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Molecular epidemiology of viral meningitis in children in south east of Caspian Sea, Iran

Fatemeh Cheraghali¹ (MD), Abdolvahab Moradi² (Ph.D), Somayeh Azadfar²(M.Sc), Naeme Javid² (M.Sc), Alijan Tabarraei^{1,2}(Ph.D)

1. Infectious Diseases Research Centre; Department of pediatrics; Golestan University of Medical Sciences
2. Infectious Diseases Research Centre; Department of Microbiology, Golestan University of Medical Sciences.

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ABSTRACT

Viruses are the most common causes of aseptic meningitis. Early detection, treatment and management of viral meningitis are priority. This study aimed to evaluate common viral meningitis in children referred to Taleghani pediatrics hospital in Gorgan, south east of Caspian Sea, Iran. In this descriptive study CSF and blood samples were taken from 40 children with negative bacterial culture who were referred with meningitis symptoms since Jun 2008 till Sep 2010. Samples were used for viral, biochemical and cytological assays. DNA extraction was done by high pure viral nucleic acid kit of viral nucleic acid from CSF. PCR and Real-time PCR were performed for detection of viruses. Demographic, clinical, biochemical and cytological data were collected and entered in SPSS version 18. All cases with $p < 0.05$ were considered as significant. In overall 12 (30%) viruses were detected by distribution of 5 (41/7%) Enterovirus, 4 (33/3%) Herpes simplex virus-1 (HSV-1) and 3 (25%) Mumps virus. Patients aged between 1 month to 10 years old with mean of 3 years old of which 92/5% were living in urban area. All positive cases showed fever and CSF Pleosytosis with no bacterial growth, gram staining and urinary tract infection. In conclusion, the results showed that clinical and biochemical analyses are not sufficient for certain diagnosis of meningitis in children and molecular assay is recommended to apply for early detection, treatment and management of viral meningitis.

1. Introduction

Viral meningitis is an inflammation of the meninges caused mainly by viruses and also often referred to as aseptic meningitis (Cherry et al., 2009). It may be suspected on the basis of epidemiologic data, clinical features, and initial CSF studies, but clinical features cannot reliably differentiate viral and bacterial meningitis (Logan, 2008; Wu et al., 2002). Enterovirus, herpes simplex virus (HSV), mumps and varicella zoster virus

(VZV) are the most common cause of aseptic meningitis. Currently, about 75-90% of viral meningitis cases are caused by nonpolio enteroviruses and disease characteristics, clinical manifestations, and epidemiology generally mimic those of enteroviral infections (Corless et al., 2002). Herpes simplex viruses collectively cause about 4% of viral meningitis cases with more common case of HSV-2 meningitis. However, recent data show diversion of common herpes virus meningitis cases from HSV-2 to HSV-1 (Razonable, 2010; Kupila et

*Corresponding author: Dr. Alijan Tabarraei

Tel: Tel: +989112733321, Fax: +981714440225

E mail: alijant@yahoo.com

al., 2006). Mumps meningitis occurs in up to 10% of all mumps patients, more often in males. Mumps meningitis is one of the commonest causes of viral meningitis in populations not immunized against this virus, estimated to occur in between 10 to 30% of those infected (Chadwick, 2006). Varicella-zoster virus (VZV) may lead to various neurological complications such as aseptic meningitis and encephalitis. Among these complications, aseptic meningitis is quite rare, and there are few reports in literature (Lee et al., 1996). VZV meningitis occurs in 0.5%-2.5% of the patients, and they usually show a full recovery without developing other complications (Kim et al., 1994; Cohen et al., 1999).

The incidence of viral meningitis are depend on different factors as it drops with age and the incidence during the first year of life being 20 times greater than children and adults. On the other hand, depending on the type of viral pathogen such as mumps viruses, the ratio of affected males to females can vary (Wan, 2011).

Before the introduction of molecular techniques, laboratory diagnosis of viral infections of the central nervous system (CNS) relied on virus isolation in cell culture, detection of specific antibody production in cerebrospinal fluid (CSF), or, for encephalitis caused by herpes simplex virus (HSV), viral antigen detection in tissue from brain biopsy specimens. With the exception of the last procedure, which is highly invasive, the impact of laboratory diagnosis on acute patient management was relatively small because of the time taken for virus replication to produce a characteristic cytopathic effect in cell culture or for the development of a specific antibody response. Deficiencies in traditional laboratory techniques with regard to the diagnosis of viral CNS infection have meant that in many patients a clinical diagnosis of viral meningitis is made without supportive laboratory evidence of a viral etiology. The use of PCR for the diagnosis of CNS disease has been well evaluated for HSV encephalitis and enterovirus meningitis. The use of this highly sensitive technique has increased our understanding of the etiological role of viruses in CNS disease (Read et al., 1999). For example, it is now recognised that both HSV-2 and VZV are common causes of aseptic meningitis in adults even without a rash. Prior to the advent of PCR, definitive diagnosis of CNS viral infection was dependent upon either virus isolation from CSF

or brain biopsy, or the demonstration of a virus specific intrathecal antibody response. Laboratory investigations such as viral culture although specific, lack sensitivity and frequently fail to provide results within a clinically useful period (Davies et al., 2005). Molecular techniques, such as RT-PCR, nested PCR, real-time PCR, multiplex PCR and nucleotide sequencing, have been increasingly used for detection of these agents in CSF (Archimbaud et al., 2004; Leitch et al., 2009). These methods are more sensitive, compared to cell culture, allowing the detection of a small number of copies of the viral genome present in clinical specimens, with high specificity and fast turnaround time (Benschop et al. 2010). It has been clear that fast and accurate identification of aspecific viral pathogen may affect treatment and prognosis of viral meningitis (Wan, 2011). Instead of sensitivity and specificity of culture in traditional diagnostic assay, its time consuming and low sensitivity in diagnosis of some viral agents such as enteroviruses lead to new technology. It should be mentioned that in as many as one third of cases, no causative agents are identified. The number of methods is increasing with new testing methodologies. PCR is the method of choice for rapid, sensitive, and specific identification of viruses (Fatahzadeh et al., 2007). Regarding the importance of applying new techniques for viral meningitis detection, molecular diagnosis are becoming pioneer and understanding of feature of epidemiology, pathogenesis, management, prognosis, therapy and prevention of viral meningitis developed (Balganesh et al., 2000).

There has been a few molecular study of viral meningitis in Ira. This study aimed to show distribution of aseptic meningitis caused by common Viruses using PCR and real time-PCR assays in children referred to Taleghani pediatrics hospital in South East of Caspian Sea, Gorgan, Iran.

2. Matherials and Methods

This study performed on the children referred to Taleghani pediatrics hospital with meningitis symptoms since Jun 2008 till Sep 2010. The study population included children of 1 to 10 years old. Demographic, clinical, biochemical and cytological data were collected. Three separated tubes of CSF (3-5 ml) were transported to the laboratory. One of the tubes was used for bacterial culture; second tube

for biochemical analyses and the last one for cell count and molecular detection. Blood and urine samples were taken for microscopic, biochemical, cell count and bacterial growth assays as well. Forty children with suspected aseptic meningitis with the negative bacterial cultures and CSF Pleocytosis entered in our study. Viral nucleic acid was purified from 200 μ L CSF samples according to instructions protocol by High Pure Viral Nucleic Acid Kit. Nucleic acid was stored in -80°C . Primers sequences were used in this study are listed in table 1. Distilled water was used for the negative control and a sample taken from the Virology Reference of Keyvan laboratory in Tehran was used for positive control. Mixture of PCR for HSV-1 was including: 3 μ l (1/5 mM) MgCl₂, 5 μ l buffer PCR1x, 0/5 μ l (0/2 mM) dNTP, 0/125 μ l primers (sense + anti sense) and 0/5 μ l enzyme Taq DNA polymerase (2/5U/ml) (Fermentase, Germany). To the final volume of 50 μ l by adding ddH₂O. Mixtures of PCR for HSV-2 was including: 3 μ l (1/5 mM) MgCl₂, 5 μ l buffer PCR1x, 0/5 μ l (0/2 mM) dNTP, 0/1 μ l primers (sense + anti sense) and 0/5 μ l enzyme Taq DNA polymerase (2/5 U/ml) (Fermentase, Germany). To the final volume of 50 μ l by adding ddH₂O. Thermocycler programs for HSV-1 and HSV-2 were identical including 94 $^{\circ}\text{C}$ for one minute one cycle, 94 $^{\circ}\text{C}$ for 20 seconds, 50 $^{\circ}\text{C}$ for 20 seconds, 72 $^{\circ}\text{C}$ for 20 seconds 33 cycles and 72 $^{\circ}\text{C}$ for one minute. VZV primer sequences used in this study are listed in table 1. Mixture of PCR for VZV was including: 3 μ l (25 mM) MgCl₂, 5 μ l buffer PCR10x, 0/5 μ l (10 mM) dNTP, 1 μ l primers (sense + anti sense) and 0/5 μ l enzyme Taq

DNA polymerase (5U/ml) (Fermentase, Germany). To the final volume of 50 μ l by adding dd H₂O. Thermocycler programs for VZV was included 94 $^{\circ}\text{C}$ for one minute one cycle, 94 $^{\circ}\text{C}$ for 20 seconds, 60 $^{\circ}\text{C}$ for 20 seconds, 72 $^{\circ}\text{C}$ for 20 seconds 33 cycles and 72 $^{\circ}\text{C}$ for one minute. The PCR product was run on 2% agarose gel by gel electrophoresis and was stained with ethidium bromide. In the vicinity of the positive and negative controls, samples and DNA ladder were read. Mumps primer sequences used in this study are listed in table 1. At first for Real time PCR, for detection of mumps DNA, cDNA was made by M-MLV Reverse transcriptase kit (BIONEER) and then Real-time PCR was performed based on SYBR Green/ROX q PCR Master Mix (2X) kit (Fermentase, Germany) using ABI system 7300. Real time PCR protocol was included 50 $^{\circ}\text{C}$ for 2 minute one cycle, 95 $^{\circ}\text{C}$ for 10 minute one cycle, 95 $^{\circ}\text{C}$ for 15 seconds and 60 $^{\circ}\text{C}$ for one minute 40 cycles. Entroviruses primer sequences used in this study are listed in table 1. At first for Real time PCR for detection DNA mumps by M-MLV Reverse transcriptase (BIONEER) kit making cDNA was make and then Real-time PCR was performed based on SYBR Green/ROX q PCR Master Mix (2X) (Fermentase, Germany) kit using ABI system 7300. Protocol Real time PCR whit ABI system 7300 device, was included 50 $^{\circ}\text{C}$ for 2 minute one cycle, 95 $^{\circ}\text{C}$ for 10 minute one cycle, 95 $^{\circ}\text{C}$ for 15 seconds and 60 $^{\circ}\text{C}$ for one minute 40 cycles. Data were entered in SPSS 18 and statistical analysis performed with Chi Square test. All cases with $p < 0.05$ was considered as significant.

Table 1. Primer sequences used for PCR and Real time PCR

Virus	Primer sequence	Reference
HSV-1	5' CGA AGA CGT CCG GAA ACA AAC 3' 5' CGG TGC TCC AGG ATA AAA 3'	Read et al., 1999
HSV-2	5'GGA CGA GGC CCG AAA GCA CA3' 5' CGG TGC TCC AGG ATA AA 3'	Read et al., 1999
Entrovirus	5'-ACACGGACACCCAAAGTAGTCGGTTCC-3 5'-TCCGGCCCTGAATGCGGCTAATCC-3	Read et al., 1999
VZV	5'-ACGGGTCTTGCCGGAGCTGGT-3 5'-AATGCCGTGACCACCAAGTATAAT-3	Read et al., 1999
Mumps	5'-CAGGATCCAATTCAAGCACA-3 5'-AATCTTGGTGTCCATCCCC-3	Palacios et al., 2000

3. Result

Of 40 samples examined in this study, 27 (60%) were belonging to males, while the rest 18 cases

(40%) were from females. Children aged between 1 month to 10 years old with mean age of 3 years old. Children were divided into 4 groups based on their

ages: 1-3 month 4 cases (10%), 3-12 month 11 cases (27.5%), 1-2 year 6 cases (15%) and Upper 2 years old 19 cases (47.5%). Of all patients 37 (92.5%) were residing in urban area, while the other 3 were from rural areas. Out of 40 children with aseptic meningitis, a virus was detected in 12 (30%) cases, of which 4 (33.3%) were HSV-1, 3 (25%) mumps and 5 (41.7%) enteroviruses (Table 2).

Clinically, all children would have shown fever (100%), vomiting (70%) and headache (55%) as common symptoms, and of specific symptoms fontanel (10%), Kernig (15%), Brudzinski (10%) and redor (20%) were seen. Of them one cases with rash and another one with Pharyngitis (2/5%) were seen, while none of cases were suffering from hepatosplenomegaly and lymphadenopathy. The molecular technique confirmed HSV-1, mumps and enterovirus in 2, 1 and 1 cases of those who had

headache, respectively. Vomiting was reported in 3 cases of HSV-1, all mumps and 2 cases of enterovirus. Five months infant positive HSV-1 and 4.5 months infant positive enterovirus showed Fontanel. Biochemical evaluation of confirmed positive cases revealed a higher than normal range of ESR in all cases (100%) and a negative case of CRP was observed as well. CSF glucose was normal in all positive cases except in 1 enterovirus positive case, which was lower than normal range. CSF protein was normal or higher than normal in all viral cases. The mean WBC in blood and CSF was 14152 and 273.17 per microliter, respectively. PMN preferred observation was seen in CSF cell analysis of 1 HSV-1, 1 enterovirus and 2 mumps positive cases. Statistically, significant correlation was found between data obtained from biochemical analysis and molecular detection of viruses.

Table 2. Frequency of viral meningitis in children referred to Taleghani pediatrics hospital in Gorgan according to the age and sex

		PCR result					
		Enterovirus	HSV-1	HSV-2	VZV	Mumps	Positive cases
Age group	1-3 month	2(40%)	0	0	0	0	2
	3-12 month	1(20%)	1(25%)	0	0	1(33/3%)	3
	1-2 year	1(20%)	1(25%)	0	0	1(33/3%)	3
	>2 year	1(20%)	2(50%)	0	0	1(33/3%)	4
Sex	male	3	2	0	0	2	7
	female	2	2	0	0	1	5
Total viruses		5(41/7%)	4(33/3%)	0	0	3(25%)	12(30%)

4. Discussion

Viral or aseptic meningitis is the most common infection of the CNS (Hom, 2011). Viruses are the common causes of acute aseptic meningitis and management of life threatening, and treatable causes such as herpes simplex virus (HSV) is possible by more rapid diagnostic tests. Efficiency and sensitivity of diagnostic methods are necessary in central nervous system for management of virus infections (Davis, 2008). According to reports from the Centers for Disease Control (CDC), inpatient hospitalizations resulting from viral meningitis range from 25,000-50,000 each year in the United States. An incidence of viral meningitis has been

estimated in 11 per 100,000 populations per year (Wan, 2011). Studies from Finland have estimated the incidence of viral meningitis to be 19 per 100,000 populations in children aged 1-4 years. This is in significant contrast to 219 cases per 100,000 populations estimated for children younger than age 1 year old (Wan, 2011).

Among 40 children suspected with aseptic meningitis in this study, 12 cases (30%) were detected to be infected by viruses using PCR and Real-Time PCR. This rate of viral meningitis is than that reported from South of Iran with 30 (46%) cases detected by PCR, while similar to the report from China with 7 (31.8%) cases detected by RT-PCR (Alborzi *et al.*, 2011; Li *et al.*, 2006). Similar

methodology by using PCR of CSF samples has been performed in these studies. Most studies have revealed enterovirus as the most common cause of viral meningitis followed by HSV and mumps, respectively. The results reported by Hosoyaa and colleagues through PCR as the method of study is consistent with the results obtained by this study (Hosoyaa *et al.*, 1999). They detected no HSV-2 and VZV positive cases in their study.

Enteroviruses are the most common cause of viral meningitis and different studies worldwide supported this idea. Of 12 viruses detected in this study 5 patients (41.7%) were positive for enterovirus as the most common virus. Similar results have been reported from Shiraz in South of Iran with 13 (43.3%) and Tehran with 35 (35%) enterovirus by Multiplex RT-PCR. However, the study done in Canada showed a higher rate of infection with 54.3% of patients infected by enterovirus (Alborzi *et al.*, 2011; Kermanian *et al.*, 2008; Lee *et al.*, 2006). Enteroviruses are thought to affect males 1.3-1.5 times more often than females (Wan, 2011) and it is shown in this study as well.

Among samples considered for aseptic meningitis, 4 (33.3 %) samples were detected for HSV-1 without any positive case for HSV-2. It has also been similar to the study done by Vrioni and *et.al* by PCR in Greece (Vrioni *et al.*, 2007). HSV meningitis pattern is going to be changed regarding different studies showing diversion of prevalent HSV meningitis from HSV-2 to HSV-1. HSV-2 is the most common cause of genital herpes in the majority of countries (Markoulatos *et al.*, 2002), where it is responsible for approximately 85% of cases, and it is the HSV type involved in 70% of neonatal herpes (Jaffe *et al.*, 1989; Nowak *et al.*, 2003). This pattern has been shown in recent studies in Greece with determination of HSV-1 instead of HSV-2 in meningitides cases (Razonable, 2010; Sawyer *et al.*, 2008). Recent studies in Europe showing conversion of epidemiology of HSV-2 meningitis to HSV-1 as it has been seen before in Japan. HSV-1 is more frequently associated with genital herpes in Japan than other countries (Vrioni *et al.*, 2007).

As mumps virus is the most common cause of viral meningitis in unvaccinated area, it has been reported in many areas regarding vaccination program and vaccine efficacy (Alborzi *et al.*, 2011). In this study 3 cases (25%) of isolated viruses were

mumps virus according to the Real time-PCR assay. Previous studies in Iran has been ranged between 1(1%) to about 11 (36.7%) mumps cases (Alborzi *et al.*, 2011; Kermanian *et al.*, 2008). However this range is vary between 6 to 31% in other countries (Krause *et al.*, 2006; Hosoyaa *et al.*, 1999). One of mumps positive cases was under 12 month age with no vaccination against mumps Virus. Instead, 2 other children were older than 12 months with vaccine administration. Vaccination failure could be a more likely possibility in these two patients. Mumps virus is known to affect males 3 times more often than females (Read *et al.*, 1999). In this study, the rate of mumps infection was 2 times more in male than female.

Previous studies reporting that aseptic meningitis tends to occur 3 times more frequently in males as compared to females (Razonable, 2010) seems to be different from the results obtained by this study whereas rate of male attended children for viral meningitis was 1.5 times more than female. On the other hand, number of suspected meningitis infection cases was higher in children aged less than 2 years old (46.7 %) in this study.

Classic signs of meningitis are fever, headache, stiff neck, photophobia, nausea and vomiting (Read *et al.*, 1999). At least one of the main signs of fever, neck stiffness and altered mental status are in the 99-100% of patients with meningitis. In terms of clinical symptoms, all of the patients had fever; this matter had been seen in 98-100% of studied cases (Frantzidou *et al.*, 2008; Hatamian *et al.*, 2009; Modarres *et al.*, 1997). Fever seen in this study accompanied with other common symptoms such as vomiting and headache; however, headache cannot be assessed in children less than 2 years old. Fontanel was other symptoms in one HSV-1 and enterovirus positive cases as only could be justified in children aged under one year old (Logan, 2008). There were no any other symptoms such as Kernig, Brudzinsky and Redor as could be assessed in children older than 2 years old. This expresses more interoperability of the tests in this age group in consistent with other studies (Rorabaugh *et al.*, 1993; Dean *et al.*, 2003; Norbakhsh *et al.*, 2009). It means investigation of CSF is a priority in diagnosis of meningitis.

CSF cell analysis was observed in one HSV-1, one enterovirus and 2 mumps positive cases with PMN preferable, in early 24 hours of disease as it

may be preferable more lymphocytes in developed form of disease. However, WBC level in the blood is high in infections but in one HSV-1 positive child no increase in WBC was seen (Dean *et al.*, 2003). In this study, CSF and blood analysis could largely suggestive of a viral agent in the sample, but there were some exceptions as well.

5. Conclusion

Early detection by molecular methods would be considered for treatment and management of viral meningitis. Reliable and sensitive method for detecting enterovirus as the most common cause of meningitis in children in this area and mumps virus as well as treatable HSV meningitis in CSF, will reduce hospitalization and use of unnecessary therapy and improve health system.

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