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# TO DETERMINE ETIOLOGICAL AGENTS INVOLVED IN VIRAL ENCEPHALITIS BY USING NEW MOLECULAR TECHNIQUE RTPCR, TAQMAN REAL TIMEPCR- A PROSPECTIVE STUDY.

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## ABSTRACT

**INTRODUCTION:** - Present study was done to determine etiological agents involved in viral encephalitis by using new molecular technique RTPCR, Taqman Real TimePCR.

**AIMS AND OBJECTIVES: -** To know the various etiopathogenetic agents of acute viral encephalitis syndrome by using various molecular techniques in serum and CSF

**MATERIAL & METHODS: -** This is a prospective study on 120 patients, arrived at JA hospital of GR Medical with clinical suspicion of acute viral encephalitis

**CONCLUSION:-** RTPCR and Real Time RTPCR are highly specific in identifying etiological agent in acute viral encephalitis

## KEYWORDS :

## INTRODUCTION

Infections caused by viruses are exorbitantly prevalent in the world. The viruses as etiological agent of diseases have been existing in the world from time immemorial and have concurrently inflicted hazardous and lethal disease to human beings, animals, and plant alike.

In terms of mortality and morbidity viral encephalitis is one of most important viral disease world wide. Encephalitis literally means an inflammation of the brain, but it usually refers to brain inflammation caused by viruses. It is a rare disorder that occur in approximately 0.5 cases per 1,00,000 individual - most commonly in children, the elderly and people with weakened immune system (e.g. those with HIV/AIDS or cancer).

The most challenging aspect of viral encephalitis infection is its definite proper diagnosis. Now a days new molecular technique have been employed for diagnosis viz demonstration of viral antigen in serum and CSF byRT PCR, Real Time RTPCR.

The sensitivity and specificity of the reported TaqMan based real-time RT-PCR was validated with a panel of defined acute phase encephalitis serum samples and CSF sample collected from AES patient admitted in J.A.H. Gwalior.

Present study was undertaken to determined etiological agents involved in dreadful illness of viral encephalitis and changes produced by viral agents in body in terms of hematological changes and their correlation by using new molecular technique RTPCR, Taqman Real TimePCR.

## AIMS AND OBJECTIVES

- To know the incidence of acute viral encephalitis syndrome in hospital admitted patient of greater Gwalior region by various molecular techniques.
- To know the various etiopathogenetic agents of acute viral encephalitis syndrome by using various molecular techniques in serum and CSF.
- To compare the result of molecular techniques in serum and CSF with relation to various etiopathogenetic agents.
- To compare the findings of conventional CSF examination and blood tests with relation to etiopathogenetic agent.

## GENERAL REVIEW OF LITERATURE

Encephalitis, an inflammation of brain parenchyma present as diffuse and/or focal neuropsychological dysfunction. Although it primarily involves the brain, the meninges are frequently involved (meningoencephalitis).

The biggest challenge in diagnosis of acute viral encephalitis is etiological identification. Despite the use of newer technique including CSF-PCR, upto 70% cases of encephalitis remain of unknown etiology in modern assay.

The common viral etiological agent causing acute viral encephalitis in South East Asia region are:

- Enterovirus
- Herpes simplex virus
- JE (Japanese Encephalitis)
- Measles
- Varicella - Zoster virus
- Mumps
- Epstein Barr virus
- Varicella zoster virus
- HIV

## MOLECULAR DIAGNOSTIC METHODS

### PCR (Polymerase Chain Reaction)

The **polymerase chain reaction** (**PCR**) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

**Reverse Transcription PCR (RT-PCR):** for amplifying DNA from RNA. Reverse transcriptase reverse transcribes RNA into cDNA, which is then amplified by PCR.

## MATERIAL AND METHODS

The present prospective study on identifying the etiological agents in acute viral encephalitis and incidence of acute viral encephalitis in clinicaly suspected cases of acute viral encephalitis syndrome was carried out during the period from October 2010-October 2011.

This work was undertaken in Department of Pathology, Gajra Raja Medical College, Gwalior and Jayarogya Group of Hospitals (J.A.H.) Gwalior (M.P.) in association with virology division Defence Research and Development Establishment, Gwalior from October 2010-2011.

Total 120 cases were selected from patient admitted in different department of Jaya Arogya Hospital (J.A.H) Gwalior with clinical suspicion of acute viral encephalitis and fulfilling inclusion criteria. Group of 120 people were also selected as controls from patient admitted in J.A.H Gwalior with clinically suspicious diagnosis of neurological disorder other than acute viral tencephalitis and which does not full fill inclusion criteria.

**Inclusion criteria:**

- All patients with acute onset of fever and change in mental

status (including symptoms like confusion, disorientation, coma, inability to talk) and/or new onset of seizure.
- Patient with CSF pleocytosis (WBC count > 5/$mm^3$) with/without parenchymal lesion.
- Substantial meningeal enhancement as identified by brain CT, MRI.
- Presence of definite neurological dysfunction without CSF pleocytosis it include aphasia, ataxia, UMN and LMN weakness, involuntary movements, cranial nerve deficits.

**Exclusion criteria:**
Patient with simple partial seizure
In all cases detailed history was taken and clinical findings were noted. In most of the cases the following investigation were done:

**1. CSF examination:**
Routine microscopic examination,Cell estimation , Protein & Sugar estimation in CSF.

**2. Blood examination**
Haemoglobin , Packed cell volume, White blood cell count, Platelet count, ESR estimation , and Random blood sugar estimation was done

**Material used:**
Material used for study is CSF, blood and serum from clinically suspected cases of viral encephalitis.

- Sample are taken within 6 days of onset of symptoms as viral load is maximum during this period and it may reduced to less than 20% after one week.
- 2 ml of CSF and 5 ml of blood sample is used for study.
- CSF was routinely stored at $^{4o}$C or –2$^{0o}$C.
- If long term storage was anticipated specimen was stored at –70°C.
- CSF sample collected was immediately transferred to DRDE lab for viral etiological identification by RTPCR/RealTimeRTPCR.

**CSF culture (Pour plate method)**
MacConkey and Chocolate agar plates were prepared by mixing the respective ingredients.

**Molecular Diagnosis**
***(a) Extraction of total nucleic acid (RNA/DNA) [QIAamp viral nucelic acid (RNA/DNA) spin column]*** The total nucleic acid (RNA/DNA) was extracted from the samples using Qiagen Ultrasense viral total nucleic acid extraction kit (**Qiagen, Germany**) according to the manufactures's protocol.

***(b) One step reverse transcription polymerase chain reaction (RT-PCR)***
RT-PCR was carried out on all 120 RNA/DNA by a one step RT-PCR protocol using Access quick RT-PCR assay (**Promega, USA)** following the manufacturer's instruction.

***(c) Agarose Gel Electrophoresis***
The size and quantity of the PCR amplicon was confirmed by 1% agarose gel electrophoresis.

**Reverse Transcription Polymerase Chain Reaction (Real time RTPCR)**
All the sample were utilized for Real Time RT-PCR, The real time PCR assays has many advantages over conventional RT-PCR methods, including rapidity, quantitative measurement lower contamination rate, linear sensitivity, higher specificity and early standardization (Mackay et al 2002).

***Taqman probe based Real-time RT-PCR***
Taqman probe based one-step real time quantitative RT-PCR amplification was performed in the $M_x3005P$ quantitative PCR system **(Stratagene, USA**).

Further confirmation of the amplified products during initial standardization was also done by the size assessment of the Real-time RT-PCR amplicons by 1% agarose gel electrophoresis. as described by **Sambrook *et al.,* 1989**.

**RESULTS AND OBSERVATION**
A total of 120 cases were identified suffering from viral encephalitis and 120 samples were collected which included 120 CSF sample and 120 serum samples.

All the samples were stored at –20°C before being transported for analysis and thereafter stored at –70°C at Defence Research Development Establishment (DRDE), Gwalior, India.

After aseptic collection CSF and serum were taken to laboratory of J.A. Group of Hospital, where routine CSF examination, protein estimation of CSF, sugar estimation of CSF and in blood and serum haematological parameters (Hb, PCV, TLC, DLC, platelet count, random sugar) were examined.

All the CSF samples were also cultured on Mac-conkey agar and chocolate agar media, none of them showed growth of colonies after 24 hrs.

After routine examination and culture, samples were stored at –20°C and transported immediately to DRDE laboratories where newer molecular techniques were applied for identification of viral etiology.

For molecular detection total nucleic acid(RNA/DNA) was extracted by using Quiagen ultrasense kit**(Quiagen,Germany**).The nucleic acid from all cases were initially processed by RTPCR.

**RT-PCR amplification**
RT-PCR was performed on all cases for EV(enterovirus), measles, HSV-1(herpes simplex virus -1), HSV-2(herpes simplex virus-2), HSV-3,4,5,6, JE (Japanese encephalitis), measles and Varicella Zoster Virus on suspected clinical samples of acute encephalitis.

**RT-PCR positivity was obtained for enterovirus and measles virus only**.
For detection of enterovirus and measles virus RNA, RNA was initially extracted by using QIA amp viral RNA mini kit(**Quiagen Germany).** The RT PCR using N-gene specific primer set for measles virus and 5'NCR gene specific primer set for enterovirus revealed characteristic 454 bp amplicon of enterovirus in 3 positive cases and 500 bp amplicon of measles virus in 1 positive case

**Real Time RT-PCR**
Nucleic acid from all cases and control was then processed by Taqman based Real Time PCR for enterovirus,HSV-1,2,3,4,5,6, measles, westnile virus using specific primer kit as mentioned in material and method.

Out of 120 cases 7 cases were confirmed by Taqman based Real time RT-PCR. Out of 7 positive cases, 4 were for enterovirus and 3 for measles virus. No flavivirus and HSV group virus was detected by Real time RTPCR as shown in (**figure 1 and 2).**

**Sensitivity and specificity of Taqman probe based Real-time RT-PCR**
On comparative evaluation with conventional RT-PCR, Taqman based real time RT-PCR demonstrated exceptionally higher sensitivity by picking up 1 and 2 additional positive samples for entero and measles virus respectively as compared to conventional RT-PCR (as shown in **Fig. 3, 4).**

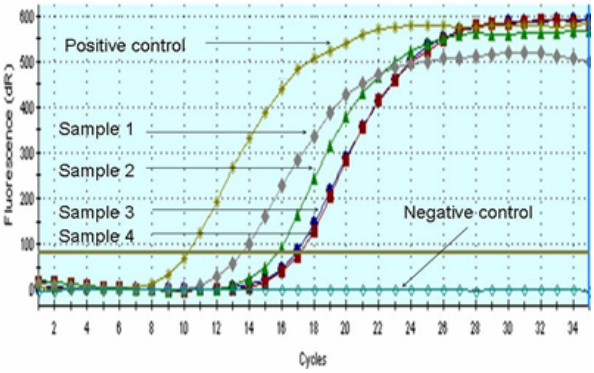



**Fig No. 1: Amplification plot shows positivity for enterovirus by Real time RT-PCR**

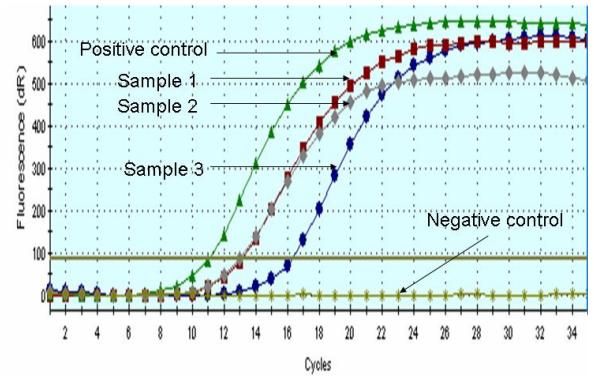
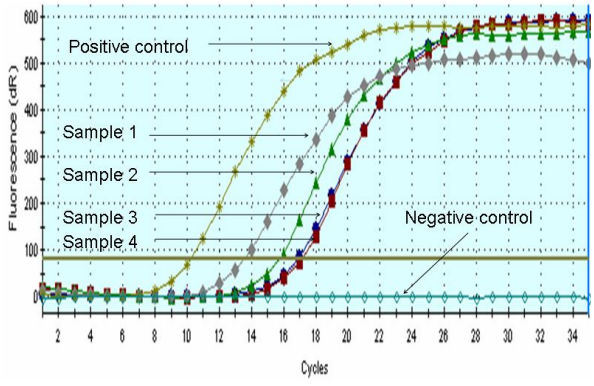



**Fig No. 2: Amplification plot shows positivity for measles virus by Real time RT-PCR**




**Comparative analysis of RT-PCR vs Real time RT-PCR for enterovirus Above figure shows that Real Time PCR is more sensitive as compare to RTPCR by identifying one more enterovirus as compare to RTPCR.**

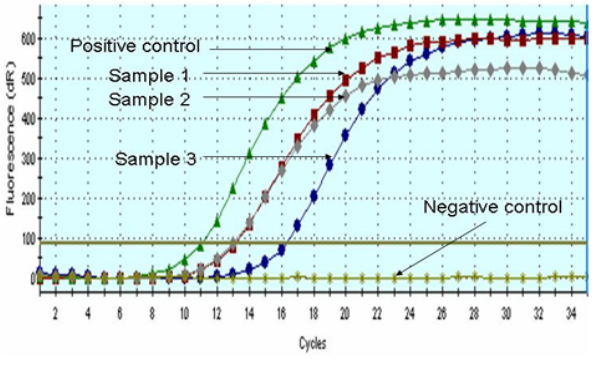



**Fig No. 4: Comparative analysis of RT-PCR vs Real time RT-PCR for measles virus. Above figure shows that Real Time RTPCR is more sensitive by identifying two more samples as compare to RTPCR.**

## OBSERVATION TABLE

**Table No. 1: Positive cases of specific viruses diagnosed by RTPCR/Real Time RTPCR**

| | EV | Measles | JE | CMV | HSV1 | HSV2 | VZV |
|---|---|---|---|---|---|---|---|
| 120 (cases) | 4 (3.33%) cases | 3(2.5%) cases | - | - | - | - | - |
| 120 (controlled) | – | – | – | – | – | – | – |

**Table No. 2: Comparison of RT-PCR and Real Time RTPCR**

| | RTPCR | Real TimePCR |
|---|---|---|
| **Total cases(120)** | **4(3.33%)** | **7(5.8%)** |
| EV(4) | 3(75%) | 4(100%) |
| Measles(3) | 1(33.33%) | 3(100%) |

Above table shows Real Time RTPCR is more sensitive in identifying EV as well as measles than RTPCR.

**Table No.3: Comparison of positivity in CSF and serum samples in all cases**

| | CSF sample | Serum sample |
|---|---|---|
| Total sample | 120(100%) | 120(100%) |
| Positive cases | 05(4.1%) | 02(1.6%) |

Above table showed that virus are diagnosed more in CSF than in serum.

**Table No.4: Comparison of RTPCR & Real Time PCR in identification of viral agents in CSF and serum of AES patients**

| | RTPCR | Real Time PCR |
|---|---|---|
| CSF | 3 (2.5%) | 5 (4.1%) |
| Serum | 1 (0.8%) | 2 (1.6%) |
| Total Cases | 120(100%) | 120(100%) |

**Table No.5: Comparison of CSF and serum sample in positive cases**

| | CSF | Serum |
|---|---|---|
| EV | 3(60%) | 1(50%) |
| Measles | 2(40%) | 1(50%) |

This table also shows that viruses are diagnosed more in CSF than in serum.

**Table No. 6: CSF routine examination in all cases**

| Total no. of CSF samples | CSF cell count | | | Differential count | |
|---|---|---|---|---|---|
| | < 5 cell/ cumm | > 5 -100 cell/ cumm | > 100 cell/ cumm | Lymphocytosis | Neutrophilia |
| 120 (cases) | 18 (15%) | 94 (78.33%) | 08 (6.66%) | 104 (86.66%) | 16 (13.33%) |
| 120 (control) | 86 (71.66%) | 30 (25%) | 04 (3.33%) | 28(23.33%) | 02(1.66%) |
| 07 (Positive) | 00 (0%) | 06 (85.7%) | 01 (14.28%) | 07(100%) | 00(0%) |

**Table No.7: Comparative chart of CSF sugar and protein estimation in total CSF sample and positive CSF samples**

| | CSF sugar | | CSF protein | | |
|---|---|---|---|---|---|
| | Normal | Reduced | Normal | Increased | Low |
| 120 (cases) | 108(90%) | 12(10%) | 36(30%) | 84(70%) | 0(0%) |
| 120 (control) | 81(67.5%) | 39(32.5%) | 89(74.1%) | 24(20%) | 07(5.8%) |
| 7 (positive cases) | 7(5.8%). | 0(0%) | 01(14.28%) | 06(85.71%) | 0(0%) |

**Table No. 8: Comparative chart of hematological parameters in our study**

| | Hb | | | TLC | | DLC | | Platelet | | Sugar | | ESR | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | < 10 gm% | 10-12 gm% | > 12 gm% | Normal (4500-11000) | Increased (>11000) | Lymphocytosis (Absolute) | Neutrophilia (Absolute) | Normal | Reduced (<150000) | Normal | Reduced | Normal | Increased |
| Total cases (120) | 34 (28.33%) | 42 (35%) | 44 (36.66%) | 73 (60.8%) | 47(39.16%) | 22(18.33%) | 06(5%) | 93(77.5%) | 27(22.5%) | 102(85%) | 18(15%) | 101( 84.5%) | 19 (15.87%) |
| Total control cases(120) | 09 (7.5%) | 73 (60.8%) | 38 (31%) | 102 (85%) | 18(15%) | 13(10.8%) | 05(4.1%) | 106(88.3%) | 14(11.66%) | 112(93.3%) | 08 (6.66%) | 113 (94.16%) | 07 (5.8%) |
| Positive cases (7) | 2(28.5%) | 4 (57.14%) | 1 (14.28%) | 6 (85.71%) | 1(14.28%) | 1(14.28%) | 0(0%) | 6(85.71%) | 1(14.28%) | 7(100%) | 0(0%) | 6 (85.71%) | 1 (14.28%) |
| EV (4) | 1 (25%) | 2 (50%) | 1 (25%) | 3 (75%) | 1(25%) | 1(25%) | 0(0%) | 4(100%) | 0(0%) | 4(100%) | 0(0%) | 4(100%) | 0(0%) |
| Measles (3) | 1 (33.33%) | 2 (66.66%) | 0 (0%) | 3 (100%) | 0(0%) | 0(0%) | 0(0%) | 2(66.66%) | 1(33.33%) | 3(100%) | 0(0%) | 2 (66.66%) | 1 (33.33%) |

**Table No. 9: Correlation of clinical features, viral load with relation to specific viral agent in Acute Encephalitis Syndrome**

| S. No. | Viral load | Viral agent | Fever | Altered sensorium | Meningeal sign | DTR | Hepatomegaly | Spleenomegaly |
|---|---|---|---|---|---|---|---|---|
| 1 | $1 \times 10^4$ | EV | + | + | + | + | - | - |
| 2 | $1 \times 10^4$ | EV | + | + | + | - | + | - |
| 3 | $3 \times 10^4$ | EV | + | + | - | + | - | + |
| 4 | $1 \times 10^2$ | EV | + | + | - | - | - | - |
| 5 | $8 \times 10^4$ | Measles | + | + | + | - | - | + |
| 6 | $6 \times 10^4$ | Measles | + | + | + | + | - | - |
| 7 | $2 \times 10^3$ | Measles | + | + | - | - | + | - |

**Table no. 10: Comparative chart showing mortality and morbidity among patients suffering from acute viral encephalitis**

| Total no. of cases | No. of patients died within 1 week | Patients with neurological sequelae |
|---|---|---|
| Cases (78) | 24(30.76%) | 20(29.48%) |
| Positive cases (7) | 5(71.42%) | 1(14.28%) |
| Enterovirus (4) | 3(75%) | 1(25%) |
| Measles (3) | 3(100%) | - |

## DISCUSSION

In our study of 120 cases of clinically suspected encephalitis patients, haematological parameters were also examined. Biggest challenge in our study was identification of specific viral etiological agents in AES patients. Now a days field of AES diagnosis has improved remarkably with application of rapid molecular diagnostic system such as RTPCR (Reverse Transcription), for early detection and identification of virus in clinical samples. Recently in addition to conventional RT-PCR, more rapid and sensitive real time RT-PCR based assay such as Taqman RTPCR, NASBA, RTLAMP and branched DNA methods are being used.

Real time RT-PCR method has several advantages over conventional PCR. Firstly real time RTPCR is more rapid and sensitive test as compared to conventional RT-PCR. The second advantage of closed and tube RT-PCR is that it is less likely to produce false positive results by contamination during sample preparation.

In this study(2011) we used and evaluated the Taqman based one step Real time RT-PCR for laboratory detection and quantitation of EV and measles virus in patient of AES. The one step Taqman based Real time RTPCR gives result within one hour in contrast to traditional viral isolation.

The findings of present study indicate that this assay was specific and was able to generate reliable result.

The Taqman based real time RT-PCR demonstrate exceptionally higher sensitivity by correctly picking 2 additional samples of enterovirus and 1 additional sample of measles virus with low copy number that were missed by RT-PCR.

In conclusion the Taqman based one step Real Time RT-PCR assay described here for detection and quantitation of enterovirus and measles virus has been shown to be simple, sensitive, specific, rapid and economic approach to surveillance and epidemiological studies. These features make it an excellent tool for laboratory detection of enterovirus and measles virus in clinical samples.

## SUMMARY AND CONCLUSION

The present study was undertaken in Department of Pathology G.R.Medical collelege and Jayarogya Group of Hospitals (J.A.H) Gwalior in association with virology division of D.R.D.E Gwalior over a period from October 2010-october2011.

1. A total of 120 patient with clinically suspected diagnosis of acute viral encephalitis, along with control group of 120 patients were tested for identification of specific viral etiological agent by RT-PCR and Real Time RT-PCR.
2. All the cases and controls were divided into 4 age group i.e.<1yr,1-10yr,10-60yr and >60yr. Majority of cases and controls belongs to age group of 1-10yrs.
3. All the cases were screened for viral etiological agents by RT-PCR and Real Time RT-PCR. Out of 120 cases 7 cases are found to be positive incidence of acute viral encephalitis is found to be 5.8%.
4. Out of 7 positive cases 4 (3.33%) cases were identified by RT-PCR while all the 7 (5.8%) cases were identified by Real Time RT-PCR.
5. Out of 7 positive cases 4 (3.33%) cases of enterovirus and 3 (2.5%) cases of measles virus are diagnosed.
6. Out of 120 CSF samples 5 cases were detected in CSF with 4.1% positivity, only 2 cases were detected in serum with 1.6% positivity. Out of 5 positive sample in CSF 3 EV(60%)and 2 measles (40%) were diagnosed while in serum 1 EV(50%)and 1 measles (50%) were detected.
7. All the CSF sample were screened for conventional CSF routine examination. Out of 120 samples, 8 sample have CSF cell count>100 cell(6.66%)while 104 samples show lymphocytosis

(86.66%)and 16 samples show neutrophilia (13.33%).Among positive samples, 6 cases(84.14%) have CSF cell count in range of 5-100 cell/mm and 1 case (14.86%)shows CSF cell count >100 cell/mm. Out of 120 samples only 12 samples (10%) show reduced sugar. CSF protein was raised in 84 (70%) samples out of 120. Out of 7 positive samples all the samples show normal CSF sugar while 6 samples (85.71%) shows increased protein.

8. All the 120 blood samples were screened for routine hematological examination. Out of 120 samples 74samples (61.66%) were ANAEMIC with Hb <12gm%, 47 samples (39.16%) showed increased total count, 22 samples (18.33%) showed absolute lymphocytosis .Platelet count was normal in 102 samples, while 18 samples (22.5%) showed reduced platelet count i.e. thrombocytopenia. Reduced blood glucose was seen in 15% samples while ESR was raised in 15.86%.

In conclusion, this evaluation and comparison of new molecular techniques RTPCR and Real Time RTPCR indicate that these test are highly specific in identifying etiological agent in acute viral encephalitis and has great potential value for use in epidemic situation .Real Time RTPCR seems to be more sensitive than RTPCR in diagnosing this dread full illness.

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