsackie virus infections spreads in day care centers and nurseries. Crowding, low socioeconomic status and poor sanitation have shown to increase the incidence of EV infections. The frequency of EV infections are more common in males than in females and their ratio appears with range of 1.2 and 2.5: 1 with male predominance higher in more severe diseases(19), for e.g. pleurodynia, hand foot andmouth disease,respiratory disease, acute hemorrhagic conjunctivitis (AHC), rash, orundifferentiated febrile illness. Transmission is by feco-oral route, direct contact.

EPIDEMIOLOGICAL SURVEILLANCE

International dataIn 1963 WHO established asystem for the collection and dissemination of informationon viral infections. Although the level of reporting may below and variable, the dataare of interest in identifyingviruses implicated in clinical syndromes. Examples from1975 to 1983,The commonest reported was CV A9.CV A16 is the main cause ofHFMD,CV B4 was associated with syndromes; CV B5 withnonparalytic CNS infections and rashes.Almost half the reported echovirus infections wereassociated with CNS disease, especially aseptic meningitis. Echoviruses

6, 9, 11, 19, and 30 are regularlyencountered during outbreaks of aseptic meningitis,sometimes accompanied by a rash in echovirus 6 and 9infections. Parechovirus 1(echovirus 22) is usually associatedwith a 'failure to thrive' syndrome in infants lessthan 1 year old and is seldom associated with CNS infection.The apparent role of CV A , B and echoviruses in paralytic diseases should be interpreted with caution because most reportsare based on virus isolation from feces alone. WHO during 1975–83llness due to enteroviruses 68-71, association ofEV70 with eye infections(20),but not pandemic nature .it is difficult to isolate EV 70 in cellculture.Enterovirus 71 is more readily isolated from clinical specimens,and its associations with CNS infections and HFMD.

3.3. Enterovirus structure and genomeGenome layout:

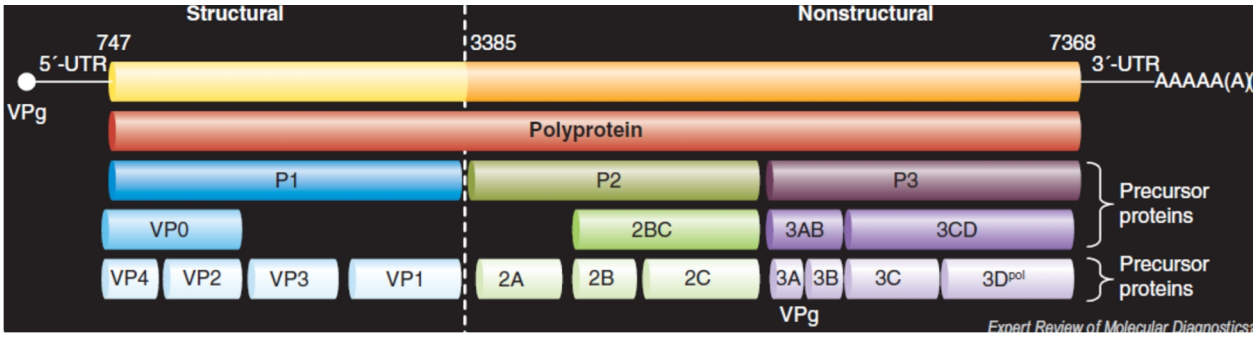


Figure 2: Genomic organization of enteroviruses and the cleavage of polyprotein (Adapted from Nasri *et al*., 2007) (14)**.**

VPg+5'UTR^{IRES-I}[1A-1B-1C-1D/2A^{pro}-2B-2C/3A-3B-3C-3D]3'UTR-poly(A)

The genome of EV is a positive sense, single-stranded RNA, 7,500 nucleotide long(21) . 5' UTR is followed by a long open reading frame ORF coding for an approximately 2,100-amino-acid polyprotein , this is followed by a short 3' UTR and a poly (A) tail (22).

Salient features of the genome are as follows:

The EV RNA is infectious ,as it is translated on entry into the cell to produce all the viral proteins required for viral replication. EV RNAgenome is unique , is covalently linked to a protein called VPg (virion protein, genome linked) at the 5` end. The VPg tyrosine linkage is linked to the viral RNA in the third amino acid from the N-terminus. VPg of 22 to 24 amino acid residues ,is encoded by a single viral gene (23), except FMDV, encodes three VPg genes . VPg is not required for infectivity of poliovirus RNA. VPg is not found on viral mRNA that is associated with cellular ribosomes and undergoing translation. Poliovirus mRNA differs from virion RNA only by the lack of VPg . VPg is removed from virion RNA by a host protein called unlinking enzyme . It is not known whether removal of VPg is a prerequisite for association with ribosomes or is a result of that association. VPg is present on nascent RNA chains of the replicative intermediate RNA and on negative-stranded RNA, which has led to the suggestion that VPg is a primer for poliovirus RNA synthesis.[25](Bouslama et al. 2007)..

5`UTR

5`UTR is the most conserved region in the genome. In PV, the first100 nt. play a role in viral replication.The 5`UTR-noncoding regions of picornaviruses are long (624 to 1,199 nucleotides). This region of genome contains sequences, which control genome replication ,translation . The 5`UTR-noncoding region contains the internal ribosome entry site (IRES) (Rousset et al. 2003) [(26)]that directs translation of the mRNA by internal ribosome binding. There are 2 major classes of picornavirus internal ribosome entry site (IRES -RNA pseudoknots).

type I IRES: found in the genomes of PV,other enteroviruses , rhinoviruses.

type II IRES: found in genomes of cardioviruses and aphthoviruses.

The GNRA (G-guanine, N- nucleotide, R-purine, A- adenine) loop and pyrimidine-rich region are conserved in both the type I and type II IRES. But the IRES of hepatoviruses differs from the type I and type II IRES constituting a third class.(Seminars in Virology 1997;8:242,255)(26) .Mirand et al. 2007(27) reported that point mutations in the 5' UTR affects virulence , temperature sensitivity, and plaque morphology (Kung etal. 2006)[28].

Open reading frame

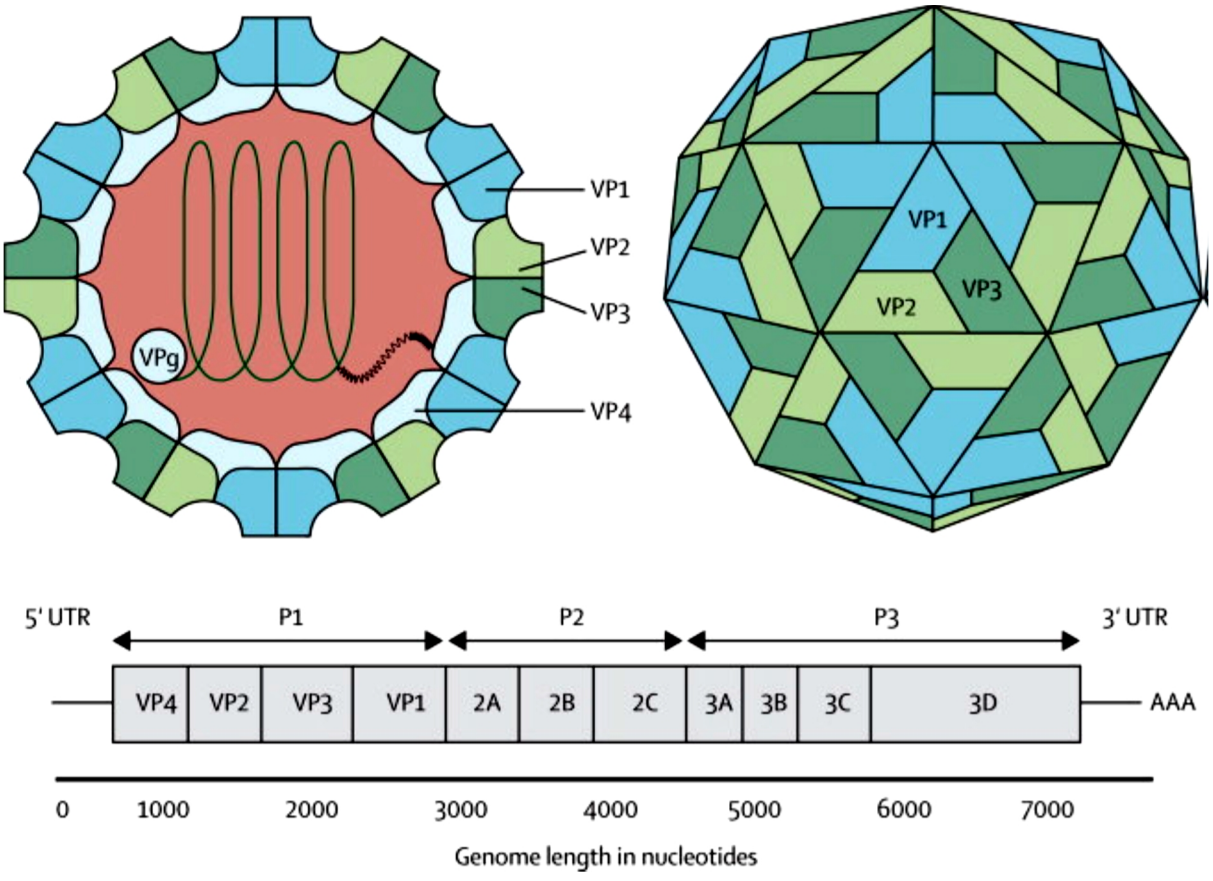


Figure 1: Scheme of the organization of the human enterovirus genomic RNA

Organization of the picornavirus genome:The genome-linked VPg protein is at the 5`UTR end, following the 5`UTR untranslated region, then the protein coding region, the 3`UTR untranslated region, and the poly(A) tail. L ,a leader protein encoded in the genomes of cardioviruses and aphthoviruses but not in other picornaviruses.

- Processing of picornavirus polyprotein: The coding region is divided into three regions, P1, P2, and P3(Gharbi etal. 2006)[29], which are separated by nascent cleavage by two viral proteinases, $2A^{pro}$ and $3C^{pro}$.
- The proteinase of VP0 cleavage not been yet identified.
- Protein **2A**,proteinases that cleaves the polyprotein between proteins VP1 and 2A, and releases the capsid protein precursor (Thompson et al. 2004)[30].

  Proteinase 2A also cleaves the cellular p220 protein, which initiates cap-dependent translation (Tamura et al. 2004)(31).
- The functions of 2B and 2C are not known, but protein 2C and its precursor 2BC have been seen in the replication complex of Polio Virus (Saitou et al. 1987)(32).
- Protein **2C** has a helicase activity.
- Protein 3AB, the small polypeptide is covalently linked to the 5' UTR of picornavirus RNA molecules as studied by Kimura etal. 1980(33).
- Protein **3C** participates in polyprotein processing (Felsenstein et al. 2003)(34), while **3D** is the RNA-dependent RNA polymerase(Kim et al. 2005)(35). The polyprotein is cleaved during translation, so that the full-length product is not observed. Cleavage is carried out by virus-encoded proteinases to yield 11 to 12

final cleavage products. Some of the uncleaved precursors also have functions during replication.

To **unify the nomenclature of picornavirus** proteins, the polyprotein has been divided into three regions: P1, P2, and P3. Aphthoviruses and cardioviruses encode a leader (L) protein before the P1 region.

- The P1 region encodes the viral capsid proteins,
- whereas the P2 and P3 regions encode proteins involved in protein processing ($2A^{pro}$, $3C^{pro}$, $3CD^{pro}$) and genome replication (2B, 2C, 3AB, $3B^{VPg}$, $3CD^{pro}$, $3D^{pol}$).

The **capsid** of poliovirus is composed of 60 protomers,each being made up of one molecule of each of the fourvirion proteins VP1, VP2, VP3, and VP4. The protomersare arranged with icosahedral symmetry. Each of the 20faces of the icosahedron is made up of three protomers,orientated so that the 12 apices of the icosahedron areoccupied by five copies of VP1 while the center of eachface is occupied by three copies each of VP2 and VP3,11–14 nm from the particle center. However, the apices ofthe icosahedron and the face at the threefold axis ofsymmetry form two elevated features separated by a cleft or ‘canyon’ that circles the apex, which, for the EV and Rhinoviruses

The structural organization of the individual proteinsthat make up the virion is similar. Eachprotein has a wedge-shaped core structure composed ofeight strands of protein,arranged in an antiparallel b-sheet array,the eight strands collectively forming a barrel (b barrel (36)). The core structure of polioviruses, rhinoviruses.The features thatare unique to the individual virus, including

antigenic sites, arise from the loops that join the different bstrands and the sequences at the N and C termini.

The narrow end of the b barrel of VP1 is located nearthe icosahedral apex of the virus while the correspondingregions of VP2 and VP3 alternate around the threefoldaxis of symmetry. VP4 forms a lattice around the inside of the pentameric apex. Interactions between adjacent pentamers include regions of secondary structure in theform of b sheets between the b barrels of VP2 and VP1.In addition to the protein components of the virus shell, the N terminus of each VP4 protein is covalently bound to a myristic acid residue (Chow et al. 1987). The myristate sequences penetrate the pentameric apex,possibly forming a framework for assembly. The picornaviruses attach to the cell via the canyon or pit regions.

3`UTR

The3`UTR-noncoding region of picornaviruses is short, ranging in length from 47 nucleotides. At the3`UTR stretch of pol(Bopegamageet al. 2003) (38),a poly adenylated tail is attached(poly A tract)

Recombinant DNA techniques

cDNA copies of genome of picornavirus are generated.it is then used to recover infectious particle either directly from DNA[39](Racaniello and Baltimore 1981) or by linking to transcription vectors like bacteriophage SP6,T7,which can generate infectious RNA.thus it helps to study the effects in a defined genetic manipulation ,on the virus phenotype

3.4 Replication:

Human are the only known natural host for human EVs, and most of their life cycle is similar to polio virus.viral entry into susceptible host cells depends on their specific receptors in host where seven different receptors have been identified for different EVs. EVs can use more than one receptor.

- CD 155 , specific receptor for polio virus,
- three integrins(α2β 1, αvβ 3, αvβ 6),
- DAC- decay accelerating factor (CD55),
- CAR- coxsackie virus-adenovirus receptor,
- intracellular adhesion molecule 1.
- EV 71 specific cellular receptors expressed scavenger receptor B2,humanP-selectin glycoprotein ligand 1 are found on WBC .
- sialic acid linked glycan,expressed in respiratory and gastro intestinal tracts and
- intercellular adhesion molecule-3-grabbing non intregrin(CD209) found only in dentritic cells of lymphoid tissue(Yang B et al 2009,Lin YW et al 2009,ZhouT et al 2006,Tee KK et al 2010, Nobusawa E et al 2006,Kew OM et al 1998). (40)(41)(42)

After EVs **binding** with specific receptors on host cell surface,it triggers conformational changes in the capsid structure and formation of pores occurs in cell membrane that is necessary for the **release** of the genome into the host cytoplasm.

the **polymerase** misincoperates one or two bases in every genome during the coping event,which explains why EVs mutate very rapidly.

The viral **protease 2A** shuts the host cellular protein synthesis. After packaging the progeny viral RNA into viral capsid in the cytoplasm, the mature infectious partical are **released** by lysing the infected cell. Once the susceptible cell is been infected,

RNA translation yields **large polyprotein** which is cleaved after translation into the virus-specific proteins. After infection, the viral RNA becomes susceptible to **RNase** within 30 to 60 minutes. Viral progeny RNA appears in cells within 3 hours after infection. The replication cycle begins when the viral RNA has entered the cell and is immediately transcribed by the viral polymerase beginning at the 3' end of the infecting viral RNA to generate a complementary RNA (cDNA). The viral progeny RNA is synthesized from the cRNA which is host dependent. The newly synthesized viral RNA is covalently linked to the VPg protein at the 5' end of the RNA, and then only the positive sense of RNA is encapsidated in the viral structural proteins to form infectiousviral particles. As the virion **assembly** has started, production of capsid protein andreplication of RNA is closely linked and integeration of viral RNA into the virionfollows within several minutes.

Morphogenesis appears to involve the combination ofviral RNA with a shell of viral proteins (VP0, VP1, VP3) during which the VP0**procapsid** protein is cleaved to yield VP2 and VP4. After final assembly, virions arereleased initially through vacuoles but after several hours escape by cell lysis and death.

Thus, the interval from cell entry to release of virions, invitro may requireapproximately 4 to 5 hours .Interior of the VirionHydrophobic PocketWithin the core of VP1, just beneath the canyon floor of many picornaviruses, is a hydrophobic tunnel or pocket. In poliovirus types 1 and 3, the pocket is believed to contain sphingosine .

An unidentified lipid has been found in human Rhinoviruses types 1A and 16, a C16 fatty acid has been modeled in the pocket of CV B3 and CV A21 is believed to carry myristic acid. In contrast, the pocket of human rhinovirus type 14 is empty .The same hydrophobic pocket is also the binding site for antipicornavirus drugs such as the WIN compounds (Titusville, NJ 1885)[17].drugs in clinical trials such as pleconaril for treatment of common colds caused by rhinoviruses may inhibit either binding or uncoating.

Myristate

Myristic acid (n-tetradecanoic acid) is covalently linked to glycine at the amino terminus of VP4 of most picornaviruses . it is an integral part of the viral capsid.The five myristyl groups extend from the N-termini of VP4 and cradle the twisted tube formed by VP3.The myristyl groups interact with the amino acid side chains of VP4 and VP3. Studies reveal Mutagenesis of VP4, as a role for myristic acid modification in virus assembly and in the stability of the capsid

3.5. Enterovirus Pathogenesis

The pathogenesis of EV infections have been studied at the molecular, cellular, and,organ system (Melnick 1990, Rueckert 1990, Rotbart et al. 1992)(45) . muchhas been learned, much more remains unexplained. EVs are transmitted by the fecal oralroute,respiratory,formites,ocular rarely transplacentally (Konstantinidou A et al)[(46)].

The incubation period vary from 3 to 10 days virus sheds up to 2 weeks from the nasopharynx and for several weeksto month in the feces. Most of the virus spread within the host was derivedfrom experimental poliovirus infections in chimpanzees more than four decades ago(Bodian 1955)(47). some replication occurs in the nasopharynx

spreading to upperrespiratory tract lymphatics,most of the virus is swallowed. Their Stability at acidic pH, is responsible for the EVs to transverse stomach to move to the site of primary infection in the lower gastrointestinal tract, distinguishes the EVs from the human Rhino virus(48).The virus traverses the lining cells, perhaps with replication but withoutapparent cytopathicity, and reaches the Peyers patches in the lamina propria, where significant viral replication occurs. A minor viremia ensues seedings numerous organsystems including the central nervous system (CNS), liver, lungs and heart. More significant replication at these sites results in a major viremia associated with the signs and symptoms of viral infection.

PVs may spread to skeletal muscle via the blood,reaching neuromuscular endplates from which the viruses ascend along nerves to the spinal cord and from there may widely disseminate within the CNS. The target tissue infected by the EVs determines the predominant disease caused by the virus. For the nonpolio EVs "leakiness"(50) in the vessels of the choroid plexus (meningitis)or of the parenchyma (encephalitis) is probably the patch by which viruses enter the CNS from the blood, probably influenced by the titer of virus in the blood, active transport ofvirus particles across the blood brain barrier has not been demonstrated. Endothelial cells may express EV receptors, which may influence tissue tropism (Huber et al. 1990)[51] andwhich if up-regulated e.g. during systemic EV infection, may facilitate virus entry intothe CNS and other organs. During clinical infections, EVs have been recovered from both

the cellular and plasma fractions of the blood (Parther et al. 1984)[52],

The virus binds to a **specific cell receptor** at a single viral capsid canyon site (Rotbart et al. 1992, Racaniello 1995)(53-54) this probablyoccurs first in the intestine, and with

subsequent progression of infection, it occurs in other target tissue sites. The human cellular receptor for PV and certain other EVs mapsto chromosome 19 (Rotbart et al. 1992, Racaniello 1995) and sensitivity of cells to PVinfection has been sublocalized to the proximal long arm of that chromosome (Siddiqueet al. 1988)(55). The PV **receptor** has been well characterized and represents a uniquemolecule within the "immunoglobulin superfamily" (Rotbart et al. 1992, Racaniello1995). Competition studies have shown that the PV, CV B1-B6, and CV A21 virus mayeach represent different receptor families (Lonberg-Holm et al. 1976)(56). At least one ECV serotype (ECV 1) binds to a cellular receptor which is a member of the integrin family of surface molecules known as decay-accelerating factor, a glycoprotein that protects cellsfrom complement-mediated lysis (Bergelson 1994)(57). Still other ECV serotypes do not bindto either the integrin or decay-accelerating factor molecules (Racaniello 1995).Preliminary binding studies also have revealed putative specific receptors for CV A and B serotypes (Bergelson et al. 1994, Roivainen et al. 1994, Racaniello 1995) it appears asif certain adenoviruses (Bergelson et al. 1994)(58). Following **attachment** of the virus,recruitment of additional cellular receptor occurs, and the virion is enveloped by cell membrane, bound new at multiple viral protomers (Rotbart et al. 1992; Racaniello 1995).A steric shift in the **capsid confirmation** occurs, resulting in extrusion of the VP4 viral protein and destabilization of the capsid structure (Guttman et al. 1977)(59). The now **uncoated RNA** is released freely into the cellular cytoplasm, where it rapidly binds to theribosomes and begins protein synthesis. A **single polypeptide** is produced, which isalmost in stantaneously **autocleaved by viral proteases** (Haller et al. 1995)(60) to

form all of the viral protein products, including those, such as the RNA-dependent RNA polymeraserequired for viral RNA replication. After 2 hr infection, all host cell protein synthesis hasbeen shut down by the EV and the cell has become a factory for viral production (Halleret al. 1995)(61). **Infectious virion cells are released** by cell lysis and spread to neighboring and distant cells via the surrounding growth medium in vitro and the blood in vivo. The **cytopathic effect** of the EVs in tissue culture cells has been well described (Hsiung 1973)(62)and remains an important diagnostic tool. Electron microscopy shows a characteristic **rounding of cells** and detachment from the tissue culture dish. a series of change occurs, alteration of nuclear morphology, chromatin margination, Ribosomal aggregates in the cytoplasm and clusters of membranous vesicles. the rounded and detached cells lysate membranous vesicles serve as the platform for viral RNA replication.

While 3serotypes of wild type PVs are neurotropic and neurovirulent, specific tropisms and virulence patterns vary among non polio Entero virus NPEVs, with certain serotypes consistentlyreported to cause specific organ disease. The common serotypes associated with CNS infections are PVs, ECV 7, 9, 11, and 30, CV B5,and EV 70 and 71 (Strikas et al. 1986, Dagan et al 1988, Rotbart 1995) (63,64,). CV in heart infection (Martino et al. 1995)(66). ECV 11, followed byseveral other ECV and CV, are the most important pathogens of neonatal EV sepsis(Abzug et al. 1999)(67) ECV 11 a common serotype causing chronic meningoencephalitis in antibody-deficient patients (Mckinney et al. 1987)(68). The presence orabsence of specific cellular receptors is unlikely to explain all heterogeneity sincevariation occurs in tropism exists even within receptor families. CV B6, for example, is a rarecause of CNS or cardiac infections, in contrast

to the other five CV B1-B5 (Melnick 1990, Martino et al. 1995). Molecular neurovirulencedeterminants among the NPEVs are now being studied in hopes of finding a genomic explanation for the increased frequency of aseptic meningitis and encephalitis observed with certain serotypes and the virtual absence of those diseases with others (Rotbart1995). CV B4 strains with pancreatic tropism and virulence are distinguished from virulent CV B4 strains by a single amino acid residue in the VP1 capsid protein (Coggana et al. 1993)(69). One mechanism of CV induced cardiomyopathy may involve the ability of the 2A protease molecule of the virus tocleave dystrophin, a cytoskeleton protein found in the heart (Badroff et al. 1999)(70).

3.6. Clinical manifestations

3.6.1. Aseptic Meningitis and Encephalitis

Rotbart 1990 in his studies showed Aseptic meningitis , a nonbacterial inflammation of meninges associatedwith acute onsetfever, headache, photophobia, and meningeal signs in the absence of signs of

parenchymal involvement .Usually negative for routine bacterial and fungal culture and CSF showing pleocytosis. Pallansch et al. 2006 studies showed EVs, the most common CNSinfection . Fever is the most common clinical symptom ranging from 38˚C-40˚with biphasic pattern, constitutional symptoms irritability anorexia, muscle aches, gastro intestinal, respiratory or lethargy and then meningeal signs (brudzinski and kernig signs)in patients with enteroviral aseptic meningitis (70,71)(Dos Santos et al. 2006, Mistchenko et al. 2006, Brunel et al. 2008). Rashes, low grade fever,sore throat, cough, photophobia, were also reported in enteroviral aseptic meningitis cases

caused by ECV 4, 6, 9 and 16 and CV A5, A9, A16 (Dos Santos et al. 2006, Frantzidou et al. 200(80). EVs are recognized as the main cause of aseptic meningitis in both children andadults in developed countries, and is identified in 85% to 95% cases in which a specific pathogen was cultured studies by Cabrerizo et al. 2008, Choi et al. 2010, Cui et al. 2010).62% infants less than 3 months old having asepticmeningitis had CV B was reported by Frantzidou et al. 2007). Enteroviruses are the most common cause of viral meningitis,which accounts for 80-%92% of aseptic meningitis.Mostof the EVs serotypes have been reported in aseptic meningitis cases (Avellon et al.2006, Zhao et al. 2006, Frantzidou et al. 2007, Brunel et al. 2008, Logotheti et al. 2009,Trallero et al. 2010)(80-85).some children present with neckstiffness and bulging fontenelle and less than 10 % presents with seizures.Acute illness has good prognosis and resolves usually without any consequences in 1-10 days with malaise and fatigue persisting for few weeks. ECV,CV A,CV B,EV are the non polio Enterovirusescausing aseptic meningitis. However the infection may progress tosymptoms of Encephalitis in some cases signifies that the brain parenchyma is infected, with the possibility ofdisturbed state of consciousness, focal neurological signs, and seizures. Encephalitis is typically a rare complication of EV infection and often presents in conjunction with ameningeal infection, resulting in a meningoencephalitis (Katz et al. 1998)(87). Sometimes EVencephalitis may also present as focal encephalitis, suggestive of HSV encephalitis(Peters et al. 1979, Whitley et al. 1989, Modlin et al. 1991)(88). In neonates, EVs infections are associated with serious complications, including encephalitis and death(Rotbart et al. 2001, Khetsuriani et al. 2006)(89). A study in New York State USA during 2005-06,Prevalence of EV aseptic meningitis/encephalitis cases was found

to be 4.9% prevalence with the most prevalent serotype CV B5, ECV18 and 6 (90)(Tavakoli et al. 2008). a fatal case ofleukoencephalitis associated with ECV 18 was reported(Brunel et al. 2007). A study from Greece, during 2003-2006, reported 22% prevalence of EVs aseptic meningitis and 9% with encephalitis cases (Frantzidou et al. 2008). ECV 9 associatedencephalitis in children from Kuwait were reported (Dalwai et al. 2009). EV 71 encephalitis cases have been observed among youngchildren in Taiwan and some other countries (Lin et al. 2002).NPEVs have been associated with outbreaks of paralytic myelitis, which usually resolves spontaneouslyincluding CV A7 (Grist et al. 1984), EV 70 (Wadia et al. 1983()91), and EV 71 (Solomon etal. 1959, Huang et al. 1999). Sporadic cases of paralysis have been reported inassociation with isolation of an EV from the stool (Grist et al. 1984, Cherry 1992), due to the known prolonged fecal shedding period of the EVs, asituation in which it is difficult to establish. Cerebellar ataxia, GuillianBarre syndrome and transverse myelitis rarely have been associated EV infections (Cherry 1992). All these associations suffer fromthe same difficulty in differentiating the pathogen of a throat or stool isolate from coincidental shedding.

3.6.2. Neonatal Sepsis

neonates appear to be at greatest risk for severe morbidity and mortality with EV infection signs and symptoms develop during first few days of life either during intrapartum or perinatal (Abzug et al. 1968, Abzug et al. 1999).59 to 68% of infected neonates are due to maternal infection (Abzug et al. 1968,Abzug et al. 1999). The timing between maternal infection and delivery appears to be critical; when there is enough time for antibody formation in the mother, positive protection of the baby occurs. But If, delivery occurs during maximal viremia and prior to adequate maternal

antibody formation, the prognosis is worse for the neonate (Abzug et al. 1968, Abzug et al. 1999). fever is ubiquitous, even in the youngest patients, with early nonspecific signs such as rash, vomiting, anorexia, and upper respiratory findings. Neurologic involvement may or may not beassociated with signs of inflammation of meninges, including nuchal rigidity and bulging of anterior fontanelle. As the neonatal disease progresses, major systemic manifestationssuch as hepatic necrosis, myocarditis, and necrotizing enterocolitis may develop (Abzuget al. 1999). DIC and other findings of "sepsis" resultin a patient with illness which may be indistinguishable from that of bacterial infection.

3.6.3. Nonspecific Febrile illness

EV infections are characterized by fever and nonspecific symptoms with or without rashes occurring subclinically annually (Moore 1982, Strikas et al. 1986). These illnesses are of significance mainly in other diseases that they mimic, including bacterial sepsis, viralexanthems and herpes simplex virus infections. Their age distribution is of clinician main concern . Most affected are younginfants in whom differentiation of viral illness from the more alarming cause of nonspecific fever and rashes is extremely difficult. Clinical manifestations include abrupt onset offever usually >39°C, irritability and fever may be biphasic (Dagan etal. 1995.). Additional symptoms, in order of decreasing frequency, include lethargy, anorexia, vomiting, diarrhea, rash, and respiratory symptoms. Signs and Symptoms do not differ in the age group between the ECV and CSV(Dagan et al. 1995)(91). Aseptic meningitis may accompany the nonspecific symptoms of EV infection in infants, and there are no clinical features which distinguish between EV infected infants with and without meningitis (Dagan et

al. 1988). The systemic, global nature of this illness results in hospitalization of many of these infants to the rule out bacterial sepsis. The duration of symptomatic illness in young infants beyond the neonatal period is usually 4 to 5 days.

3.6.4. Respiratory illnesses

EV infections causes nonspecific respiratory sign andsymptoms, which are usually mild. Pharyngitis, tonsillitis, and laryngotracheobronchitis(croup) (Chonmaitree et al. 1991). Bronchitis andpneumonia are less commonly seen. The EVs are responsible for about 15% of upper respiratory infections (URIs) for which an etiology is identified (Chonmaitree et al. 1995)Many EV serotypes are identified inrespiratory infections (Gristet al. 1978, Moore 1982). The clinical manifestations of EV associated URIs, are indistinguishable from other respiratoryviruses which includes fever, apnea, and cyanosis. . Pneumonia due to the EVs has been associated with numerous serotypes ininfants and children (Cherry 1992).(94)The laboratory findings usually are a normalleukocyte count although extreme leukocytosis is occasionally encountered. Chest X raymay show perihilar infiltrates. mortality in infants and young children seen.Histopathology of the lungs reveals thickening and infiltration of the alveolar septabut no necrosis or giant cells. In adults, bronchopneumonia associated withCV B3 and ECV 9 is seen(Cherry 1992, Chonmaitree et al. 1995).

3.6.4. Herpangina

Group A CV is the most common causes of herpangina, but the syndrome hasbeen reported with the coxsackie B viruses and the echoviruses as well (Cherry et al.1965). The highest incidence is among children 1 to 7 year old (Pichichero et al. 1998)(96),but infection has also been described in neonates and adults. There is usually an abruptonset

of fever associated with a sore throat, dysphagia, and malaise. One fourth of thepatients may have vomiting and abdominal pain. Early in the illness, grayish whitevesicles measuring 1 to 4 mm in diameter appear over the posterior portion of the palate, uvula, tonsillar pillars, and occasionally the oropharynx. These vesicles are discrete, surrounded by erythema and usually number fewer than 20. The vesicles usually rupture, leaving punched out ulcer that may enlarge slightly, while new vesicles may appear.

There may be also mild cervical adenopathy, headache, myalgia, arthralgia, and rarely parotitis or aseptic meningitis. Coryza and other respiratory symptoms are lacking and clinical laboratory studies are usually normal. The fever lasts 1 to 4 days; local andsystemic symptoms begin to improve in 4 to 5 days, and recovery is usually complete

within a week of onset (Parrott et al. 1951, Cherry et al. 1965, Pichichero et al. 1998).

3.6.6. Hand-Foot-and-Mouth Disease

Contagious disease which is characterised by pharyngeal , oral ulceration ,vesicular rashes of palms and soles that heals without crusting. This differentiates them clinically from from the vesicles of pox ,apathous ulcer and herpes.in varicella lesions seen in trunks and rare in palms an soles and orally.it is associated with CV A16 the most common, other serotypes like CV B1,EV 71 ,CV A6 are often isolated (Cherry 1992). children younger than 5 years are affected but can spread to adults too. The disease is usually mild, and the onset is associated with asore throat with or without a low grade fever. Usually mild,self limiting, resolve by one week. Rarely palmar, plantar and groin lesions appear, particularly those due to EV 71, have been associatedwith

diffuse systemic and neurologic disease like aseptic meningitis, encephalitis, AFP, death(Ho et al. 1999, Huang et al. 1999).

3.6.7. Pleurodynia(also known as Epidemic myalgia, Bornholm disease, Devil's grip)

Fever and severe paroxysmal stabbing chest pain oftenreferred to the lower ribs or the sternum of abrupt onset (Kantor et al. 1962, Grist et al. 1978, Ikeda et al.1993) or preceded by prodromal symptoms like headache, anorexia, malaise, vague myalgia lasting 2 days to 14 days. CV B members are the

usual causes of pleurodynia. Deep breathing, coughing, and other movement accentuates the pain, which isdescribed as knife like stabbing, catching or smothering. Abdominal pain seen in half of the patients but may be chief complaint in children. Fever is usuallyabout 38ºC -40ºC. Muscle tenderness is usually not prominent. There is no frank myositis ormuscle swelling. The chest X ray is typically normal it is often self limiting with complete recovery .relapses can occur

3.6.7. Acute Hemorrhagic Conjunctivitis (AHC)

AHC is a highly contagious disease of eye characterized by conjunctivitis, keratitis, foreign body sensation, pain, respiratory symptoms and associated severe neurological symptoms including acute flaccid paralysis (Abzug et al. 1968). After an incubation period of 24 hours it is characterised by sudden onset of bilateral conjunctivitis, with congestion, photophobia,eye discharge and pain.CV A24 EV 70 are the main enterovirus serotype associated with outbreaks of acute subconjuctival hemorrhagic conjunctivitis worldwide, which may be pin point or large. This may or may not be associated with general symptoms like fever, headaches It usually resolves by 10 days without any permanent sequlae, but can rarely cause paralysis. However, echovirus 7

and 11, coxsackievirus B1 and B2 have been also reported in conjunctivitis cases (Abzug et al. 1995).CV A24v, an antigenic variant of CV A24 strainwas first isolated in Singapore from an outbreak of AHC in 1970 (Abzug et al. 1999).

It has caused several epidemics and outbreaks of AHC in different countries of the world including India, China, Nepal, Malaysia, Taiwan, Korea, Japan, Caribbean, Tunisia, Spain, Cuba, Brazil and France. In India, CV A24v has been reportedin several epidemics and outbreak of AHC in different part of the country including Vellore (1979), Delhi (1988), Uttar Pradesh (1994), Chennai (1999), Gujarat and Maharashtra (2003) and Mumbai (2007) since after the first epidemic in 1971 (Bodian 1955, Caggana et al. 1993).

3.6.8. Acute Myocarditis

The EVs are one of the most commonly identified etiologies of viral myocarditis, affecting the serous layer of pericardium and heart muscle. About 25-35% of cases of EV myocarditis is found , based on serologic, nucleicacid hybridization, and PCR based studies of end myocardial biopsy and autopsy specimens (Martino et al. 1995).CV B(60), being more common with myocardial involvement, CV A and CV B combination can also cause myocarditis. Clinical manifestations reflect the regions and extent of the cardiac involvement. Symptoms may vary from mild palpitations , chest pains, arrhythmias ,congestive cardiac failure(61),sudden loss of consciousness due to abnormal rythm of heart ,sudden death reflect prominent involvement of the conducting system.

Electrocardiographic findings include evolution of early S-T segment elevation and T wave inversion to intermediate stage normalization and then late stage recurrence of T

wave inversion (Matrino et al. 1995). ECHO finding most commonly is suggestive of global akinesia,left ventricular dilation and left ventricular dysfunction(64)blood tests detects Myocarditis enzyme. Magnetic resonance imaging(MRI) may help in establishing diagnosis of myocarditis. End myocardial biopsy remains the "gold standard" for confirming the histopathologic diagnosis of myocarditis (Matrino et al. 1995).Most patients recover uneventfully, many have residual ECG abnormalities for months to years. Smaller percentages of patients develop congestive heart failure, chronic myocarditis, or dilated

cardiomyopathy(97).

3.6.10. Gastrointestinal illness

EVs derive name from their site of replication and shedding in the gastrointestinal tract. but enteric illness (vomiting and diarrhea) is usually a minor manifestation of EV infections (**Cherry 1992**). Neither CV nor ECV have been epidemiologically implicated as important primary causes of acute gastroenteritis. Therefore Isolation of an EV from the feces of a patient with gastroenteritis must be interpreted with caution. The CV B have been rarely associated with acute abdominal pain and mesenteric adenitis, which may mimic acute appendicitis. (Cherry 1992).

3.6.11. Juvenile-Onset Diabetes Mellitus

The worldwide incidence ranges from 3 - 30per 100,000 persons per year. Many serological studies have found high titers of antibodies to CV B in children with diabetes than in controls (Barrettconnor1985, Rewers et al. 1995). An occasional patient dying of diabetic ketoacidosis had CV isolated from the pancreas and elsewhere at

autopsy (Yoon et al. 1979). EV RNA has been identified by PCR in the serum of children with new onset diabetes mellitus (Clernents et al. 1995).Maternal EV infections during pregnancy has been correlated with the later development of diabetes in offspring (Dahiquist et al. 1995). Occasional patients have developed anti islet cell antibodies in close proximity to acute EV infection (Lonnrot et al. 1998). observations from animal model studies of diabetes suggests that an EV infection in a genetically susceptible host results in an exaggerated autoimmune response and destruction of pancreatic islet cells (99)(Rewers et al. 1995), (Barrett-Connor 1985).

3.6.12. Inflammatory Myositis

Like an autoimmune response to EV infection is felt totrigger diabetes , there is also evidence for such a mechanism in dermatomyositisand polymyositis with serologic studies (Travers et al. 1977). In addition, EV like particles in muscle biopsy specimens has been seen by electron microscopy from these patients (Chou et al. 1970). PCR ,dot blot hybridization, in situ studies of muscle biopsy suggest the presence of EV RNA (Rosenberg et al. 1989). But other investigators, failed to find EV using the same molecular techniques (Leon-Monzon et al. 1992).50% of agammaglobulinemic.

Patients with chronic CNS EV infections also develop myositis (McKinney et al. 1987). In these patients, cultivable virus was recovered from muscle-tissue.

3.7 Spaces and coverings of CNS :

Brain and spinal cord have 2 protective coverings the outer bone and inner membrane covering the meninges. meninges is collectively of 3membrane layers surrounding the brain and spinal cord.

Anatomical structures	locations	Key features
Epidural space	Inside the skull, outside the dura	Connective tissues and fat cushion
Dura mater	outer most membrane	White fibrous tissue, adheres to skull
Subdural space	Between dura mater and arachnoid membrane	Lubricating serous fluid
Arachnoid membrane	Between duramater and piamater Arachnoid villi absorbs CSF	Delicate cobweb like membrane covering brain and spinal cord
Subarachnoid space	Below the arachnoid membrane	CSF 125-150ml in adults
Piamater	Below the subarachnoid space	Adheres to the brain and spinal cord outer surface ,blood vessels +

Pia and arachnoid membrane together is called leptomeninges CSF Choroid plexus present in lateral ventrical,3rd and 4th ventricles of brain secretes CSF at the rate of 500ml/day.it carries essential metabolites and removes wastes as it circulates the brain,ventricles and spinal cord as it moves within the subarachnoid space.the entire volume of CSF is being exchanged every 3-4 hours. CSF circulation provides us valuable information about chemical and cellular changes if any infection in

subarachnoid space. Normal csf pressur 65-195mm of water average 130mmof watrin lying position in normal healthy person. csf absortion is by arachnoid villi which acts like valves which allows CSF TO FLOW into the blood of venous sinuses but prevents backward flow of blood ,this valvular like action happens when SF pressureis 1.5mm Hg greater than the blood pressure in the venous sinuses

Routes of CNS infection

The BBB-blood brain barrier maintance homeostasis of brain by restricting the chemicals, bacteria, fungi, virus of blood entering the CNS. For any infection the BBB must be penetrated

Haematogenous spread(common)

Ddirect extension from infected site

Anatomic defectsin CNS structures

Direct intraneural,it travels along nerves(rabies ,HSV)

Disease of CNS

1.Meningitis

2.Encephalitis/Meningoencephalitis

3.Brain abcess

1.Meningitis: meningitis is the infection within subarachnoid space or throughout leptomeninges. Based on host response to microorganisms it is divided into

a**.purulent meningitis**- mainly bacterial causing acute inflammatory exudates and PMNL. ventricles are usually affected, **in neonates**-E.coli, Listeria monocytogenes, before Hib vaccine-Haemophilus influenza type b, less commonly by Elizabethikingia meningoseptica (nursey out breaks) **in adults** N.meningitidis, Streptococcus

pneumonia(PCV13-children and adults,PPSV23- for adults >65yrs and children over 2yrs-protects),less commonly Staphylococcus aureus, Klebsiella, Pseudomonas species, Acinetobacter, Salmonella Sps. Spirochetal meningitis by Treponima Palladium, Naegleria fowleri, Acanthamoeba, Balamuthaiya cause Meningoencephalitis, Angiostrongylus cantonensis, Gnathostoma spp, Baylisascaris procyonis, Toxacara, Taenia solium cause esinophilic meningitis

b.**aseptic meningitis:** usually viral characterised by increased in lymphocytes and ther mono nuclear cells(pleocytosis)in CSF, but glucose levels remain normal or slightly elevated. bacterial and fungal cultures negative.it is usually self limiting.Symptoms may headache, fever, neckstiffness, nausea, vomiting

fever: sudden onset, may range from 38-40 ˚C.

Headache: usually frontal or retro orbital, may be associated with photophobia, pain on moving eyes

Neck rigidity: neck resist passive flexion indicating meningeal irritation.2 classical signs.

Kernigs sign-thigh is flexed on the abdomen, with the patient in supine position. When flexed knee attempts to extend, pain is elicited if meningeal irritation present.

Brudzinskis sign-when neck is flexed, spontaneous flexion of hips and knee is elicited in patient lying supine.

Seizures :frequently occurs in encephalitis cases were brain parenchyma are affected.it can be general, focal or status epilepticus

Altered sensorium: depressed level of consciousness ranging from mild lethargy to coma, hallucination, agitation, personality changes, behavioural disorder and psycoticstate. coma, marked confusion suggests encephalitis.

Focal neurological deficit: aphasia, ataxia, involuntary movements, upper motor or lower motor neuron weakness, cranial nerve deficit

Signs of increased ICP-deteriorating level of consciousness, papiloedema, dilated poorly reactive pupil,6^{th} nerve palsy, decerebrate posturing, CUSHING reflex(irregular respiration, bradycardia, hypertension).cerebral herniation is a diasterous complication.

Clinical triad of meningitis: fever, headache, nuchial rigidity, may not present in all cases. But in more than 75% patient level of consciousness may vary from lethargy to coma .nausea, vomiting, photophobia.

CSF collected from arachnoid space. a sterile wide bore needle inserted between fourth and fifth lumbar vertebrae and CSF is allowed to drip into a dry sterile container. A ventricular puncture may sometimes performed to collect CSF from infants (monica cheeseburg vol.1 pg116).advise should be given to report to laboratory before performing lumbar puncture, so that they will be ready to receive and examine the sample immediately. Any delay in examining CSF will result in lower WBC counts and falsely low glucose content due to glycolysis

Take 3 sterile ,dry ,screw capped containers with no additives.

- label no.1 and collect 1ml CSF -for chemistry studies ,glucose, protein count, immunology studies. These tests are not affected by blood or bacteria that may result during spinal tap procedure.

- label no.2-for microbiology culture, large fluid can be concentrated which can help detection even if low number of agents present

  label no.3-for cell count and differential. If any capillary blood due to traumatic spinal tap wont be present in the 3 tube which will help us rule out intracranial haemorrhage.

  sent samples with request to laboratory

  Report Appearance of CSF

  CSF is reported normal when it appears clear, contains no more than 5 WBC $*10^6$ /L(CSF begins to appear turbid >200 WBC$*10^6$ /L and protein concentration not raised by pandy test-measures total globulin in areas where total proteins cant be measured

  Clear ,slightly turbid,definitely purulent looking(don't centrifuge if pus looking)other wise centrifuge label no.2 sample one part, take supernant and use for serology and to check glucose and protein,do gram stain with sediment smear after alcohol fix. If CSF contains blood-could be traumatic or subarachnoid haemorrhage (after centrifuging-if its yellow red-xanthochromia-cns haemorrhage)If CSF contains clot-high protein(>20mg%) concentration with increased fibrinogen. It can occur with pyogenic meningitis ,cob web appearance on CSF standing for 24 hours- TB meningitis) Uncentrifuged CSF- for cell count studies

  Differential diagnosis of acute meningitis and meningo encephalitis

  non polio enteroviruses, Herpes viruses: HSV, VZV, CMV, EBV Arboviruses: JEV, dengue, West nile, St. louis encephalitis virus, Chikungunya, Scrub typhus, Leptospirosis, Cerebral malaria, Acute encephalopathy: Typhoid, Shigella, Varicella, Reye syndrome, Immune mediated: ADEM—acute demyelinating encephalomyelitis(post measles/post varicella encephalitis, Neuro imaging of

Enterovirus encephalitis has characteristic lesion locations in the posterior portions of the brain stem, substantia nigra, dentate nucleus and within the anterior horns of spinal cord. Recognition of these findings in the presence of suggestive clinical presentation can help to establish the diagnosis of enterovirus encephalomyelitis.

3.8. Laboratory Diagnosis

Specimen collection

EVs are mainly transmitted by respiratory and fecal-oral routes and therefore respiratory epithelium and GIT are the primary site of EV infections .stool, rectal swabs, CSF, throat swabs, conjunctival swabs, blood(serum, plasma),BAL, brain tissue, heart tissue and fluid, lesion material depending on type of infection which varies from often mild to serious disease like aseptic meningitis, encephalitis, paralytic poliomyelitis, pericarditis, myocarditis, respiratory illness. specimen should be collected within 4 days of onset of onset of symptoms. But viral shedding in faeces may be upto 1month,and more than 2weeks post onset in respiratory specimens. Except for faeces and body fluids all specimens should be transported in VTM. Sterile plastic screw cap containers should be used. Specimen should be transported immediate or in ice packs

3.7.1. Viral Isolation

Isolation of EVs by cell culture remains the gold standard for diagnosis. the Nobel Prize receivers John Enders and his colleagues(1954) successfully propagated PV in a continuous cell culture, paved the way for vaccine development (Enders et al. 1989).Not all EVs can be isolated by cell culture, many of them grows in a range of mammalian cell lines and a single cell line is not optimal for all EV serotypes. Few of them include

- primary African green, rhesus monkey cynomolgus monkey kidney cells :the traditional first choice for EV isolation, have good sensitivity for PV, CV, and ECV.
- RD cells line :derived from a human rhabdomyosarcoma are the most sensitive for detection of Coxsackievirus group B strains, EC V and PV (73).
- Madin darby canine kidney (MDCK)
- Human dipoid cell lines(MRC-5,WI-38,SF)
- Human embryonic kidney (HEK)
- Human embryonic fibroblast(HEF)
- Human epithelial carcinoma(HEp-2)(Cincinnati-PV,CV B grows well in this cell line)
- L20B cell line: a genetically engineered mouse cell line expresses human poliovirus receptor, helps PV detection.

All speciemens should be inoculated in each cell line chosen, in duplicates and examine daily for any charactreristic CPE like visible cell rounding, refractility, degeneration or detachment from surface, destroying the entire monolayer of cells. Maintenance, purity, authenticity, stability of cell lines should be given strict attention because of contamination with bacteria, fungi which will kill the cell lines and mycoplasma contaminates cell culture without inhibiting cell growth. Other viruses can contaminate cell lines producing their CPE. But isolation and recognition of CPE requires a high level of expertise and is labor-intensive. Some EV serotypes like CV group A, do not grow in cell culture (75)(Melnick et al. 1964, Hsiung 1973,Lipson et al. 1988, Rotbart 1999). And about 25 to 25% specimens from characteristic EV infection patient of any serotype will be negative by cell culture(76) (Chonmaitree et al. 1982) because of antibody neutralization in the body. Other causes could be ofinadequate collection,

handling , processing of the samples or intrinsic insensitivity to the cell lines, used. It is reported that mean isolation times for EVs from CSF range from 3.7 to 8.2 days (Rotbart et al 1999) .rhino virus(RV) having similar CPE,but are acid labile ,grows at 30˚C,and grows well in MRC-5,IFA cant be used to confirm RV in cell culture but PCR can be used. HAV different from other picornaviruses having trophism for liver tissue is also transmitted faeco orally . they are heat stable and are the only group of hepatic viruses that can be grown in culture.

The EV growth isolation and identification in cell culture is based on appearance of characteristic CPE, clinical history, source of sample, and is confirmed by IFA and neutralisation using serotype specific antisera or intersecting pools of antisera (lyophilized).

Shell vial culture method

1.Virus isolation should be done into three continuous cell lines; RD (human rhabdomyosarcoma), HEp2 (human epithelium larynx) and L20B (mouse fibroblast cells expressing the poliovirus receptor CD155) to maximize EV detection.

2.All three cell lines should be grown in Eagle's minimal essential growth medium (EMEM) containing 10% foetal calf serum with penicillin and streptomycin (0.1 mg/ml each) in flat-bottom culture tubes).

3.After development of confluent monolayer, growth medium should be decanted from culture tube and samples should be inoculated.

4.The inoculated tubes were then centrifuged at 1000 × g for 30 min at room temperature and incubated for further 30 min at 36.5°C.

5.After incubation, 1 ml of EMEM containing 2% foetal calf serum (maintenance medium) should be added in each tube and

6. again incubated at 36.5°C for 2 days.

7.The tubes should be observed on the next day for bacterial contamination and toxicity. After 2 days all the tubes were kept at -70°C for RNA extraction

Organ suspensions for virus isolation in tissue cultures

1.Snap-frozen tissues were freeze–thawed twice

2.10% suspensions in PBS were prepared by sonification (Bopegamage et al., 2003)(98).

3. Penicillin (50 units/ml) and 40 mg streptomycin were added.

4.Suspensions were incubated overnight at 4ºC, centrifuged at 1500 *g* for 30 min at 4ºC, passed through a 0.45 mm Millipore filter and frozen at -80ºC.

Quantification of infectious virus in organs

1.Tenfold serial dilution of the sera and organs should be prepared.

2. add 100 µl should be added (eight wells per dilution) to monolayers of HEp-2 cells grown on 96-well flat-bottom microtitre plates

3. incubated at 37ºC in a CO2 incubator.

4.Virus titres should be expressed as TCID50 values, calculated by the Karber method.

5. The results should be read at 4–7 days after infection.

6.To confirm that CPE was not due to toxicity, each sample should be passaged further in HEp-2 cells

General consideration of central nervous system:infections involving CNS is considered medical emergency, a basic understanding of anatomy and physiology will help us the microbiologist to ensure appropriate specimen collection, processing and interpret laboratory results

3.7.3. Serotyping and Serologic Assays

- **Neutralisation**: Having more than 100 EV serotypes, neutralisation with type specific antisera would be very difficult. Then Neutralisation with intersecting pools of lyophilized antisera, the gold standard for serotype determination was prepared by mixing different combination of individuals antisera and type specific equine hyper immune sera . The EV isolates should be incubated with each antisera pool one by one and again inoculated into suitable cell line and after several days of incubation, neutralisation pattern distinct for each EV serotype is seen in the designed pools. neutralisation with type specific confirms the serotype (established by Lim and Benyesh Melnick-Melnick et al. 1973).it is also called **LBM pool antisera** scheme having 8 different pools(A-H),containing anti sera against 42 enteroviral serotypes(75)and additional 7pools(J-P) containing antisera against 19 CV A serotypes , which is used to identify the non type able viruses.(95)) . Antisera pool developed by national institute of public health and environment were used to identify EV 68-71(96).being expensive, time consuming process, with frequent isolation of untypeable EV strains mainly because of new emerging serotypes which are not included in intersecting anti sera and according to WHO not all clinical isolates should be tested with antisera, limits the use of intersecting antisera pool for sero typing of EV

- **IFA** indirect immunofluorescence assay is simple and rapid than the traditional neutralization test used for serotyping.(97).type specific monoclonal antibody binds to specific serotype, detected by fluorescent conjugate. developing monoclonal antibody for more than 100 serotypes is expensive and practically very difficult with the ever emerging EV serotypes
- **immunoassays** plays a limited role and is complicated in EV diagnosis by the presence of large number of serotypes , the lack of a common single antigen,

  . If EV is recovered in patient feces or throat with unusual clinical manifestations, fourfold rise in EV antibody titer to that serotype in paired sera is diagnostic. In patient with suspected EV meningitis or other acute illness, serologic testing will not be useful.

Virus titration

1.Virus titre should be measured on RD/HEp-2 cells by endpoint titration as per standard procedure.

2.In brief, RD/HEp-2 cell should be grown in 96 well (103 cells per well) tissue culture plates

3.Ten fold serial dilutions of the virus through 10−9 were made in 2% MEM.

4.To the 65-70% confluent cell monolayer, 100 µl of each serial dilution was inoculated into four wells of each well and

5.plate was incubated at 36.50C with 5% CO2.

6.Plain MEM should be used as a negative control. After 5 days of incubation, the titres should be determined by an endpoint micromethod and should be expressed in log10 .50% tissue culture infective dose units (TCID50) per ml.

MTT Assay

MTT measures mitochondrial enzyme activity inside living cells. The quantitative measurement of cell damage (cytotoxicity) should be estimated by cell viability using the MTT assay (Mosmann et al. 1983)(101)

1.cells maintained after 24 hr post infection should be incubated with 50 µl of MTT (1 mg/ml in PBS) for 4 hr.

2.After 4 hr the media was discarded from the plate, and 50 µl of dimethyl sulfoxide (DMSO) should be added to the wells and mixed thoroughly to dissolve the dark blue formazan crystals produced by viable cells.

3.Absorbance should be read on an ELISA reader, using a test wavelength of 540 nm and a reference wavelength of 650 nm.

4. Each value should be estimated by the following equation (absorbance of the sample-absorbance of the blank) / (absorbance of the uninfected absorbance of the blank) X 100. Each experiment was done in duplicate.

Rct marker test

Reproductive capacities at different temperatures (Rct marker) should be evaluated by the Rct test. The Rct value is defined as the difference between the log10 virus titer of a viral stock measured at the optimal temperature (37°C) and that of the supraoptimal temperature (40°C).each virus stock decimally diluted through 10−9. A 0.1- ml volume of medium containing 4 × 104 RD/HEp-2 cells added into each well of two 96-well plastic plates. A 0.1 ml volume of each virus dilution should inoculated into four wells of each of the two plates. One plate was incubated at 37°C and the other at 40°C. After 5 days of incubation at the appropriate temperatures, the titers were determined by as

previously described method in section 4.10. Viruses were considered to be thermosensitive (ts) if the Rct value (between 37°C and 40°C) was greater or equal to 2.00 and thermoresistant (not-ts) when the Rct value was less than 2.00 (Pilaka et al.2011).

Animal inoculation :

CV group A are difficult to isolate from cell culture as they do not grow well in it(Melnick et al. 1964, Hsiung 1973,Lipson et al. 1988, Rotbart 1999).The choice of primary isolation of them are inoculating the sample intracerebrally in suckling mice. other entero viruses and CV B can also be grown in suckling mice. Group A CV produces generalised myositis and flaccid paralysis in suckling mice whereas, CV B viruses produces focal myositis, spastic paralysis, also affects other organs like heart,pancreas, skeletal muscles, brain and causes necrosis of brown fat. With the availability of molecular PCR techniques ,animal inoculation studies are rarely used nowadays because of technical difficulties and maintenance of animals.

Mice innoculaion

1.BALB/c mice (10-15 days old) Mice should be divided into two groups for clinical and environmental strain.

2.After the virus titer determination, specified dose of virus i.e. 3 x 106 PFU should be inoculated in BALB/c mice by intracereberal and oral route.

3. Sterile PBS should be inoculated in age and sex matched control mice. Mice should be monitored daily for sign and symptoms.

4.Mice should be sacrificed at the same intervals after 5, 10, 15 and 20 days post inoculation. At each time point 6 mice (2 for clinical strain, 2 for environmental strain

and 2 for control group) should be sacrificed and bled retro-orbitally followed by euthanization by cervical dislocation. Blood should be obtained by cardiac puncture. Portions of heart, pancreas, thymus, spleen, small and large intestine should be obtained, washed in PBS and stored at -80ºC or fixed in 10% formaldehyde for histological studies.

EV and molecular typing

In 1984, Hyypia et al 1984 reported in study about successful detection of multiple EV serotypes with single molecular probes by two laboratories). After 6 years of numerous investigators, extending their observations using cDNA probes, RNA probes and oligomeric probes(Rotbart 1991).It showed genetic homology among EVs extended beyond the few serotypes for which information of genome sequence was known during that time. Though EVs were detected in body fluids during experiments (Rotbart et al. 1985), the sensitivity in clinical specimens was about33% or less. lowtiter of EVs ,particularly in CSF from aseptic meningitis patients, which contained as few as 1 to 10 titerable virions per ml was limiting variable in these hybridization based assays (Rotbart 1999). A group specific EV PCRwhich discrimination of polioviruses from the nonpolio EVs in clinical specimens and Strain specific PCR for studying genetic shiftsand drifts of individual EV strains and specific molecular virulencedeterminants are used for epidemiological studies.3 sets of universal PCR primer probes for the EVs were reported 1989 - 1990, each with high specificity for the EVs and EV serotypes (Hyypia et al. 1989, Chapman et al.1990)detected at highly conserved regions of the 5' UTR noncoding region of the viralgenome and were designed for RT-PCR. EV RT PCR showed 100% specific and consistently more than culture (Rotbart et al. 1994). Then

by modifying one of the primers-probes sets they adapted aone step microwell based colorimetric RT PCR kit which performed well in 5 hr in routine diagnostic virology (Rotbart et al. 1994). The impact of availability of accurate diagnosis of EV infections in less than a day had significant quality in patient management and cost . these test may become clinically important when antiviral therapy becomes available and monitoring of the viral load in response to therapy become desirable. following molecular assays based on RT PCR for EV ,recent advances include nucleic acid hybridization,gene sequencing. RT PCR amplifies the viral genome and the amplified product can be typed using sequencing or non sequencing method. Initially the non sequencing typing method were used which were easy to perform using RFLP restriction fragment length polymorphism analysis to detect and identify the amplicon(83) and hybridization by type specific probes(84)targeting the 5`UTR ,the most conserved region in EV genome and VP1region the non structural capsid region. Both showed good results in typing and differentiating intratypic variants of poliovirus wild and vaccine strain because PV has limited sero types(85). But they failed with non polio EV typing because of point mutations modifying the restriction sites and the 5`UTRregion is not variable enough to discriminate between serotypes. This led to sequencing of amplicons. Here the RT PCR with primers binds to the conserved regions of genome of EV serotypes, but flanks a variable region, then sequencing of amplicons are done by comparing these sequence with gene bank accessible with internet. Limitation in typing method occurs when recombination occurs in EV genome, for this parts of capsid region can be sequenced as mutation of this occurs at a low frequency.

Molecular typing helps identify new strains and variants and informs about circulating strains to prevent transmission and control out breaks. It also differentiate PV and NPEV and EV serotype

3.8. Prevention

Vaccines are available only for the polioviruses, and they provide no protectionagainst the nonpolio EV serotypes. There has been extensive research to develop vaccine against EV 71.several candidates for EV 71 including recombinant proteins and synthetic peptides, virus like particles(VLPs),live attenuated and inactivated whole virus, DNA vaccines. High levels of IgG antibody specific to EV 71 VP1 can be induced by immunization with EV 71 VP1 protein expressed in *Escherichia coli* followed by expression in mammalian cell line, yeast, or baculovirus system(Premanand B et al), attenuated salmonella enteric serovar typhimurium expressing VP1gene and transgenic tomatoes expressing VP1 gene all under trial. The first vaccine against EV71 was manufactured by the Institute of Medical Biology at the Chinese Academy of Medical Sciences which was approved by the Food and Drug Administration (FDA) in December 2015. Another EV71 vaccine manufactured by Sinovac Biotech was approved by Chinese FDA in January 2016 studies by (Yi E-J, Shin Y-J et al)

3.9. Treatment

As with other viral pathogens, there are several steps in the replication cycle of the picornaviruses that are potential targets in antiviral therapy (Rotbart 1985). Cell susceptibility viral attachment, viral uncoating, viral RNA replication and viral protein synthesis have all been studied as targets of antipicornaviral compounds.

3.9.1. Interferon

Interferon is potent selective mediators of cellular changes which induce a number of antiviral antiproliferative and immunological effects of all which collectively affect host cell susceptibility to picornavirus infection (Rotbart et al. 1998). Clinically, children with acute EV meningitis have significant elevations in endogenous interferon levels in the CSF (Ichimura et al. 1985, Chonmaitree et al. 1991), which may be important in recovery from the infection. Although alpha
interferon itself is a very potent inhibitor of picornavirus infection, additive or synergistic protective effects are seen when it is used in conjunction with humoral antibodies and macrophages to eliminate picornavirus infections (Rotbart 2000). Despite in vitro efficacy and limited clinical utility in infections due to the closely related rhinoviruses, interferons have not been clinically evaluated in EV infections.

3.9.2. Immunoglobulins

The primary mechanism of clearance of EVs by the host is via humoral immunity. Patients who lack antibody because of congenital or acquired immunodefeciencies are uniquely susceptible to infections with the EVs (McKinney et al. 1987). Similarly, normal neonates are at high risk for severe EV disease because of a relative deficiency of EV antibodies (Abzug et al. 1968, Modlin et al. 1981). Antibodies act by binding to EVs and preventing attachment and binding to host cells, which correlates with neutralization of EVs observed in cell cultures treated with antibody.

Immune serum globulin has been used prophylactically and therapeutically. Neonates may develop an overwhelming sepsis syndrome from transplacental or peripartum acquisition of EV infection and the high mortality rate of this disease .

Anecdotal reports of clinical success withmaternal serum or plasma (Jantausch et al. 1995) or commercial immunoglobulin preparations (Johnston et al. 1989) against a variety of EV serotypes causing neonatal sepsis have been reported; other reports describe progressive disease and death despite such therapy (Wong et al. 1989). As Therapeutic efficacy in established EV meningoencephalitis in antibody deficient patients has only been anecdotally studied.

3.9.3. Capsid- Inhibiting Compounds

Capsid inhibiting compounds block viral uncoationg and/or viral attachment to host cell receptors. The resolved three dimensional structures of the EVs reveal a "canyon" formed by the junctions of VP1 and VP3. Beneath the canyon lies a pore, this leads to a hydrophobic pocket into which a variety of diverse hydrophobic compounds and integrates. Although the compounds integrate into a virus capsid it affect the function of the virus capsid.

Pleconaril (3-13,5-dimethyl-4-[3-methyl-5-isoxazolyl0 propyl]phenyl]-5-(trifluromethyl)-1,2,4-oxadiazole) is the first of a new generation of metabolically stable capsid function inhibitors.It is broad spectrum and potent anti EV and anti rhinovirus activity and is highly orally-bioavailable (Kearns et al. 1999,
Pevear et al. 1999). High levels of pleconaril are achieved in the CNS and in the nasal epithelial tract (M. McKinley, personal communication).Attention to fluid balance is necessary to avoid or ameliorate the syndrome of inappropriate antidiuretic hormone or brain edema. Electrolytes , urine and serum osmolarity may require monitoring. Brain edema is a rare complication of EV meningitis but is readily managed with mannitol. Seizures may result from fever alone or may reflect direct viral or indirect inflammatory

damage of brain parenchyma. Phenytoin or phenobarbital is the preferred agents for managing this complication. Rapidly progressive deterioration requiring more intensive support speaks strongly against an EV etiology, and other potentially treatable causes must be immediately considered.

Treatment for the neonate with sepsis or the child or adult with myocarditis is symptomatic. Maintenance of blood pressure is, of course, paramount in each of those syndromes. Steroids have been widely debated in the therapy of myocarditis but are now felt to be contraindicated in most cases (Martino et al. 1995). No significant benefit has been reported for other immunosuppressive classes of drugs either and some are clearly harmful in animal models of viral myocarditis (Rotbart et al. 2001).

Adequate hydration is the only indicated therapy in children with herpangia and hand foot and mouth syndrome due to the EVs. Other respiratory manifestations of EV infections are managed symptomatically.

MATERIALS AND METHODS

The present study was conducted at Tirunelveli Medical College, Tirunelveli Tirunelveli District, Tamilnadu from March to June 2018 to detect the presence of enteroviruses in the inpatient meningitis and meningoencephalitis populationtested negative for dengue fever by using commercially prepared PCR kits. The results obtained werestatistically analysed and results were interpreted.

STUDY DESIGN: Prospective

STUDY AREA: Department of Microbiology -Tirunelveli Medical College

Department of Medicine- Tirunelveli Medical College

Department of Paediatrics- Tirunelveli Medical College

STUDY PERIOD: 1 year

SPECIMEN: CSF samples

SAMPLE SIZE: 50

STUDY POPULATION:

INCLUSION CRITERIA

- children and adults years with clinical signs suggestive of meningitis or meningoencephalitis admitted in Paediatric and Medicine wards of Tirunelveli Medical College.
- Patient with acute fever and symptoms with an onset< 14days before coming to hospital,
- a neurological illness and >1 of the following signs(a change in mental status including confusion, disorientation, coma, inability to talk, photophobia, headache or meningitis, new onset seizures excluding simple febrile seizures)

EXCLUSION CRITERIA

- patients proven positive for other circulating virus etiology like Dengue, Japanese encephalitis
- laboratory confirmed malaria
- CSF: positive for bacterial and fungal culture
- previous history of neurological condition
- those with other known infectious illnesses (TB/ HIV/ HCV/ malaria / typhoid) or children with febrile seizure or
- old cases of meningitis or meningo encephalitis will be excluded from the study.

Study place and test material

A total of 50 CSF samples were collected for this study from patients admitted in the Departments of Medicine and Pediatrics, Tirunelveli Medical College Hospital Tirunelveli.

Test Kits

The Real time reverse transcriptase PCR kit

Equipments

The following equipments were used :

Table top centrifuge (Remi, R8C), Incubator (Tempo) , PCR machine (Rouche,)

The controls used were those provided in the respective kits

METHODS

General safety precautions and ethical clearance

The CSF samples were collected, transported, stored and processed using all safety

precautions throughout the course of this study. Gloves and masks were worn during processing of the samples. Storage of CSF samples at -70°C . During withdrawal of samples due care was given to follow aseptic precautions by using sterile disposable needles and discarded according to Biomedical Waste Management Rules-2016 to ensure patient safety.

The institutional ethical clearance was obtained for this studying.

Collection of CSF samples:

The following steps were followed during collection of specimens

1. Patient name, age, gender, address was noted
2. Following strict aseptic precaution, lumbar puncture was done in suspected meningitis/ meningoencephalitis on admission by physician and transported to laboratory without delay in 3 tubes for biochemistry, microbiology, cytology in that order examination. any delay in examining CSF will result in lower WBC counts and falsely low glucose content due to glycolysis

3.2-3 ml CSF needed but if only small amount of CSF is collected ,priority of testing should be discussed with physician. they inform us prior to collection of CSF samples, necessary arrangement for sample receiving and processing will be done immediately

The contaminated materials/supplies were discarded in designated containers.

The samples received at the laboratories were then processed.

Examination of CSF

- **Macroscopic appearance: clear** in normal and viral infection, **blood stained**/xanthochromia in CNS haemorrhagic condition may be of any cause. Traumatic tap should be kept in mind and **turbidity** is seen in bacterial cause.
- **Biochemical CSF analysis**:

CSF glucose (normal range 45-80mg/dl in adults and) it usually normal in viral aetiology which distinguish bacterial cause where glucose levels are reduced.

CSF protein (normal range15-40 mg/dl),mild elevated protein in viral aetiology and increased levels seen in bacterial cause

- **Cytological CSF examination**: normally acellular (<5 leucocytes / µl). pleocytosis (upto 1000 leukocytes / µl)- polymorph mononuclear cell predominate initially suggesting acute presentation, leukocytes predominates in chronic condition.
- **Direct microscopy**: Gram stained by heaped method to rule out bacterial and fungal cause using Gram stain, Indianink, giemsa ,KOH ,calcoflor
- **Culture and antimicrobial susceptibility testing** :routine culture for bacteria and fungus will be done,if any growth it will be processed and reported. Culture positive samples were not taken for study. Viral culture though gold standard was not done due to non availability

Serology: Dengue NS1.dengue IgM and JE IgM ELISA detecting IgM antibodies was done to rule out dengue, JE in fever cases and suspected meningitis /meningoencephalitis cases. Positive cases were excluded

Extraction of RNA from samples

Materials required:

Pure fast Viral nucleic acid RNA minispin prep kit (HELINI BIO MOLECULES), containing

- Carrier RNA
- Proteinase K
- Viral lysis buffer
- Elution buffer
- Wash buffers 1
- Wash buffer 2
- Spin columns with collection tubes

  Carrier RNA and protienase K should be stored in -20˚C, others store in room temperature

  Reagents preparation

	Wash buffer 1	Wash buffer 2
Concentrated buffer	18ml	12ml
Ethanol(96-100%)	12ml	48ml
Total volume	30ml	60 ml

Materials not provided with the kit:

- Biosafety cabinet (BSL 2B)
- Real time PCR machine

- Centrifuge with rotor for 1.5 reaction tubes
- Vortex mixer
- Water bath
- Sterile Milli-Q water
- Rack
- 1.5ml/2ml centrifuge tube
- Micro-Pipettes(0.5-10µl,10-100µl, 100-1000µl)
- Disposable filter plugged pipette tips (2-20µl,10-100µl, 100-1000µl)
- 1.5mL Micro centrifuge tubes
- 0.2ml PCR tubes/8 well strip
- Discard jar
- Disposable nitrile gloves
- Absolute alcohol

Enterovirus RNA extraction was performed as per manufacturer's instructions.

Step 1: Add20 of proteinase k to the bottom of fresh 1.5ml microcentrifuge tubes labelled appropriately.

Step2: Add 200 of CSF

Step 3:Add 200 of lysis buffer and pulse vortexed for 15 seconds

Step 4: Add 5 of carrier DNA(add 5 of internal control template)

Step5;Mix immediately by brief vortex and centrifuge few seconds to bring down drops to bottom of tube

Step 6: Incubate at 56 C for 10 minutes in water bath

Step 7:After the incubation, add 220µL each of absolute alcohol (96 - 100%) to the micro centrifuge tubes and pulse vortexed for 30 seconds and spin briefly few seconds to bring down drops to bottom of the tube

Step8;Transfer into the correspondingly labelled Purefast spin columns. The spin column-collection tube assembly was spun at 8,000 rpm for 1 minute and. discard flow through and place the collection tube back into the same collection tube.

Step 9:Add 500 µL each of wash buffer 1 (ethanol added) to the Purefast spin columns. The spin column-collection tube assembly was spun at 8,000 rpm for 1 minute and discard the flow through and place the spin column back into same collection tube

Step 10 ;Add 500 µL each of wash buffer 2 to the Purefast spin columns. It was centrifuged at 10,000 rpm for 1 minutes and discard the flow through and place the spin column back into same collection tube

Step 11:Repeat wash buffer 2 again

Step 12: Discard collection tube and insert purefast spin column into fresh 1.5 ml micro centrifuge tube. Centrifuge at 12000 rpm for 2mint empty spin(to avoid any residual ethanol).discard 1.5ml micro centrifuge tube.

Step 13;Transfer purefast spin column into fresh 1.5 ml microcentrifuge tube

Step 14:Add elution buffer to center of pure fast spin column membrane and this was incubated at room temperature for 2 minute

Step 15: Centrifuge at 10,000 rpm for 1 minute and the spin columns were discarded. The microcentrifuge tubes now containing the extract were used subsequently for PCR.

Reaction assay

Mastermix preparation should be done in the clean room

Materials required:

- Real time PCR system with software

-Cooling block

Detection mix components

- One step RT-PCR Master Mix
- RT-Enzyme mix
- Enterovirus primer probe mix
- IC Primer probe mix

Loading of PCR plate:

1) µL each of the appropriate master mix and µL each of the RNA extracts was added to appropriate blocks and placed in thermal cycling plate as per the PCR template addition protocol.

samples.

4) The blocks was loaded into the real time PCR system and the program was started after setting the cycling.

Centrifuged PCR blocks before placing into thermal cycler, should be no bubble in the reaction mix, it will interfere with fluorescence detection.

Negative control(NTC)

Add 10 µl of nuclease free water

Qualitative positive control set up

Add 10 µl of positive control

Set amplification protocol

	Step	Time	Temperature
	Reverse transcriptase	20 mints	50˚C
	Taq enzyme activation	15 mints	95˚C
45 cycles	Denaturation	20 sec	95˚C
	Annealing / data collection	20 sec	56˚C
	Extension	20 sec	72˚C

Enterovirus – FAM channel

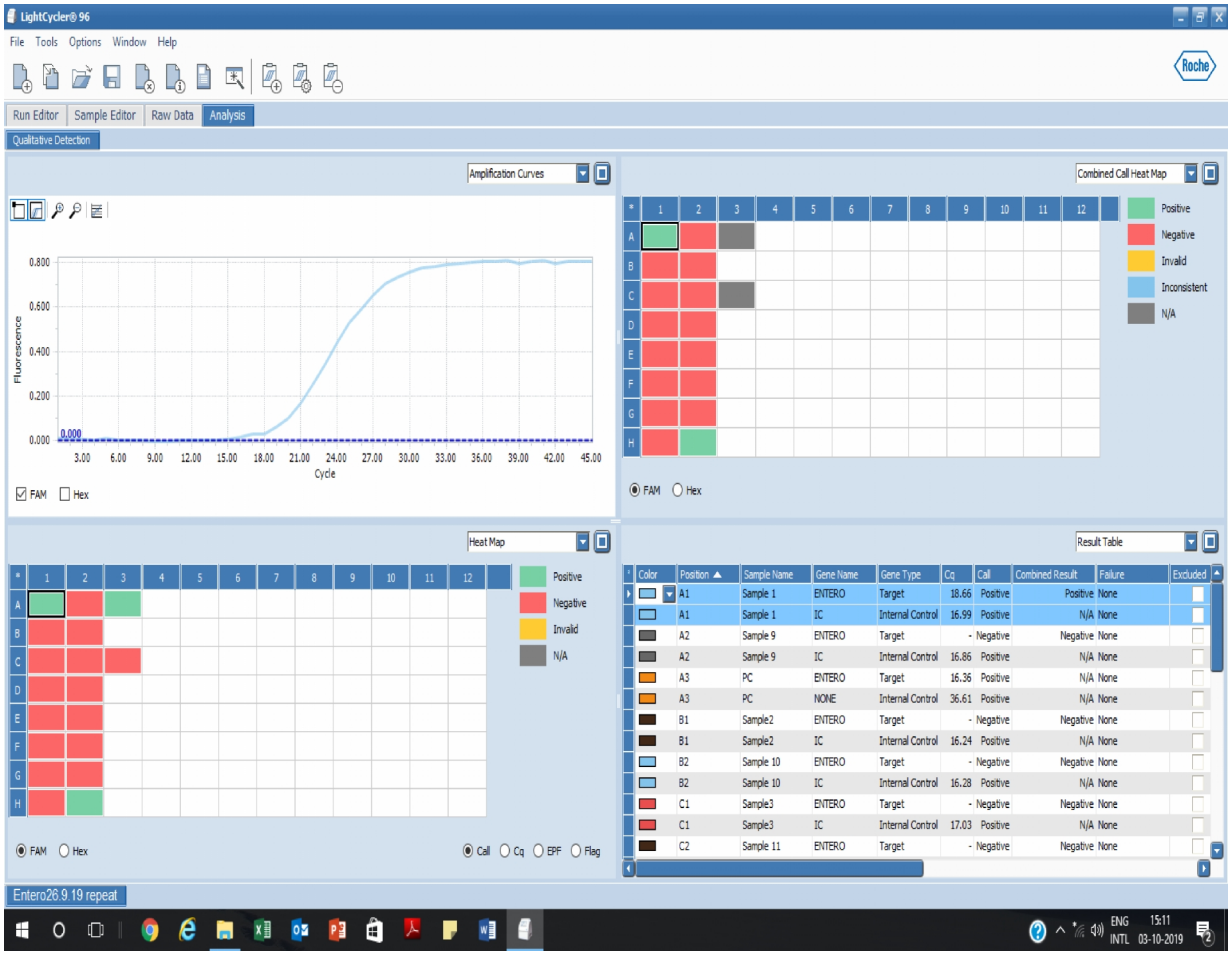
Internal control-HEX channel

Target region of viral RNA sequence is reverse transcribed tp single stranded complementary DNA. primer extension with RNA dependent DNA polymerase. After the reverse transcription step, mixture is heated to denature the RNA-cDNA .on cooling the upstream primers starts to anneal the cDNA strand. A second polymerase catalyses the extension of the synthesis of second strand in the presence of deoxynucleotide molecules exposing primer target sequences. This repeated process of heating cooling primer annealing and extension more amplified amplicons of the target is produced, which is detected by the detection system.

Statistical analysis(SEE)

1. Continuous variables were recorded as the mean and standard deviation for hypothesis test p value of <0.05 were considered statistically significant. Statistical computation were performed by SPSS statistics was entered into Microsoft excel(windows 7,version 2007) and analyses were done using the

Statistical Package for Social Sciences (SPSS) for windows software(trial version 22.0;spss inc Chicago).descriptive statistics such as mean and standard deviation (SD) for continuous variablesand frequencies and percentage were calculated for categorial variables, frequencies and percentages were calculated for categorical variables.to check for associated factors with Enterovirus positivity chi square test was used.in case of lesser expected counts fishers exact test was used .bars and pie charts were used for visual representation of data. level of significance was set at 0.05.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Result Table
Color Position Sample Name Gene Name Gene Type Cq Call Combined Result Failure Excluded
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A1 Sample 1 IC Internal Control 16.99 Positive N/A None
A2 Sample 9 ENTERO Target - Negative Negative None
A2 Sample 9 IC Internal Control 16.86 Positive N/A None
A3 PC ENTERO Target 16.36 Positive N/A None
A3 PC NONE Internal Control 36.61 Positive N/A None
B1 Sample2 ENTERO Target - Negative Negative None
B1 Sample2 IC Internal Control 16.24 Positive N/A None
B2 Sample 10 ENTERO Target - Negative Negative None
B2 Sample 10 IC Internal Control 16.28 Positive N/A None
C1 Sample3 ENTERO Target - Negative Negative None
C1 Sample3 IC Internal Control 17.03 Positive N/A None
C2 Sample 11 ENTERO Target - Negative Negative None
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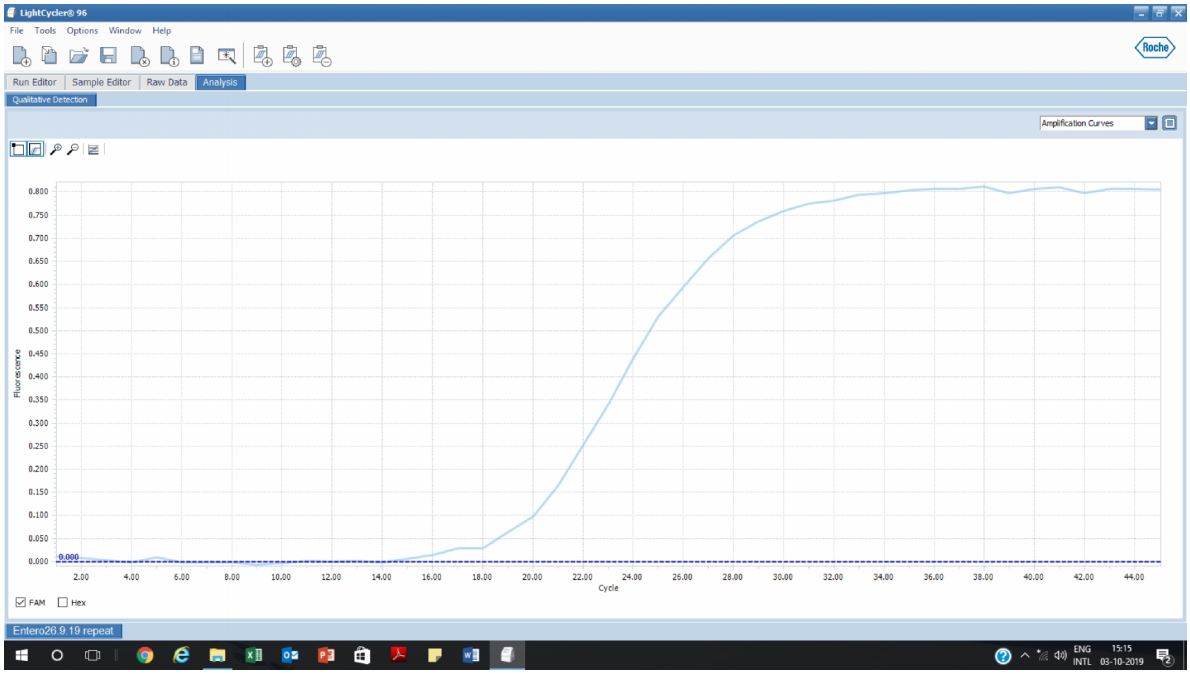
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RESULTS

The study population included 50 clinically suspected cases of acute meningitis and meningo encephalitis who were admitted in SNN, PICU, IMCU, Neurology wards of The Tirunelveli Medical College, Tirunelveli. A total of 50 CSF samples were collected from clinically diagnosed suspected patients of mengitis and meningo encephalitis patients.it satisfied the inclusion and exclusion criteria. Molecular study with q RT PCR to detect the presence of EVs was done

I.SOCIO DEMOGRAPHIC FACTORS

Table 1: Age distribution of study population:

Age	Frequency	Percentage
<1month	4	8%
<1 year	8	16%
1-5 years	7	14%
6-15 years	7	14%
16 -40years	10	20%
41-60 years	9	18%
61- 80 years	5	10%
Total	50	100%
Mean	25	
Standard deviation	23	
Range	4 days – 75 years	

Among the study population 4 (8%) patients were in < 1month group,8 (16%) were in <1year group, 7 (14%) patients were in 1-5 years, 7 (14%) patients were in 6-15 years

age group , 10 (20%) patients were in 16-40 age group , 9 (18%) patients were in 41-60 year group , 5 (10%) patients were in 61-80 age group.

Chart 1: Age distribution of study population

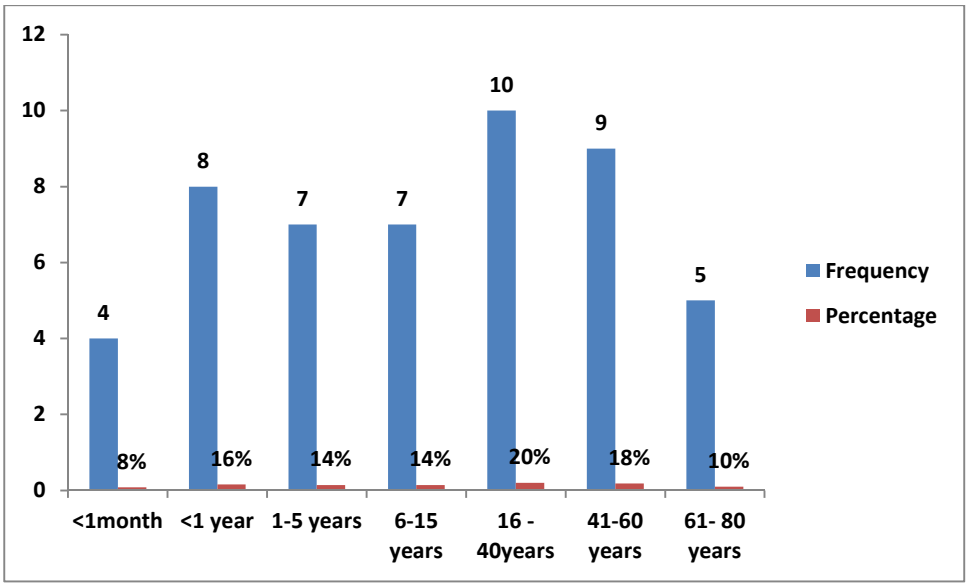


Table 2: Gender distribution of study population:

Age	Frequency	Percentage
Male	27	54%
Female	23	46%
Total	50	100%

Among 50 patients included in study population, 27(54%) were male and 23(46%) were females.Males were affected little more than females.male and female ratio was 1.2:1

Chart 2: Gender distribution of study population:

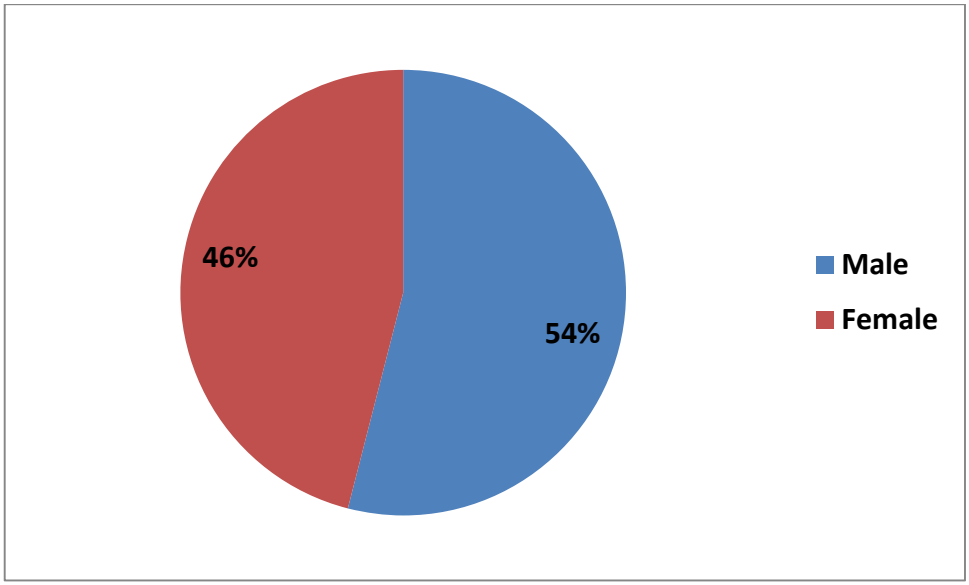


Table 3: Wardwisedistribution of study population:

Age	Frequency	Percentage
PICU	20	40%
IMCU	17	34%
Neurology	9	18%
SNN	3	6%
Total	50	100%

Among the study population 20(40%) were admitted in PICU ward, 17 (34%) in IMCU ward ,9(18%) from neurology ward, 3 (6%) from SNN ward

Chart 3: Ward distribution of study population:

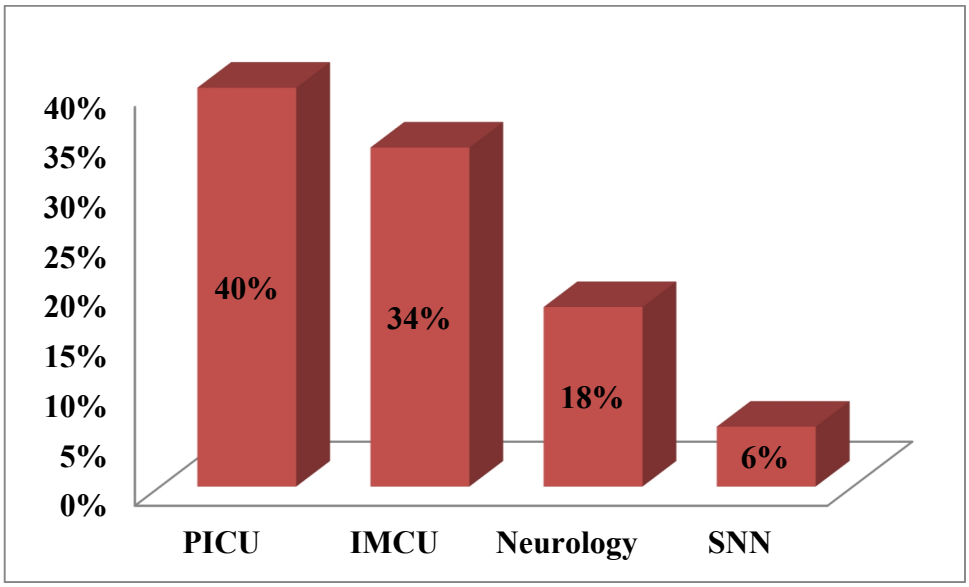
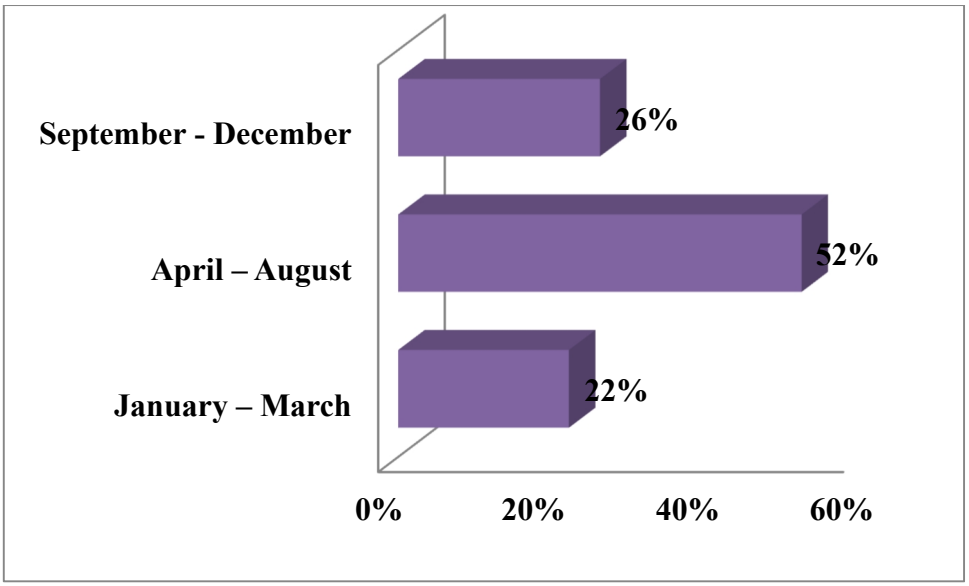


Table 4: Month at which study population was on admitted in hospital:

Age	Frequency	Percentage
January – March	11	22%
April – July	26	52%
August - December	13	26%
Total	50	100%

II.CLINICAL FEATURES

Chart 4: Month at which study population was admitted in hospital:



Among study population most of the patients 52% were admitted during summer month(april to august),26% during September to December and 22% during January to march during 2018-2019

Table 5a: Fever distribution of study population:

Fever	Frequency	Percentage
Present	50	100%
Absent	0	0.0%
Total	50	100%

The presenting clinical feature included fever in all 50 cases

Chart 4: Fever Among the study population:

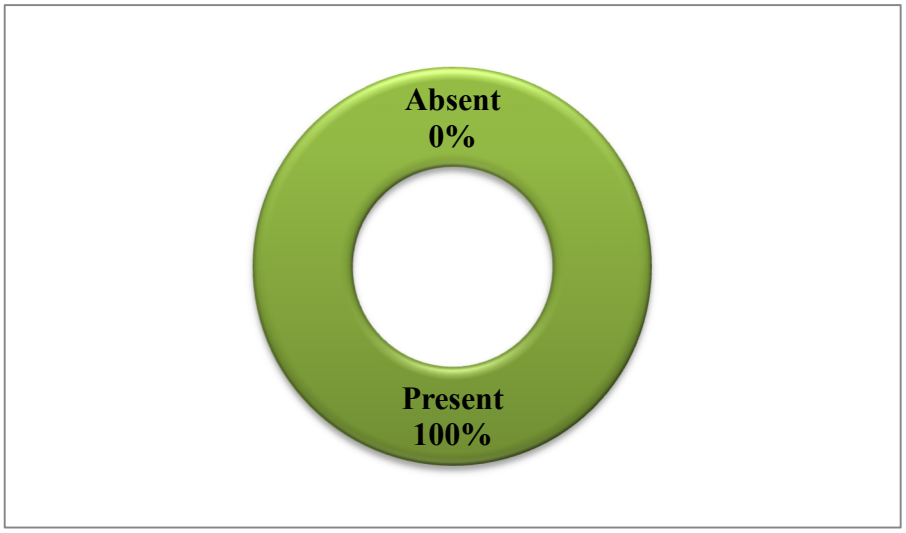


Table 5b:Symptoms and signs of study group at time of admission :

Clinical features	Frequency	Percentage
Fever	50	100%
Headache	38	76%
Vomiting	10	20%
Lethargy	23	46%
Neck rigidity	12	24%
Photophobia	23	46%
Rashes	-	-
Pharyngitis / upper respiratory tract infection	18	36%
Diarrohea	7	14%
Flaccid paralysis	Nil	0
Dysphagia/Dysarthria/Dysphonia / Facial weakness.	0	0

Fever was present in all 50 patients,headache in 38 patients, vomiting in 30 patients,lethargy in 23 patients,neck rigidity in 12 patients ,18 patients had URTI,15 patients had diarrohea,1 patient had sepsis like syndrome at time of admission

Chart 5b:Symptoms and signs of study group at time of admission

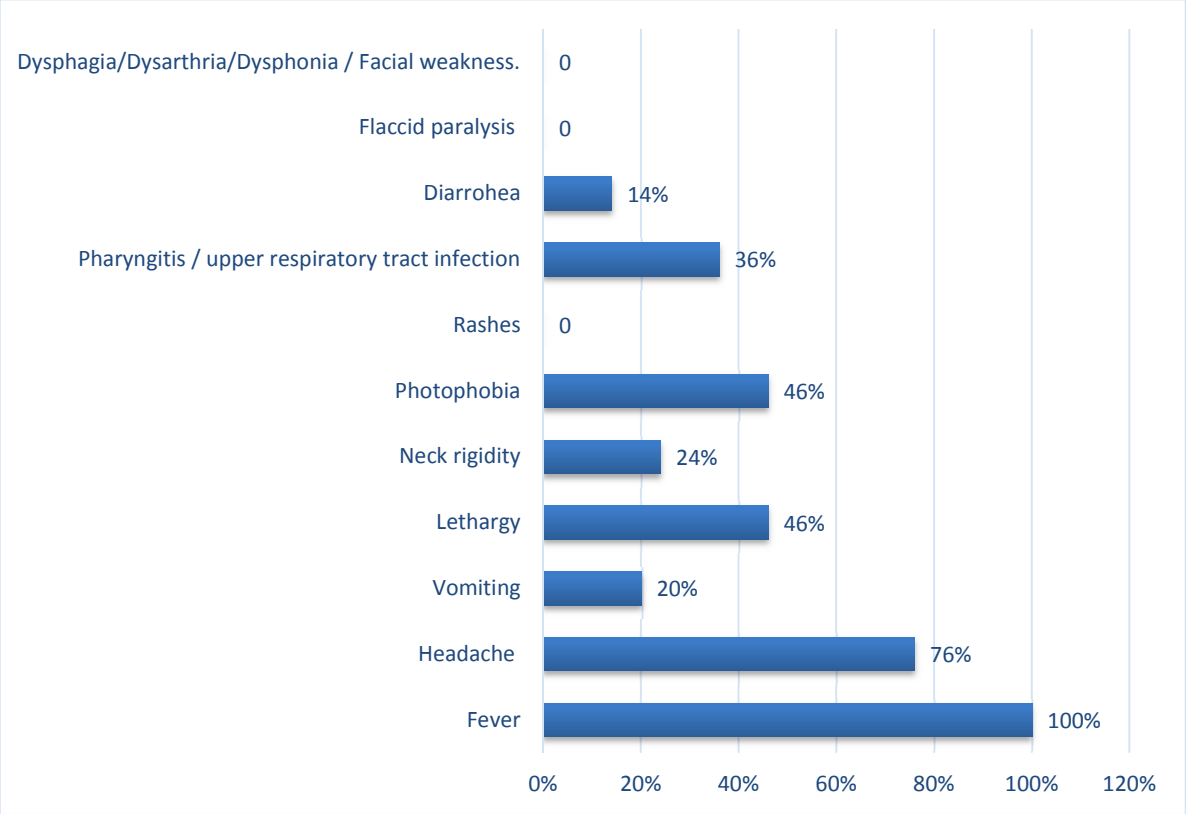


Table 5c: Signs of brain parenchymal involvement among study population at time of admission :

Clinical features	Frequency	Percentage
Seizures	23	46%
Altered sensorium	24	48%
Coma	3	6%

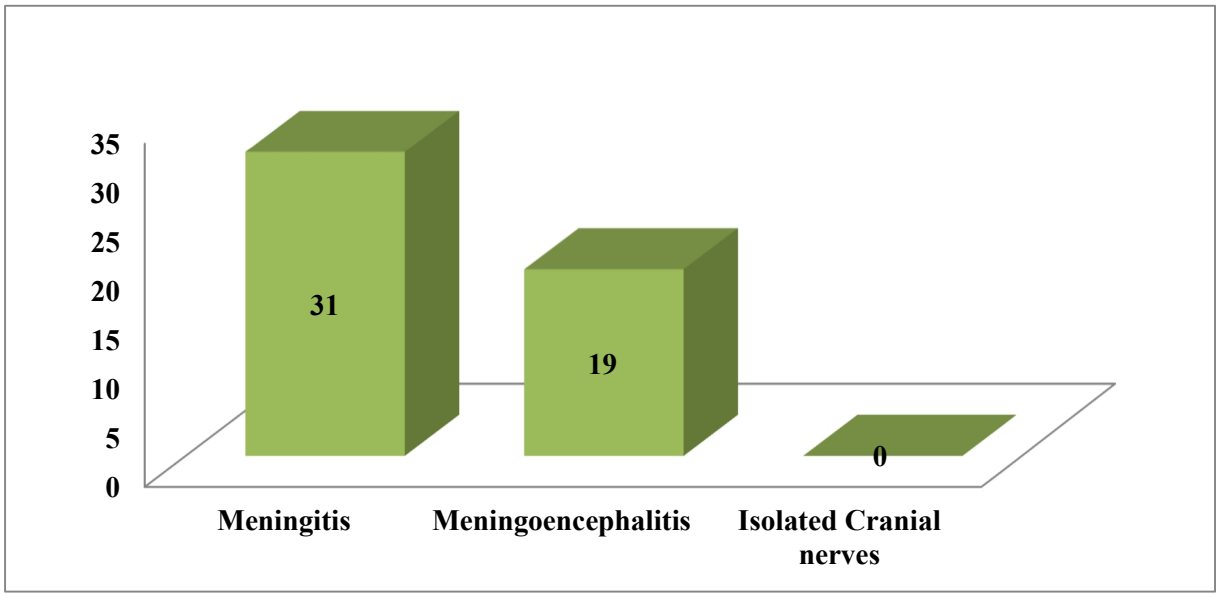
23 patients had seizures ,24 had altered sensorium at time of admission

Table5d: Clinical diagnosis of the study population:

Diagnosis	Frequency	Percentage
Meningitis	31	62%
meningoencephalitis	19	38%
Isolated cranial nerves involvement	-	-
Total	50	100%

Among the 50 study population 31 patients were diagnosed of meningitis and 19 patients were diagnosed as meningoencephalitis and there was no isolated cranial nerve involement among the study population.

Chart 5d:Clinical diagnosis of the study population.



III.CSF profile

Table 6a: Macroscopic appearance ofCSF:

CSF	Frequency	Percentage
Clear	47	94%
Slight Turbid	2	4%
Blood stained	1	2%
Total	50	100%

Macroscopically 47 CSF samples were clear,2 samples were slightly turbid and 1 sample was slightly blood stained

Chart 6a: Macroscopic appearance of CSF :

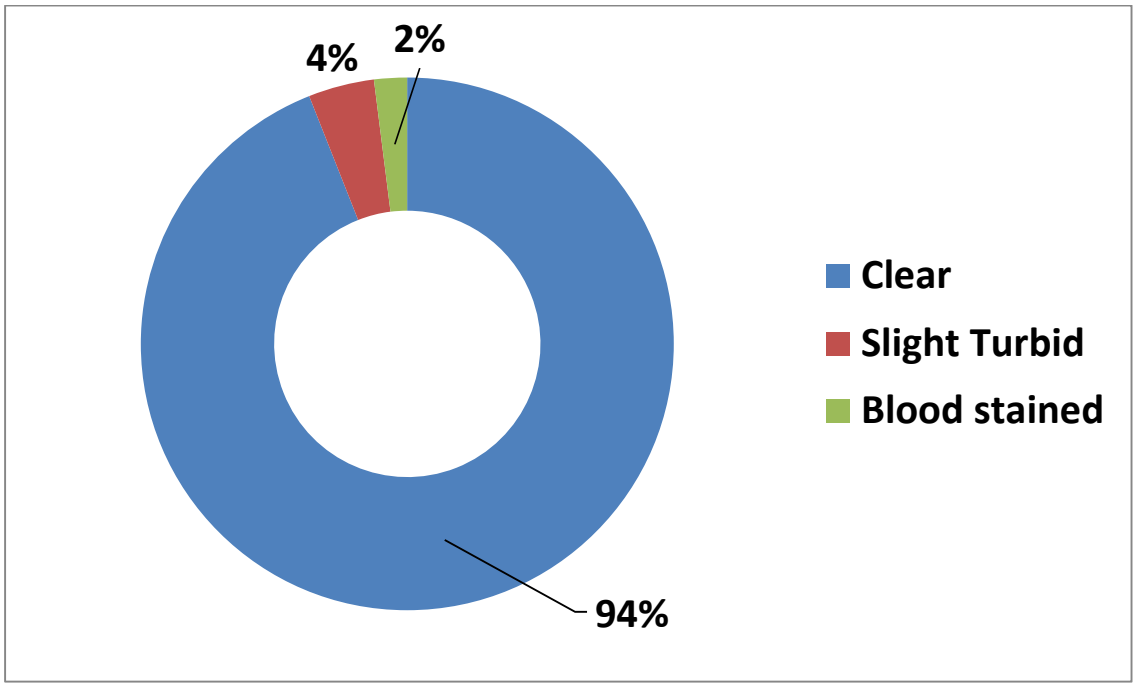


Table6b: CSF cell count Among the study Group :

CSF	Frequency	Percentage
Acellular	41	82%
1-100 mm3	9	18%
Total	50	100%

Maximum number of CSF samples were acellular 82% ,9 samples were found to have pleocytosis among 9 samples , 3 samples had neutrophil predominance and 6 samples had lymphocytic predominance.

Chart 6b: CSF cell count among study Group:

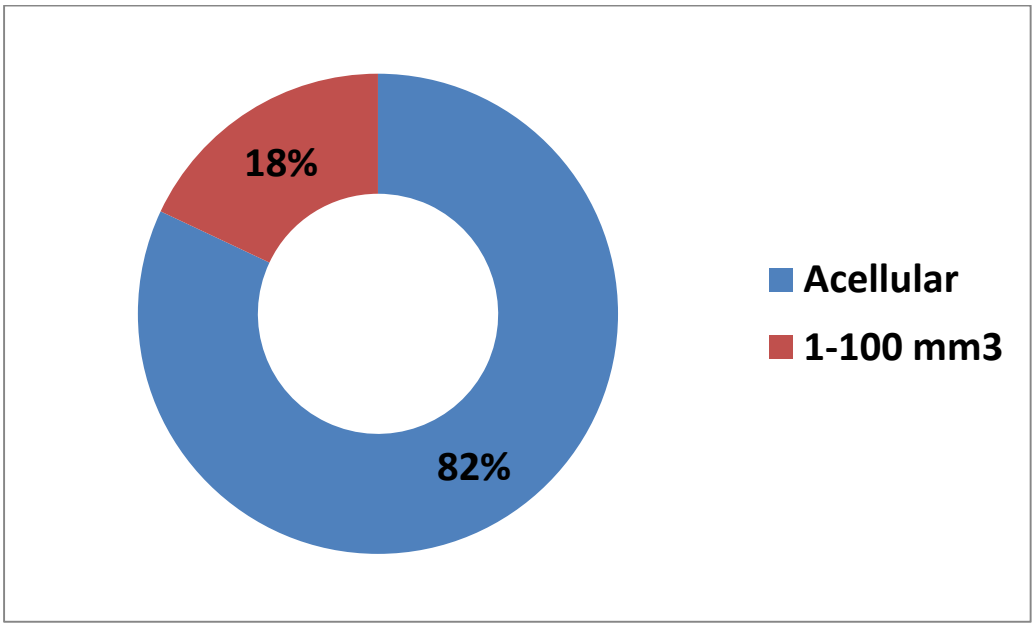


Table 6c: CSF protein levels of the study group:

CSF protein	Frequency	Percentage
Normal 15-40mg/dl	38	76%
41-100mg/dl	12	24%
>100mg/dl	-	-
Total	50	100%
Mean	30.6	
Standard deviation	22.1	
Range	9 - 96	

The CSF protein level was between <50 -100mg/dl in maximum number of clinically suspected cases. Mean 30.6 and range 9-96 mg/dl

Chart 6c: CSF protein levels of study population:

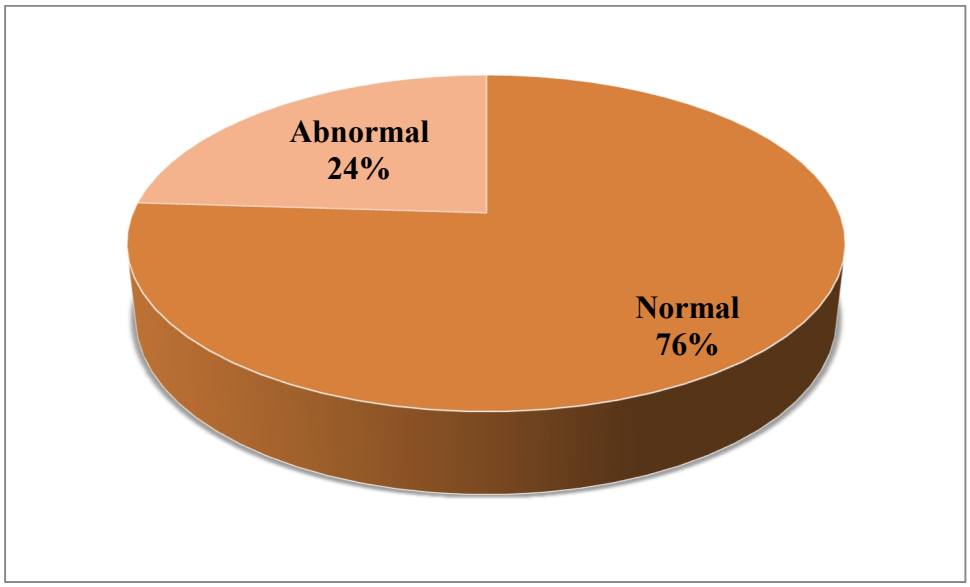
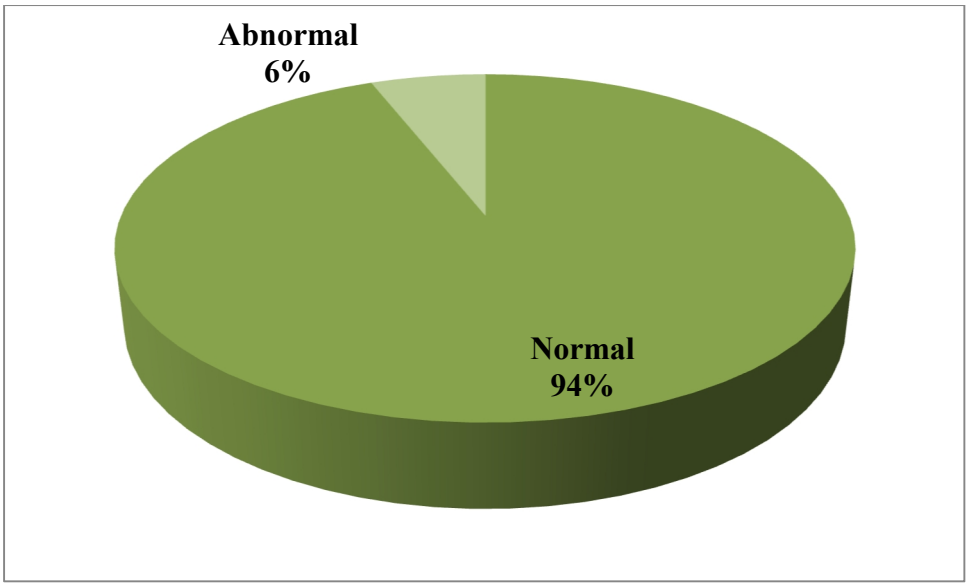


Table 6d: CSF glucose levels of study population:

CSF glucose mg/dl	Frequency	Percentage
Normal(45-80mg/dl)	47	94%
81-100mg/dl	3	6%
Total	50	100%
Mean	55	
Standard deviation	14.5	
Range	23-93	

94% of study population had glucose level 1-50mg/dl and 6% was >51-100mg/dl in neonates

Chart 6d: CSF glucose of study population:



IV. LABORATORY PROFILE

Table 7: Total WBC count distribution of study population:

WBC	Frequency	Percentage
Normal 4000-11000cells/cumm	41	82%
Slightly decreased 3000-4000 cell/cumm	9	18%
Total	50	100%

Among study population 41 patients showed normal WBC count,9 patient had slight decrease in WBC count, non presented with leucocytosis.

Chart 7a: Total WBC count of study population:

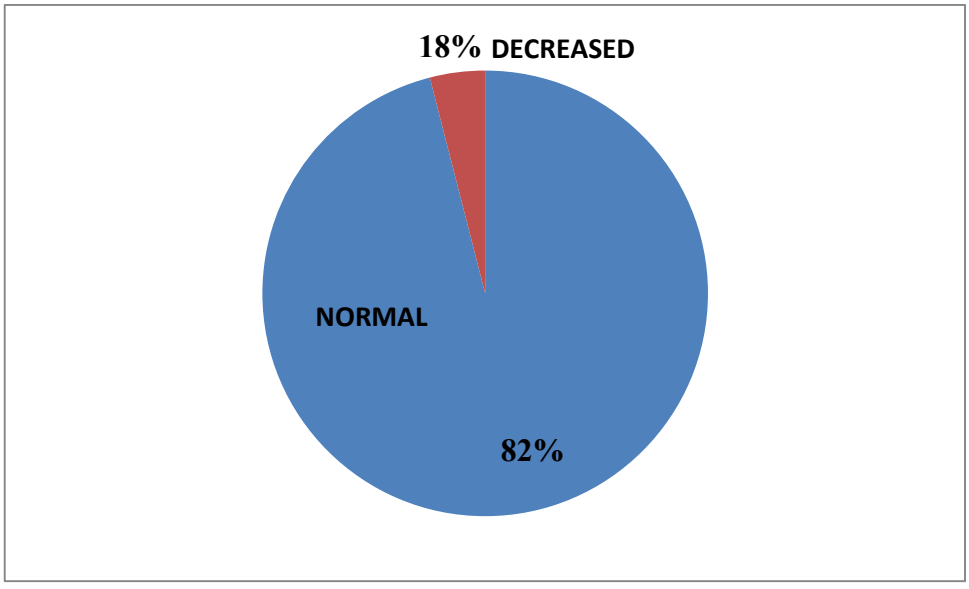


Table 7b: Serum C reactive protein (CRP) levels among study group:

CRP level	Frequency	Percentage
Normal 0.6mg/dl	47	94%
Increased >0.6mg/dl	3	6%
Total	50	100%

Among study population 3 patients had an increase in CRP

Chart 7b: C reactive protein (CRP) of study population:

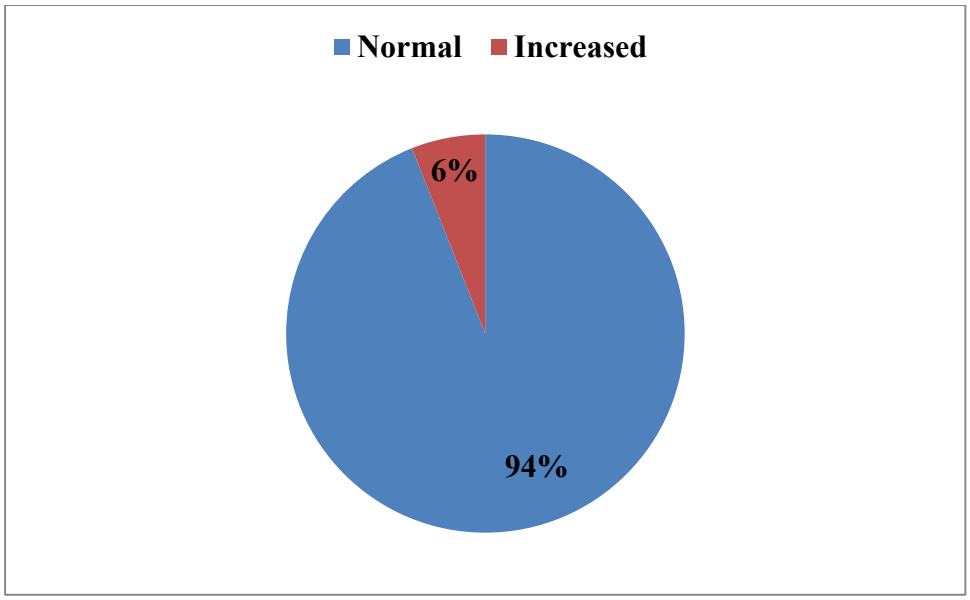


Table 8: Imaging (CT/MRI) findings of study population:

Imaging results	Frequency	Percentage
Not done	10	20%
Age related changes	24	48%
Diffuse Cerebral edema	1	2%
Temporo- parietal hypodensity	2	4%
Frontotemporal hypo attenuation	1	2%
Mastoiditis	4	8%
No changes	4	8%
Total	50	100%

Table 9: Association of Hand, foot and mouth disease (HFMD) among study group:

HFMD	Frequency	Percentage
Present	1	2%
Absent	49	98%
Total	50	100%

Chart 9: Hand, foot and mouth disease (HFMD) in the study population:

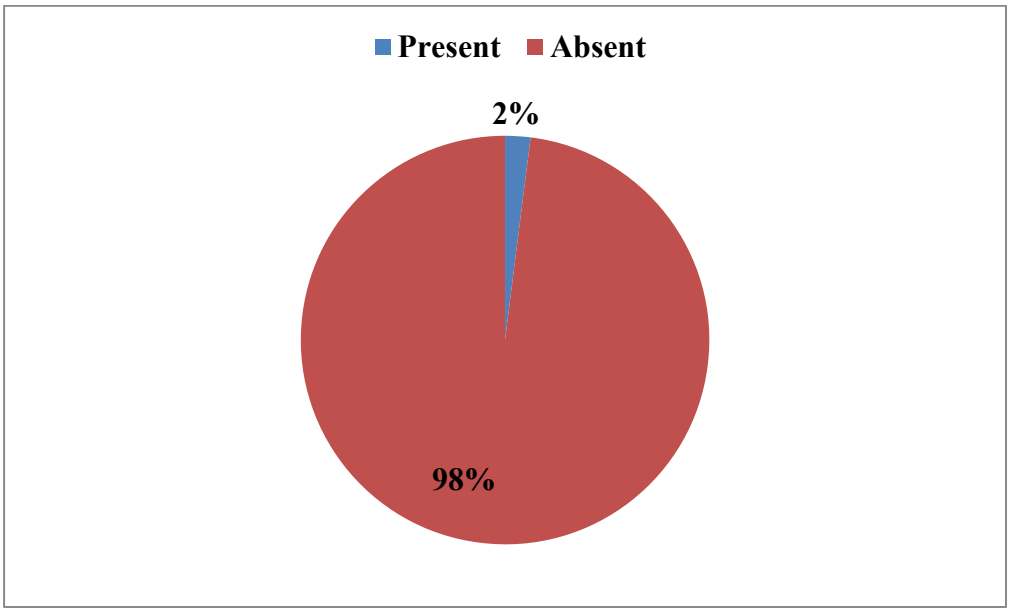


Table 10: Enterovirus positivity by RT PCR:

Enterovirus RT PCR	Frequency	Percentage
Positive	1	2%
Negative	49	98%
Total	50	100%

Chart 10: Enterovirus positivity by real RT PCR in the of study population:Among 50 suspected cases of meningitis, meningoencephalitis,evidence for presence of EV was detected in one patient by RT PCR.

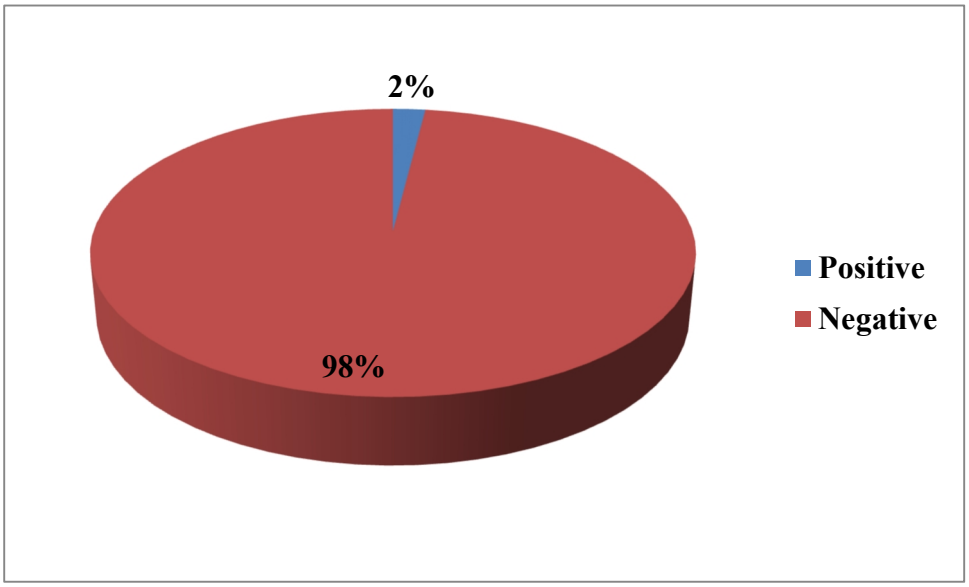


Table 11: Profile of the Enterovirus positive case:

Variable	Frequency
Age	1.5 years
Sex	Female
Clinical features Fever Seizures lethargy Neck rigidity Diarrhoea	 Present Present present Absent Present
Clinical diagnosis	Aseptic meningitis
CSF Macroscopic Glucose(mg/dl) Protein(mg/dl) Cell count	 Clear 56 70 Acellular
Hand, foot, mouth disease	Absent
Laboratory findings WBC CRP	 Normal Normal

Table 12: Factors association with enterovirus positive case:

Variable	Enterovirus positive N = 1 (%)	Enterovirus negative N = 49 (%)	P value
Age			
<18 years	1 (100%)	15 (30.6%)	0.560
18 – 40 years	0 (0.0%)	10 (20.4%)	
>40 years	0 (0.0%)	24 (49%)	
Sex			
Male	0(0.0%)	23 (45%)	0.460
Female	1 (0.100%)	27 (55%)	
Fever			
Present	1 (100%)	49 (100%)	----
Absent	0 (0.0%)	0 (0.0%)	
Headache			
Present	1 (100%)	46 (93.5%)	0.400
Absent	0 (0.0%)	3 (6.5%	
Seizures			
Present	1 (100%)	23 (53%)	0.540
Absent	0 (0.0%)	23 (47%)	
Diarrhoea			
Present	1 (100%)	6 (12%)	0.620
Absent	0(0.0%)	43 (87.5%)	
Upper Respiratory			
Present	1 (100%)	18 (37%)	0.960
Absent	0 (0.0%)	31 (63%)	
Neck rigidity			
Present	1 (100%)	12 (24.5%)	0.520
Absent	0 (0.0%)	37 (75.5%)	
Clinical presentation			
Altered sensorium	0 (0.0%)	24 (48%)	1.000
Coma	0 (0.0%)	3 (7%)	
	1 (100%)	22 (45%)	
Meningitis			
Present	1 (100%)	31(41%)	0.620
Absent	0 (0.0%)	19(39%)	
Meningoencephalitis			

Present **Absent**	0 (0.0%) 1 (100%)	19(39%) 20 (41%)	0.620
Hand.foot and mouth disease **Present** **Absent**	0 (0.0%) 1 (100%)	1 (2%) 49 (98%)	0.980
CSF **Acellular** **Lymphocytic predominant**	1 (100%) 0 (0.0%)	41 (96%) 9 (18%)	0.960
CSF glucose **Abnormal** **Normal**	0 (0.0%) 1 (100%)	3 (6%) 47 (94%)	0.940
CRP **Abnormal** **Normal**	0 (0.0%) 1 (100%)	3 (6%) 46 (94%)	0.940
WBC **Abnormal** **Normal**	0 (0.0%) 1 (100%)	9 (4%) 41 (96%)	0.960

P Value >0.05 no significant association.

DISCUSSION

Enteroviruses(EVs) have emerged as one of the important etiological agents for meningo encephalitis. Enteroviruses are RNA viruses of the family Picornaviridae that consists of more than 100 serotypes and have single positive strand genomic RNA. EV group includes 12 species of which 4 species are Human EV A-D. It comprises of Coxsackie viruses, Poliovirus, Echovirus, Parecho virus and enteroviruses 68-72.The clinical features of CNS infection due to enteroviruses can overlap bacterial and Herpes simplex virus and may be accompanied by mucocutaneous manifestations..

This is a prospective study conducted in Tirunelveli medical college, a tertiary care hospital with the aim to detect the presence of Enterovirus in CSF of 50 clinically suspected inpatients of viral meningitis and meningoencephalitis ,studied for a period of one year.

SOCIO DEMOGRAPHIC PROFILE OF STUDY POPULATION

Among the 50 study population, the mean age was 25 years and range from 4days old-75 years. In this study, there was26and 24 adults. Majority of the patients were in the 16-40 age group. Out of the 50 study participants ,1 patient was positive for EV by real time PCR. The age of the patient was 1.5 years. Enterovirus are the most common cause of CNS infection in younger children and adults (Kaminski et al.,2017;Logan and Macmahon et al.,2008;. Recent

studies by Shukla et al.,2017 however reported Herpes simplex as the most common cause of viral CNS infection in adults.

Among 50 patients included in study population, 27(54%) were male and 23(46%) were females. Males were affected more than females .Male and female ratio was 1.2:1. 21(42%) study participants were admitted in PICU ward, 3 (6%) in SNN ,9(18%) in neurology ward and 17 (34%) in IMCU ward. CSF sample was taken by lumbar puncture from the patients and received in the molecular laboratory for Enterovirus RNA detection by PCR during the study period .The Enterovirus positive patient was admitted in PICU ward in this hospital during the month of May 2018 . EV CNS infections are common in younger children and during summer months. Hong et al., 2014 reported a similar occurrence of EV CNS infection.

Clinical features

In this study Fever was present in all 50 (100%)patients which was similar to study by Chatterjee,.et al;. Headache was noted in 38(76%) patients, vomiting in 10 (20%)patients, lethargy in 23(46%) patients and neck rigidity in 12(24%) patients at time of admission. Diarrhea was present in 14 % and upper respiratory tract infection was present in 36 % of the patients. 3 adult patients presented with sudden onset headache that initially led to suspicion of subarachnoid haemorrhage but CSF tap was clear and CSF analysis showed mild pleocytosis , CT/MRI revealed normal study. Landtblom et al., 2002 stated that aseptic meningitis can cause sudden onset of headache. Enterovirus is known to cause

acute meningitis commonly than other viruses (Han et al.,2016; Hong et al.,2014;)

Among 50 clinically suspected patients,31 patients presented with meningitis and 19 patients had meningoencephalitis which were 62% and 38% respectively .The classical clinical triad of meningitis fever,neck rigidity and headache was observered in 12 patients(24%)which is similar to the findings reported by Durand ML,.et al and van de Beek D et al

There was no isolated cranial involvement in the study population. This is similar to the findings by Hong et al.,2014 & Kaminski et al.,2017 . However Messacaret al.,2015had shown cranial involvement to be common in children with EV CNS infection.Paralysis was not observed in any of the patients in this study.

ENTEROVIRUS DETECTION BY RT PCR

In this study only one patient was positive for Enterovirusby real time PCR which was only 2% . The EV positive patient was clinically diagnosed to have aseptic meningitis.The EV real time PCR kit used in this study,contained reagents and enzymes for the specific amplification of 95 bp region of EVgenome and for the detection of the specific amplicon in FAM channel . Conventional lab diagnosis of EV meningitis relies on virus isolation from cerebrospinal fluid (CSF) , followed by neutralization typing. This method is time-consuming and mostly unsuccessful, due to low titers of virus in CSF and difficulties in cell culture . Evidence for recent EV infection can also be identified from stool culture or demonstration of specific immunoglobulin M antibody , but this provides only

circumstantial evidence of an etiologic role in the central nervous system (CNS) pathology. Detection of EV CNS infection through the amplification of EV RNA from CSF by a reverse transcription-PCR RT-PCR assay can provide a definitive diagnosis of EV infection. Almost all EV serotypes have a conserved 5′ non translated region and the use of PCR with primers from this region identifies the majority of EVs that infect humans (Zoll GJ et al). RT-PCR is more sensitive and the workload is lower than for virus culture.

Many studies world wide have reported EV as a common cause of aseptic meningitis in 80-92% of patients (Han et al.,2016;Kaminski et al.,2017) . The study samples was also subjected to real RT PCR CNS panel testing for other encephalitis causing viruses, it detected 2 patients positive for adenovirus(4%) and 2 patients positive for HSV(4%).The cause of low prevalence of EV detected in this study could be due to the presence of few virus particles in CSF .The concentrationof the virus may vary in different samples like throat swab, stool samples etc during the course of the disease . In this study CSF was the only sample taken. EV can be detected by real time PCR in CSF during the early 1-4 days of onset of viral aseptic meningitis.Being a tertiary care hospital, patients would have presented late and may have been treated at home or referred late from other hospitals.Some EV infections of the CNS may not be accompanied by the presence of the virus in CSF (Read SJ et al). False-negative results may also be due to presence of inhibitory substances which interfere with the function of

the enzymes used in PCR. Retesting may be useful, but CSF is available usually in small amounts that do not allow this.

- Macroscopically 47 CSF samples were clear,2 samples were slightly turbid and 1 sample was faintly blood stained which may be a traumatic tap as the patient did not have changes suggestive of sub arachnoid haemorrhage in MRI.
- CSF ANALYSIS

  In this study CSFpleocytosis was not seen in high proportion (82%)of the study population. The patients without CSF pleocytosis were mostly young children and older patients. In the EV positive patient CSF was acellular. 3 studies reported normal CSF cell count in about 4-10% adults with EV CNS infection (Carrol et al.,2006;Graham and Murdoch et al., 2005;Ihekwaba et al.,2008;. These finding suggest that during initial stage of infection, immunological mechanisms that recruit WBC to CSF compartment may be incomplete. This is similar to the study reported by J.Ahlbrecht et al.,2018. Yun et al.,2012; that there is normal CSF cell count seen in paediatric Enteroviral CNS infection.

  CSFpleocytosis was noted in 18% of the study patients. Three patient had neutrophils predominantly and six patients showed lymphocytes and neutrophils . Martinez-Giron and Pantanowitz et al 2017 and Samejaijakul et al.,2017 reported EV CNS infection had CSF neutrophilic pleocytosis more frequently than other viral CNS infection.these 3 patients had headache ,fever,neck stiffness. Classical meningitis symptoms were less in patients who did not have CSF pleocytosis. This is similar to the reports given by Erdem et al.,2017;Wang

et al., 2014 who showed that headache and neckstiffness were less in older adults without CSF pleocytosis. More over lumbar puncture was done within one day of onset of symptoms in patients who showed no CSF pleocytosis.

The CSF protein level was between <50 mg/dl in maximum number of clinically suspected cases. Mean 30.6 and range 9-96 mg/dl. 94% of study population had CSF glucose level 45-80mg/dl and 6% had >80-100mg/dl . Further there was elevated CRP in 3 patients in age group 5-10 years old this is similarly reported by J.Ahlbrecht et al.,2018;

The EV positive patient had fever,seizures and lethargy.CSF was macroscopically clear,acellular, glucose was 56mg/dl and protein was 70 mg/dl. She had normal CRP and normal WBC counts. From this study it is obvious that EV CNS infection may occur without CSF pleocytosis.J.Ahlbrecht et al.,2018 suggested that CSF cell count analysis is not a reliable criterion for excluding an EV CNS infection. As the positivity was low, strong statistical association could not be made out for any of the parameters.

- ASSOCIATION WITH HFMD

HFMD was seen in one male child (2%) in the 1-5 years age group,which is the commonest age HFMD affects children. This finding is similar with Bending et al .1996 who reported presence ofHMFD in children of 1-4 years with EV71 encephalitis ,Xia et al.,2016 reported HFMD in age group 1-3 years.Ang et alalso reported 0-4 years as the most common group age,among HMFD children during Singapore outbreak 2001-2007.In India, during the out break of HFMD in Odisha

2013 , 5-14 years age group was affected the most. The patient with HFMD was admitted during month of July which is similar to the usual pattern.However the patient was negative for EV PCR.

Neuro imaging CT/ MRI was done in 40 patients 14 patients showed age related changes,2 patient showed temporal region enhancement,4 patients showed sinusitis, mastoiditis .1 patient showed cerebral oedema. EV patient did not show signs of CNS infection, therefore neuroimaging may not positive be a useful tool in diagnosis of EV CNS infection. Similar reports were given by shukla et al., 2017; Frantzidou et al.,2008 &Nowak et al.,2003 .

Limitation of the study is the small sample size due to high cost of molecular technology.

This study high lights the use of PCR testing for EV in CSF samples when a viral CNS infection is suspected, even if the patients had normal CSF cell count. Enterovirus PCR can shorten the duration of hospital stay and improve management of patients.

SUMMARY

- The study was conducted among 50 suspected cases of viral meningo encephalitis RT PCR was done on CSF.
- Majority of the study population were in 16-40 age group,with range 4days to 75 years.
- There was slight male prepondence in this study.
- 21 patients were admitted in PICU ward,3 in SNN,9 in Neurosurgery,17 in IMCU.
- Fever was present in all patients.
- 76 % of patients had head ache, and 24% patients had neck rigidity
- 31 patients had clinical diagnosis of aseptic meningitis and 19 had meningo encephalitis.
- None of the patients presented with paralysis or isolated cranial nerve involvement.
- CSF pleocytosis was not seen in a high population of study population, CSF protein was between 50-100 mg/dl in maximum number of patients.94%of study population had CSF glucose levels between 45-80 mg/dl
- Hand foot mouth disease was found in one patient.
- The positive patient was 1 year 6months old female and was admitted in PICU ward during may month. The Enterovirus positive patient had fever,

seizures, lethargy, diarrhoea. CSF was macroscopically clear ,acellular, CSF glucose was 56mg/dl and CSF protein was 70 mg/dl there was slight raised CSF protein levels pointing to viral aetiology, she had normal CRP and total WBC count,

- The EV PCR of CSF can provide a definitive diagnosis and shorten the duration of hospital stay for the patients

CONCLUSION

The initial presentations of symptom and clinical manifestation of Enterovirus CNS infection often make specific diagnosis difficult and uncertain. EVs with their ever evolving serotypes is the commonest cause of meningitis, though each serotype may be associated with or without many syndromes like conjunctivitis, myocarditis, HFMD, meningitis, encephalitis, clinical presentation is not constant. They are ubiquitous in nature, some EVs can predominate in particular area and others introduced periodically causing epidemics or sporadic. As prognosis and treatment modality can be determined with aetiological agent and type of infection, early diagnosis can reduce morbidity and mortality.

The objective of this study was to detect Entero virus with realtime RT PCR – which amplified the 5`UTR the highly conserved region of Entero viruses among 50 clinically diagnosed suspected cases of meningoencephalitis and encephalitis in the study population among which 1 patient was positive for EV PCR which showed about 2% prevalence in our region. The patient recovered within 7-10 days of hospital stay. RT PCR detecting EVs RNA in CSF ,is becoming a standard practice in laboratory. They with their speed, accuracy, sensitivity and specificity and quick turn around time than the traditional gold standard cell culture, when used in diagnostic workup will provide a definitive diagnosis and improves management of patients.

BIBLIOGRAPHY

1. Themes UFO. Enteroviruses: Polioviruses, Coxsackieviruses, Echoviruses, and Newer Enteroviruses [Internet]. Basicmedical Key. 2016 [cited 2018 Apr 3]. Available from: https://basicmedicalkey.com/enteroviruses-polioviruses-coxsackieviruses-echoviruses-and-newer-enteroviruses/
2. Rotbart HA. Enteroviral infections of the central nervous system. Clin Infect Dis Off Publ Infect Dis Soc Am. 1995 Apr;20(4):971–81.
3. R Stalkup J, Chilukuri S. Enterovirus infections: A review of clinical presentation, diagnosis, and treatment. Vol. 20. 2002. 217 p.
4. Lo C-W, Wu K-G, Lin M-C, Chen C-J, Ho DM-T, Tang R-B, et al. Application of a Molecular Method for the Classification of Human Enteroviruses and its Correlation with Clinical Manifestations. J Microbiol Immunol Infect. 2010 Oct 1;43(5):354–9.
5. Intratypic genome variability of echovirus type 30 in part of the VP4/VP2 coding region | SpringerLink [Internet]. [cited 2018 Apr 3]. Available from: https://link.springer.com/article/10.1007/BF01718164
6. Hyypiä T, Hovi T, Knowles NJ, Stanway G. Classification of enteroviruses based on molecular and biological properties. J Gen Virol. 1997 Jan;78 (Pt 1):1–11.
7. Lugo D, Krogstad P. Enteroviruses in the Early 21st Century: New Manifestations and Challenges. Curr Opin Pediatr. 2016 Feb;28(1):107–13.
8. Melnick, J. L. 1996. Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer nenteroviruses, p. 655-712. In B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J.L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus (ed.), Virology, 3rd ed. Lippincott-Raven, Philadelphia, Pa.

9. Moore, M. 1982. Enteroviral disease in the United States, 1970-1979. J. Infect. Dis. 146:103-108
10. Ten.Yoke-Fun, C., and S. AbuBakar. 2006. Phylogenetic evidence for inter-typic recombination inthe emergence of human enterovirus 71 subgenotypes. BMC Microbiol. 6:74-85..
11. Mirand A., C. Henquell, C. Archimbaud, H. Peigue-Lafeuille, and J.L. Bailly. 2007. Emergence of recent echovirus 30 lineages is marked by serial genetic recombination events. J Gen Virol. 88:166-76.
12. Kung, C.M, C.C. King, C.N. Lee, L.M. Huang, P.I. Lee, and C.L. Kao. 2007. Differences in replication capacity between enterovirus 71 isolates obtained from patients with encephalitis and those obtained from patients with herpangina in Taiwan. J Med Virol. 79:60-8.
13. Gharbi, J., R. el Hiar, M. Ben M'hadheb, H. Jaïdane, L. Bouslama, S. N'saïbia, and M. Aouni.2006. Nucleotide sequences of IRES domains IV and V of natural ECHO virus type 11 isolates with different replicative capacity phenotypes. Virus Genes. 2006. 32:269-76.
14. Rotbart, H. A. 1995. Meningitis and encephalitis, p.271-289. In H. A. Rotbart (ed.), Human Enterovirus Infections. ASM Press, Washington, D.C.
15. Mistchenko, A.S., M. Viegas, M.P. Latta, and P.R. Barrero. 2006. Molecular and epidemiologic analysis of enterovirus B neurological infection in Argentine children. J Clin Virol.37:293-9.
16. Chowdhry R., A. Shukla, T. Datta, and T.N. Dhole. 2005. Rapid detection of sewage samplepolioviruses by integrated cell culture polymerase chain reaction. Arch Environ Occup Health. 60: 223-8.
17. Bouslama, L., D. Nasri, L. Chollet, K. Belguith, T. Bourlet, M. Aouni, B. Pozzetto, and S. Pillet. 2007. Natural recombination event within the capsid genomic region leading to a chimeric strain of human enterovirus B. J. Virol.81: 8944–8952

18. Rousset, D., M. Rakoto-Andrianarivelo, R. Razafindratsimandresy, B. Randriamanalina, S. Guillot, J. Balanant, P. Mauclere, and F. Delpeyroux. 2003. Recombinant vaccine-derived poliovirus in Madagascar. Emerg. Infect. Dis. 9:885-887
19. Nix W.A., M.S. Oberste, and M.A. Pallansch. 2006. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical
20. Fields BN, Knipe DM, Howley PM. Fields virology. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins; 2013.
21. Kung, C.M, C.C. King, C.N. Lee, L.M. Huang, P.I. Lee, and C.L. Kao. 2007. Differences in replication capacity between enterovirus 71 isolates obtained from patients with encephalitis and those obtained from patients with herpangina in Taiwan. J Med Virol. 79:60-8.
22. Racaniello, V. R. 1995. Early events in infection: receptor binding and cell entry, p. 73-93. In H. A. Rotbart (ed.), Human Enterovirus Infections. ASM Press, Washington, D.C.
23. Zhang, A., R. G. Nanni, D. A. Oren, E. J. Rozhon, and E. Arnold. 1992. Three dimensional structure activity relationships for antiviral agents that interact with picornavirus capsids.Semin. Virol. 3:453-4716.
24. Thivierge B, Delage G., 1982. [Infections of the central nervous system caused by enterovirus:223 cases seen at a pediatric hospital between 1973 and 1981]. Can Med Assoc J. 1;127(11):1097-102.
25. Rotbart HA, Webster AD; Pleconaril Treatment Registry Group. 2001. Treatment of potentially life-threatening enterovirus infections with pleconaril. Clin Infect Dis. 15;32(2):228-35. Epub 2001 Jan 15.
26. Rueckert, R. R. 1990. Picornaviruses and their replication, p. 507-548. In B. N. Fields, D. M Knipe, R. Chanock, M. Hirsch, J. Melnick, T. Monath, and B. Roizman (ed.), Fields Virology. Raven Press, New York N.Y.

27. Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting,position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673- 4680.
28. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.specimens. J Clin Microbiol. 44: 2698-70412.
29. Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol 16:111-120.
30. Felsenstein, J. 1993. PHYLIP: Phylogeny inference package, version 3.5 c. Distributed by Department of Genetics, University of Washington. Seattle, USA.
31. Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. J Immunol methods 65:55-63.
32. Bopegamage, S., M. Borsanyiova, A. Vargova, A. Petrovicova, M. Benkovicova, and P. Gomolcak. 2003. Coxsackievirus infection of mice. I. Viral kinetics and histopathological changes in mice experimentally infected with coxsackieviruses B3 and B4 by oral route. Acta Virol 47:245–251.
33. Melnick, J. L. 1990. Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses, p. 549-605. In B. N. Fields, D. M. Knipe, R. Chanock, M. Hirsch, J. Melnick, T. Monath, and B. Roizman (ed.), Fields virology. Raven Ross, New York,N.Y.
34. Rueckert, R. R. 1990. Picornaviruses and their replication, p. 507-548. In B. N. Fields, D. M. Knipe, R. Chanock, M. Hirsch, J. Melnick, T. Monath, and B. Roizman (ed.), Fields Virology. Raven Press, New York N.Y.

35. Rotbart, H. A., and K. kirkegard. 1992. Picornavirus pathogenesis: viral access, attachment, andentry into susceptible cells. Semin. Virol. 3:483-499. 20 Huber SA, Haisch C, Lodge PA. 1990. Functional diversity in vascular endothelial cells: role in coxsackievirus tropism. J Virol. 64(9):4516-2.
36. Prather, S. L., R. Dagan, J. A. Jenista, and M. A. Menegus. 1984. The isolation of enteroviruses from blood: a comparison of four processing methods. J. Med. Virol.14:221-227.
37. Bodian, D. 1955. Emerging concept of poliomyelitis infection. Science 122:105-108.
38. Sicinski P, Rowinski J, Warchoł JB, Jarzabek Z, Gut W, Szczygieł B, Bielecki K, Koch G. 1990. Poliovirus type 1 enters the human host through intestinal M cells. Gastroenterology. 98(1):56-8
39. Wyatt, H. V., 1976. Provocation poliomyelitis and entry of poliovirus to the CNS. Med Hypotheses. 2(6):269-74
40. Ren, R., and V. R. Racaneillo. 1992. Human poliovirus receptor gene expression and poliovirustissue tropism in transgenic mice. J. Virol. 66:296-304
41. Huber SA, Haisch C, Lodge PA. 1990. Functional diversity in vascular endothelial cells: role in coxsackievirus tropism. J Virol. 64(9):4516-2.
42. Dagan, R., and M. A. Menegus. 1992. Replication of enteroviruses in human mononuclear cells.Isr. J. Med. Sci. 28:369-372.
43. Rotbart, H. A., and K. kirkegard. 1992. Picornavirus pathogenesis: viral access, attachment, andentry into susceptible cells. Semin. Virol. 3:483-499.
44. Racaniello, V. R. 1995. Early events in infection: receptor binding and cell entry, p. 73-93. InH. A. Rotbart (ed.), Human Enterovirus Infections. ASM Press, Washington, D.C.28.Lonberg-Holm K, Crowell RL,

Philipson L. 1976 Unrelated animal viruses share receptors.Nature. 26; 259(5545):679-81

45. Bergelson, j. M., M. P. Shepley, B. M. Chan, M. E. Hemler, and R. W. Finberg. 1992. Identification of the integrin VLA-2 as a receptor for echovirus 1. Science 255: 1718-1720.
46. Guttman N, Baltimore D. 1977 A plasma membrane component able to bind and alter virions of poliovirus type 1: studies on cell-free alteration using a simplified assay. Virology. 1:82(1):25-36.
47. Haller, A. A., and B. L. Semier. 1995. Translation and host cell shutoff, p. 113-133. In H. A.Rotbart (ed.), Human Enterovirus Infections. ASM Press, Washington, D.C.
48. Hsiung, G. O. 1973. Enteroviruses, p. 25-36. In G. D. Hsiung (ed.), Diagnostic Virology. YaleUniversity Press, New Haven, Conn.
49. Dagan R, Jenista JA, Menegus MA. 1988. Association of clinical presentation, laboratory findings, and virus serotypes with the presence of meningitis in hospitalized infants withenterovirus infection. J Pediatr. 113(6):975-8.
50. Martino, T. A., P. Liu, M. Pediatric, and M. J. Sole. 1995. Enteroviral myocarditis and dilated cardiomyopathy: a review of clinical and experimental studies, p. 291-351. In H. A. Rotbart (ed.), Human Enterovirus Infections. ASM Press, Washington, D.C.
51. Abzug, M. J., M. J. Levin, and H. A. Rotbart. 1968. Profile of enterovirus disease in the first two weeks of life. Pediatr. Infect. Dis. J. 12:1201-1206
52. McKinney, R. E., Jr., S. L. Katz, and C. M. Wilfert. 1987. Chronic enteroviral meningoencephalitis in agammaglobulinemic patients. Rev. Infect. Dis. 9:334-356.
53. Hellen, C. U. T., and E. Wimmer. 1995. Enterovirus genetics, p. 25-72. In H. A. Rotbart (ed.),Human Enterovirus Infections. ASM Press, Washington, D.C.

54. Minor, P. D., G. Dunn, A. Phillips, G. D. Westrop, K. Wareham, and J. W. Almond. 1989.Attenuation and reversion of the Sabin type 3 vaccine strain, p. 307-318. In B. L. Semler and E. Ehrenfeld(ed.), Molecular Aspects of Picornavirus Infection and Detection. American Society for Microbiology, Washington, D.C.
55. Minor, P. D., G. Dunn, A. John, M. Ferguson, and J. P. Icenogle. 1986. Antigenic and molecular evolution of the vaccine strain of type 3 poliovirus during the period of excretion by a primary vaccine. J. Gen. Virol. 67:693-706.
56. Caggana M, Chan P, Ramsingh A. 1993. Identification of a single amino acid residue in the capsid protein VP1 of coxsackievirus B4 that determines the virulent phenotype. J. Virol. 67:4797-803
57. Badorff C, Lee GH, Lamphear BJ, Martone ME, Campbell KP, Rhoads RE, Knowlton KU.1999. Enteroviral protease 2A cleaves dystrophin: evidence of cytoskeletal disruption in an acquired cardiomyopathy. Nat Med. 5(3):320-6
58. Grist NR, Bell EJ. 1984. Paralytic poliomyelitis and nonpolio enteroviruses: studies in Scotland. Rev Infect Dis. 6 Suppl 2:S385-6.
59. Modlin JF, Dagan R, Berlin LE, Virshup DM, Yolken RH, Menegus M. 1991. Focal encephalitis with enterovirus infections. Pediatrics. 88(4):841-5.
60. Rotbart HA, Webster AD; Pleconaril Treatment Registry Group. 2001. Treatment of potentially life-threatening enterovirus infections with pleconaril. Clin Infect Dis. 15;32(2):228-35.Epub 2001 Jan 15.
61. Whitley, R. J., Cobbs CG, Alford CA Jr, Soong SJ, Hirsch MS, Connor JD, Corey L, Hanley. 1 DF, Levin M, Powell D. A., 1989. Diseases that mimic herpes simplex encephalitis. Diagnosis, presentation, and outcome. NIAD Collaborative Antiviral Study Group. JAMA. 14;262(2):234-9.

62. Solomon P, Weinstein L, Chang T. W., Artenstein M. S., Ambrose C.T. 1959. Epidemiologic, clinical, and laboratory features of an epidemic of type 9 ECHO virus meningitis. J Pediatr. 55:609-19.
63. Pallansch MA, Oberste MS (2006) Coxsackieviruses, echoviruses and other enteroviruses. In: Gorbach SL, Bartlett JG, Blacklow NR, editors. Infectious diseases. Philadelphia: Lippincott Williams &Wilkins.
64. Wadia NH, Katrak SM, Misra VP, Wadia PN, Miyamura K, Hashimoto K, Ogino T, Hikiji T,Kono R. 1983. Polio-like motor paralysis associated with acute hemorrhagic conjunctivitis in an outbreak in 1981 in Bombay, India: clinical and serologic studies. J Infect Dis. 147(4):660-8.
65. Cherry, J. D. 1992. Polioviruses (poliomyelitis), coxsackieviruses, echoviruses, and enteroviruses, p. 1705-1753. In R. D. Feigin and J. D. Cherry (ed.), Textbook of pediatric Infectious Diseases, 3rd ed. The W. B. Saunders Co., Philadelphia, PaTao Z, Wang H, Li Y, Liu G, Xu A, Lin X, et al. Molecular Epidemiology of Human Enterovirus Associated with Aseptic Meningitis in Shandong Province, China, 2006–2012. PLoS ONE [Internet]. 2014 Feb 21 [cited 2018 Aug 26];9(2). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3931826/
66. Kumar A, Shukla D, Kumar R, Idris MZ, Misra UK, Dhole TN. Molecular Epidemiological Study of Enteroviruses Associated with Encephalitis in Children from India. J Clin Microbiol. 2012 Nov 1;50(11):3509–12.
67. CHERRY J. Enteroviruses ; Polioviruses, Coxsackieviruses, Echoviruses, and Enteroviruses. Textb Pediatr Infect Dis. 1992;1705–53.
68. Gaaloul I, Riabi S, Harrath R, Hunter T, Hamda K, Ghzala A, et al. Coxsackievirus B detection in cases of myocarditis, myopericarditis, pericarditis and dilated cardiomyopathy in hospitalized patients. Mol Med Rep [Internet]. 2014 Sep 18 [cited 2016 Oct 25]; Available from: http://www.spandidos-publications.com/10.3892/mmr.2014.2578 101

69. Joy J, Rao YY, Raveendranath M, Shanmugam J, Balakrishnan KG. Coxsackie viral myocarditis: a clinical and echocardiographic study. Indian Heart J. 1990 Dec;42(6):441–4.
70. Nejmi S, Gaudin OG, Chomel JJ, Baaj A, Sohier R, Bosshard S. Isolation of a virus responsible for an outbreak of acute haemorrhagic conjunctivitis in Morocco. J Hyg (Lond). 1974 Apr;72(2):181–3.
71. Maitreyi RS, Dar L, Muthukumar A, Vajpayee M, Xess I, Vajpayee RB, et al. Acute hemorrhagic conjunctivitis due to enterovirus 70 in India. Emerg Infect Dis. 1999;5(2):267.
72. Lévêque N, Huguet P, Norder H, Chomel J-J. [Enteroviruses responsible for acute hemorrhagic conjunctivitis]. Med Mal Infect. 2010 Apr;40(4):212–8.
73. Ghazali O, Chua KB, Ng KP, Hooi PS, Pallansch MA, Oberste MS, et al. An outbreak of acute haemorrhagic conjunctivitis in Melaka, Malaysia. Singapore Med J. 2003 Oct;44(10):511–6.
74. Nikonov OS, Chernykh ES, Garber MB, Nikonova EY. Enteroviruses: Classification, Diseases They Cause, and Approaches to Development of Antiviral Drugs. Biochem Biokhimiia. 2017 Dec;82(13):1615–31.
75. Gopalkrishna V, Patil PR, Kolhapure RM, Bilaiya H, Fulmali PV, Deolankar RP. Outbreak of acute hemorrhagic conjunctivitis in Maharashtra and Gujarat states of India, caused by Coxsackie virus A-24 variant. J Med Virol. 2007 Jun;79(6):748–53.
76. Palacios G, Oberste MS. Enteroviruses as agents of emerging infectious diseases. J Neurovirol. 2005 Oct;11(5):424–33.
77. Konstantinidou A, Anninos H, Spanakis N, Kotsiakis X, Syridou G, Tsakris A, et al. Transplacental infection of Coxsackievirus B3 pathological findings in the fetus. J Med Virol. 2007 Jun;79(6):754–7.
78. Rotbart HA. Viral meningitis. Semin Neurol. 2000;20(3):277–92.

79. Huang H-I, Shih S-R. Neurotropic Enterovirus Infections in the Central Nervous System. Viruses. 2015 Nov 24;7(11):6051–66.
80. Noor A, Krilov LR. Enterovirus Infections. Pediatr Rev. 2016 Dec 1;37(12):505–15.
81. Hsu C-H, Lu C-Y, Shao P-L, Lee P-I, Kao C-L, Chung M-Y, et al. Epidemiologic and clinical features of non-polio enteroviral infections in northern Taiwan in 2008. J Microbiol Immunol Infect. 2011 Aug 1;44(4):265–73. 102
82. Pons-Salort M, Parker EPK, Grassly NC. The epidemiology of non-polio enteroviruses: recent advances and outstanding questions. Curr Opin Infect Dis. 2015 Oct;28(5):479–87.
83. McMinn PC. An overview of the evolution of enterovirus 71 and its clinical and public health significance. FEMS Microbiol Rev. 2002 Mar;26(1):91–107.
84. Ramirez-Fort MK, Downing C, Doan HQ, Benoist F, Oberste MS, Khan F, et al. Coxsackievirus A6 associated hand, foot and mouth disease in adults: Clinical presentation and review of the literature. J Clin Virol. 2014 Aug 1;60(4):381–6.
85. Rao PK, Veena K, Jagadishchandra H, Bhat SS, Shetty SR. Hand, Foot and Mouth Disease: Changing Indian Scenario. Int J Clin Pediatr Dent. 2012;5(3):220–2.
86. Logan SAE, MacMahon E. Viral meningitis. BMJ. 2008 Jan 5;336(7634):36–40. 54. Sawyer MH. Enterovirus infections: diagnosis and treatment. Pediatr Infect Dis J. 1999 Dec;18(12):1033–9; quiz 1040.
87. Viral Meningitis: Background, Pathophysiology, Etiology. 2016 Oct 12 [cited 2016 Nov 8]; Available from: http://emedicine.medscape.com/article/1168529-overview

88. Chonmaitree T, Baldwin CD, Lucia HL. Role of the virology laboratory in diagnosis and management of patients with central nervous system disease. Clin Microbiol Rev. 1989 Jan;2(1):1–14.
89. Wildin S, Chonmaitree T. The Importance of the Virology Laboratory in the Diagnosis and Management of Viral Meningitis. Am J Dis Child. 1987 Apr 1;141(4):454–7.
90. Irani DN. Aseptic meningitis and viral myelitis. Neurol Clin. 2008 Aug;26(3):635–55, vii–viii.
91. Arbustini E, Porcu E, Bellini O, Grasso M, Pilotto A, Bello BD, et al. Enteroviral infection causing fatal myocarditis and subclinical myopathy. Heart. 2000 Jan 1;83(1):86–90.
92. Lee W-S, Lee KJ, Kwon JE, Oh MS, Kim JE, Cho EJ, et al. Acute viral myopericarditis presenting as a transient effusive-constrictive pericarditis caused by coinfection with coxsackieviruses A4 and B3. Korean J Intern Med. 2012 Jun;27(2):216–20.
93. Fujioka S, Koide H, Kitaura Y, Deguchi H, Kawamura K, Hirai K. Molecular detection and differentiation of enteroviruses in endomyocardial biopsies and pericardial effusions from dilated cardiomyopathy and myocarditis. Am Heart J. 1996 Apr;131(4):760–5. 103
94. Shanmugam J, Raveendranath M, Balakrishnan KG. Isolation of ECHO virus type-22 from a child with acute myopericarditis--a case report. Indian Heart J. 1986 Feb;38(1):79–80.
95. LN et al. Acute myocardial infarction spurred by myopericarditis in a young female patient: Coxsackie B2 to blame. - PubMed - NCBI [Internet]. [cited 2018 Jun 21]. Available from: https://www.ncbi.nlm.nih.gov/pubmed/21446385
96. Pinamonti B, Alberti E, Cigalotto A, Dreas L, Salvi A, Silvestri F, et al. Echocardiographic findings in myocarditis. Am J Cardiol. 1988 Aug 1;62(4):285–91.

97. Muir P, Nicholson F, Illavia SJ, McNeil TS, Ajetunmobi JF, Dunn H, et al. Serological and molecular evidence of enterovirus infection in patients with end-stage dilated cardiomyopathy. Heart. 1996 Sep;76(3):243–9.
98. Acute Hemorrhagic Conjunctivitis: Background, Etiology, Epidemiology. 2017 Oct 31 [cited 2018 Jun 22]; Available from: https://emedicine.medscape.com/article/1203216-overview
99. Wright PW, Strauss GH, Langford MP. Acute hemorrhagic conjunctivitis. Am Fam Physician. 1992 Jan;45(1):173–8.
100. Wadia NH, Wadia PN, Katrak SM, Misra VP. A study of the neurological disorder associated with acute haemorrhagic conjunctivitis due to enterovirus 70. J Neurol Neurosurg Psychiatry. 1983 Jul;46(7):599–610.
101. Langford MP, Anders EA, Burch MA. Acute hemorrhagic conjunctivitis: anti-coxsackievirus A24 variant secretory immunoglobulin A in acute and convalescent tear. Clin Ophthalmol Auckl NZ. 2015 Sep 10;9:1665–73.
102. Pichichero ME, McLinn S, Rotbart HA, Menegus MA, Cascino M, Reidenberg BE. Clinical and economic impact of enterovirus illness in private pediatric practice. Pediatrics. 1998 Nov;102(5):1126–34.
103. Ooi MH, Wong SC, Lewthwaite P, Cardosa MJ, Solomon T. Clinical features, diagnosis, and management of enterovirus 71. Lancet Neurol. 2010 Nov;9(11):1097–105.
104. Wood DJ, Hull B. L20B cells simplify culture of polioviruses from clinical samples. J Med Virol. 1999 Jun;58(2):188–92.
105. Schmidt NJ, Ho HH, Lennette EH. Propagation and isolation of group A coxsackieviruses in RD cells. J Clin Microbiol. 1975 Sep;2(3):183–5.
106. Dahling DR, Berg G, Berman D. BGM, a continuous cell line more sensitive than primary rhesus and African green kidney cells for the recovery of viruses from water. Health Lab Sci. 1974 Oct;11(4):275–82. 104

107. Muir P, Kämmerer U, Korn K, Mulders MN, Pöyry T, Weissbrich B, et al. Molecular Typing of Enteroviruses: Current Status and Future Requirements. Clin Microbiol Rev. 1998 Jan;11(1):202–27.

108. Chonmaitree T, Ford C, Sanders C, Lucia HL. Comparison of cell cultures for rapid isolation of enteroviruses. J Clin Microbiol. 1988 Dec;26(12):2576–80.

109. Hambling MH, Davis PM, Macrae AD. The typing of enteroviruses in tissue culture by neutralization with composite antiserum pools. J Hyg (Lond). 1963 Dec;61(4):479–84.

110. Hyypiä T, Kallajoki M, Maaronen M, Stanway G, Kandolf R, Auvinen P, et al. Pathogenetic differences between coxsackie A and B virus infections in newborn mice. Virus Res. 1993 Feb 1;27:71–8.

111. Bendig J, Earl P. The Lim Benyesh-Melnick antiserum pools for serotyping human enterovirus cell culture isolates--still useful, but may fail to identify current strains of echovirus 18. J Virol Methods. 2005 Jul;127(1):96–9.

112. Melnick JL, Schmidt NJ, Hampil B, Ho HH. Lyophilized combination pools of enterovirus equine antisera: preparation and test procedures for the identification of field strains of 19 group A coxsackievirus serotypes. Intervirology. 1977;8(3):172–81.

113. Zheng H, Zhang Y, Liu L, Lu J, Guo X, Li H, et al. Isolation and Characterization of a Highly Mutated Chinese Isolate of Enterovirus B84 from a Patient with Acute Flaccid Paralysis. Sci Rep. 2016 Aug 8;6:31059.

114. Rigonan AS, Mann L, Chonmaitree T. Use of Monoclonal Antibodies To Identify Serotypes of Enterovirus Isolates. J Clin Microbiol. 1998 Jul;36(7):1877–81.

115. Konstantinidou A, Anninos H, Spanakis N, Kotsiakis X, Syridou G, Tsakris A, et al. Transplacental infection of Coxsackievirus B3 pathological findings in the fetus. J Med Virol. 2007 Jun;79(6):754–7.

116. Read S J, Jeffery K J M, Bangham C R M. Aseptic meningitis and encephalitis: the role of PCR in the diagnostic laboratory. J ClinMicrobiol. 1997;35:691–696. [PMC free article] [PubMed] [Google Scholar]

117. Zoll G J, Melchers W J G, Kopecka H, Jambroes G, van der Poel H J A, Galama J M D. General primer-mediated polymerase chain reaction for detection of enteroviruses: application for diagnostic routine and persistent infections. J ClinMicrobiol. 1992;30:160–165. [PMC free article] [PubMed] [Google Scholar]

118. Hong HL, Lee EM, Sung H, Kang JK, Lee SA, Choi SH. Clinical features, outcomes, and cerebrospinal fluid findings in adult patients with central nervous system (CNS) infections caused by varicella-zoster virus: comparison with enterovirus CNS infections. J Med Virol 2014;86(12):2049–54.

119. Kaminski M, Grummel V, Hoffmann D, Berthele A, Hemmer B. The spectrum of aseptic central nervous system infections in southern Germany — demographic, clinical and laboratory findings. Eur J Neurol 2017;24(8):1062–70.

120. Han SH, Choi HY, Kim JM, Park KR, Youn YC, Shin HW. Etiology of aseptic meningitis and clinical characteristics in immune-competent adults. J Med Virol 2016;88 (1):175–9.

121. Shukla B, Aguilera EA, Salazar L, Wootton SH, Kaewpoowat Q, Hasbun R. Aseptic meningitis in adults and children: diagnostic and management challenges. J Clin Virol 2017;94:110–4.

122. Landtblom AM, Fridriksson S, Boivie J, Hillman J, Johansson G, Johansson I. Sudden onset headache: a prospective study of features, incidence and causes. Cephalalgia 2002;22(5):354–60.

123. Carrol ED, Beadsworth MB, Jenkins N, Ratcliffe L, Ashton I, Crowley B, et al. Clinical and diagnostic findings of an echovirus meningitis outbreak in the north west of England. Postgrad Med J 2006;82(963):60–4.
124. Graham AK, Murdoch DR. Association between cerebrospinal fluid pleocytosis and enteroviral meningitis. J Clin Microbiol 2005;43(3):1491
125. Ihekwaba UK, Kudesia G, McKendrick MW. Clinical features of viral meningitis in adults: significant differences in cerebrospinal fluid findings among herpes simplex virus, varicella zoster virus, and enterovirus infections. Clin Infect Dis 2008;47(6):783–9.
126. Martinez-Giron R, Pantanowitz L. Cerebrospinal fluid cytology in nonmalignant aseptic meningeal disorders. Diagn Cytopathol 2017;45(11):1020–9.
127. Jaijakul S, Salazar L, Wootton SH, Aguilera E, Hasbun R. The clinical significance of neutrophilic pleocytosis in cerebrospinal fluid in patients with viral central nervous system infections. Int J Infect Dis 2017;59:77–81.
128. Yun KW, Choi EH, Cheon DS, Lee J, Choi CW, Hwang H, et al. Enteroviral meningitis without pleocytosis in children. Arch Dis Child 2012;97(10):874–8.

PROFORMA FOR ENTEROVIRUSES MENINGITIS,

MENINGOENCEPHALITIS

Name: Age/Sex: Ward:

IP/OP NO:

Address: Sample Receiving Time In Lab:

Phone no:

1.Fever duration

2.seizures/dowsy/headache/photophobia/coma/altered consciousness/any cranial nerve palsy

3.Rashes-Vesicles/Papules/Spasmodic/Rib pain& fever

4.Pharyngitis/URTI

5.Diarrhoea/Vomitting

6.Others:

Haemorrhagic conjunctivitis

Myopericarditis

Guillain barre syndrome

Flaccid paralysis

Symptoms of shock

Haemorrhagic manifestations

H/O Similar episode in home or school

INVESTIGATONS:

Hb CSF

TC	Protein
DC	Glucose
HCT%	Cell count
Platlet count	Globulin
LFT	
Blood culture	
Malaria/JE/Dengue	
USG	
CSR	
CT,MRI	
Molecular diagnosis	
RT-PCR	
Other reports if any	

நோயாளிகளுக்கு அறிவிப்பு மற்றும் ஒப்புதல் படிவம்
(மருத்துவ ஆய்வில் பங்கேற்பதற்கு)

ஆய்வு செய்யப்படும் தலைப்பு:
பங்கு பெறுவரின் பெயர்:
பங்கு பெறுவரின் வயது:

		பங்கு பெறுவர் இதனை ✓ குறிக்கவும்
1.	நான் மேலே குறிப்பிட்டுள்ள மருத்துவ ஆய்வின் விவரங்களை படித்து புரிந்து கொண்டேன். என்னுடைய சந்தேகங்களை கேட்கவும், அதற்கான தகுந்த விளக்கங்களை பெறவும் வாய்ப்பளிக்கப்பட்டுள்ளது என அறிந்து கொண்டேன்.	☐
2.	நான் இவ்வாய்வில் தன்னிச்சையாக தான் பங்கேற்கிறேன். எந்த காரணத்தினாலோ எந்த கட்டத்திலும், எந்த சட்ட சிக்கலுக்கும் உட்படாமல் நான் இவ்வாய்வில் இருந்து விலகி கொள்ளலாம் என்றும் அறிந்து கொண்டேன்.	☐
3.	இந்த ஆய்வு சம்பந்தமாகவோ, இதை சார்ந்து மேலும் ஆய்வு மேற்காள்ளும் போதும் இந்த ஆய்வில் பங்குபெறும் மருத்துவர் என்னுடைய மருத்துவ அறிக்கைகளை பார்ப்பதற்கு என் அனுமதி தேவையில்லை என அறிந்து கொள்கிறேன். நான் ஆய்வில் இருந்து விலகிக் கொண்டாலும் இது பொருந்தும் என அறிகிறேன்.	☐
4.	இந்த ஆய்வின் மூலம் கிடைக்கும் தகவலையோ, முடிவையோ பயன்படுத்திக் கொள்ள மறுக்க மாட்டேன்.	☐
5.	இந்த ஆய்வில் பங்கு கொள்ள ஒப்புக் கொள்கிறேன் எனக்கு கொடுக்கப்பட்டஅறிவுரைகளின் படி நடந்து கொள்வதுடன், ஆய்வை மேற்கொள்ளும் மருத்துவ அணிக்கு உண்மையுடன் இருப்பேன் என்று உறுதியளிக்கிறேன். என் உடல் நலம் பாதிக்கப்பட்டாலோ, அல்லது எதிர்பாராத, வழக்கத்திற்கு மாறான நோய்குறி தென்பட்டாலோ உடனே இதை மருத்துவ அணியிடம் தெரிவிப்பேன் என உறுதி அளிக்றேன்.	☐

பங்கேற்பவரின் கையொப்பம் / ..... இடம் .....
கட்டைவிரல் ரேகை
பங்கேற்பவரின் பெயர் மற்றும் விலாசம் .....
ஆய்வாளரின் கையொப்பம் /..... இடம் .....
ஆய்வாளரின் பெயர் .....
மையம் .....
கல்வியறிவு இல்லாதவற்கு (கைரேகை வைத்தவர்களுக்கு) இது அவசியம் தேவை
சாட்சியின் கையொப்பம் /..... இடம் .....
பெயர் மற்றும் விலாசம் ....

Master chart

IP No.	Age	Sex	Ward	Fever (Yes/No)	Headache (Yes/No)	Seizures (Yes/No)	Neck rigidity (Yes/No)	Altered sensorium / Lethargy / Behavioural changes / Coma (Yes/No)	Appearance of CSF	CSF Sugar mmol	CSF protein mg /dl	CSF Cell count	month of admission	HFMD	neuro imaging	diarrhoea	upper respiratory tract infection	vomitting	sepsis like syndrome	photophobia	RT PCR POSITIVE
10002	4	Mch	PICU	yes	yes	yes	yes	altered sensorium	clear	55	18	acellular	Jan-18	no	not done	no	yes	no	no	no	negative
19700	23	F	IMCU	yes	yes	no	no	altered sensorium	clear	57	18	acellular	Jan18	no	not done	no	yes	no	no	no	negative
54893	10	Fch	PICU	yes	yes	yes	yes	altered sensorium	clear	41	25	acellular	Jan-18	no	sinusitis	no		no	no	no	negative
37941	46	M	IMCU	yes	yes	no	yes	oss of consciousness	slight turbid	51	21	lymphocyte predominant	Jan-18	no	temperopa rietal enhancem	no	yes	no	no	no	negative
62047	8	Fch	PICU	yes	yes	no	no	altered sensorium	clear	54	19	a cellular	Feb18	no	not done	yes	yes	no	no	no	negative
61430	9	Fch	PICU	yes	yes	yes	yes	altered sensorium	clear	43	28	a cellular	Feb-18	no	sinusitis	yes	no	no	no	no	negative
64862	30	M	IMCU	yes	yes	no	no	altered sensorium	clear	55	32	acellular	Feb18	no	not done	no	yes	no	no	no	negative
64862	1y 6m	Mch	PICU	yes	-	yes	-	lethargy	clear	65	32	a cellular	Mar18	no	not done	no	yes	no	no	no	negative
63341	10	Fch	PICU	yes	yes	yes	yes	altered sensorium	clear	50	22	a cellular	Mar-18	no	sinusitis	no	no	no	no	yes	negative
67008	8	Fch	PICU	yes	yes	yes	yes	altered sensorium	clear	75	48	a cellular	Mar-18	no	not done	no	yes	no	no	yes	negative
20191	75	M	IMCU	yes	No	no	no	altered sensorium	clear	85	45	a cellular	Mar18	no	age related changes	no	no	no	no	yes	negative
16005	7	Mch	PICU	yes	Yes	no	yes	altered sensorium	clear	48	32	a cellular	Apr-18	no	sinusitis	no	yes	no	no	yes	negative
18332	46	M	IMCU	yes	yes	no	yes	altered sensorium	clear	65	18	utrophil predomina	Apr-18	no	age related changes	yes	yes	no	no	yes	negative
10001	1y6m	Mch	PICU	yes	-	yes	-	lethargy	clear	65	27	a cellular	April18	no	not done	no	yes	no	no	no	negative
12583	3	Fch	PICU	yes	yes	no	yes	lethargy	clear	58	22	a cellular	Apr-18	no	not done	no	no	no	no	no	negative
19458	20	M	IMCU	yes	yes	no	no	oss of consciousness	clear	65	35	phocytes predomir	Apr-18	no	temperopa rietal	no	yes	no	no	no	negative
57790	3	F	Neurology	yes	yes	yes	yes		clear	52	23	a cellular	Apr-18	no	not done	no	no	no	no	no	negative
46883	18	M	IMCU	yes	yes	no	no	altered sensorium	clear	41	9	a cellular	may18	no	not done	no	yes	no	no	yes	negative
18775	71	M	IMCU	yes	No	no	no	altered sensorium	slight turbid	51	22	acellular	may18	no	age related changes	no	no	no	no	yes	negative
57254	1yr6m	Fch	PICU	yes	-	yes	-	lethargy	clear	56	70	acellular	may18	no	not done	yes	no	no	no	-	positive
52658	55	M	Neurology	yes	yes	no	no	altered sensorium	clear	59	15	acellular	may18	no	age related changes	no	yes	no	no	yes	negative
53266	9m	Fch	PICU	yes	-	yes	-	lethargy	clear	57	11	acellular	may18	no	not done	yes	yes	no	no	no	negative
52434	45	F	IMCU	yes	yes	no	no	altered sensorium	clear	80	20	phocytes predomin	may18	no	age related changes	no	no	yes	no	yes	negative
52705	55	M	IMCU	yes	yes	no	no	altered sensorium	clear	93	38	phocyte predomina	may18	no	age related changes	no	yes	no	no	yes	negative
52711	30	F	ICCU	yes	yes	no	no	altered sensorium	clear	44	20	acellular	Jun-18	no	not done	no	yes	no	no	yes	negative
50009	9	Fch	PICU	yes	yes	yes	yes	altered sensorium	clear	23	15	acellular	june18	no	cerebral edema	no	no	yes	no	yes	negative
52325	9m	Mch	PICU	yes	-	yes	-	lethargy	clear	23	15	acellular	Jun-18	no	not done	no	yes	no	no	no	negative
53393	40d	Mch	PICU	yes	-		-	lethargy	clear	56	15	acellular	Jun-18	no	not done	no	no	no	no	no	negative
56175	45	M	IMCU	yes	yes	no	no	altered sensorium	clear	45	85	acellular	june18	no	age related changes	no	yes	yes	no	yes	negative
57295	35	F	Neurology	yes	yes	no	no	altered sensorium	clear	56	23	acellular	june18	no	not done	no	no	no	no	yes	negative
57802	2days /365	F	SNN	yes	-	yes	-	lethargy	clear	42	60	acellular	july18	no	not done	no	yes	no	no	no	negative
57014	39	F	IMCU	yes	yes	no	no	altered sensorium	clear	58	12	acellular	july18	no	age related changes	no	no	yes	no	yes	negative
56062	8m	Mch	PICU	yes	-	yes	-	lethargy	clear	59	28	acellular	Jul-18	no	not done	no	no	no	no	yes	negative

IP No.	Age	Sex	Ward	Fever (Yes/No)	Headache (Yes/No)	Seizures (Yes/No)	Neck rigidity (Yes/No)	Altered sensorium / Lethargy / Behavioural changes / Coma (Yes/No)	Appearance of CSF	CSF Sugar mmol	CSF protein mg /dl	CSF Cell count	month of admission	HFMD	neuro imaging	diarrhoea	upper respiratory tract infection	vomitting	sepsis like syndrome	photophobia	RT PCR POSITIVE
57663	40	M	IMCU	yes	yes	no	no	altered sensorium	clear	65	85	acellular	July18	no	age related changes	no	yes	yes	no	no	negative
57545	2m	Mch	PICU	yes	-	yes	-	altered sensorium	clear	47	50	acellular	july18	no	not done	yes	no	yes	no	no	negative
55536	70	F	IMCU	yes	no	no	no	altered sensorium	clear	26	20	acellular	july18	no	age related changes	no	no	yes	no	yes	negative
17525	1	Mch	PICU	yes	-	yes	no	altered sensorium	clear	85	37	acellular	july18	present	not done	no	yes	yes	no	no	negative
62766	60	M	Neurology	yes	no	no	no	altered sensorium	clear	57	10	phocyte predomin	aug18	no	age related changes	no	no	yes	no	no	negative
52400	58	F	IMCU	yes	yes	-	-	altered sensorium	clear	72	10	acellular	aug18	no	age related changes	no	yes	no	no	yes	negative
66871	64	M	Neurology	yes	yes	yes	no	altered sensorium	clear	45	20	phocyte predomina	aug18	no	age related changes	no	no	no	yes	yes	negative
67288	8m	Fch	Neurology	yes	-	yes	-	lethargy	blood stained		52	acellular	sep18	no	not done	yes	no	no	no	no	negative
68112	6m	Fch	Neurology	yes	-	yes	-	lethargy	clear	56	15	acellular	sep18	no	not done	no	no	no	no	no	negative
69473	18	M	IMCU	yes	yes	no	yes	altered sensorium	clear	52	17	utrophil predomina	sep18	no	not done	no	yes	no	no	yes	negative
68851	11m	Mch	PICU	yes	-	yes	-	lethargy	clear	53	14	acellular	sep18	no	not done	no	yes	no	no	no	negative
33557	3y9m	Fch	PICU	yes	yes	yes	yes	lethargy	clear	52	17	acellular	oct18	no	not done	no	no	no	no	no	negative
65854	56	M	Neurology	yes	yes	no	no	altered sensorium	clear	60	41	acellular	oct18	no	age related changes	no	no	no	no	yes	negative
67078	55	M	Neurology	yes	yes	no	no	altered sensorium	clear	38	21	acellular	nov18	no	age related changes	no	yes	yes	no	yes	negative
67211	4d/365	F	SNN	yes	-	yes	-	lethargy	clear	35	93	acellular	nov18	no	not done	no	no	no	no	no	negative
53423	20	F	IMCU	yes	yes	no	yes	altered sensorium	clear	65	12	utrophil predomina	dec18	no	not done	no	no	no	no	yes	negative
67312	5d	M	SNN	yes	-	yes	-	lethargy	clear	45	96	acellular	dec18	no	not done	no	no	no	no	no	negative