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Epidemiological features of viral encephalitis in Cangzhou of China with use of multiplex RT-PCR for five RNA viruses

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Objective: With use of multiplex RT-PCR testing the five RNA viruses associated with viral encephalitis, the aim of research is to find out the epidemiological features of viral encephalitis in Cangzhou of China.
Methods: Patients hospitalized in Cangzhou central hospital with the diagnosis of viral encephalitis from January 2010 to December 2012 were retrospectively analyzed. The sensitivity and specificity of multiplex RT-PCR was compared with ELISA through testing CSF samples of enterovirus (EVs), Japanese encephalitis virus (JEV), mumps virus (MUV), measles virus (MV) and rubella virus (RV).
Results: Disease incidence of viral encephalitis in Cangzhou of China was 18.6 per 100 thousand, and the main pathogen focused on EVs, MUV, JEV, MV and RV, which positive rate were 27.8%, 14.4%, 12.2%, 6.7% and 3.3% respectively. The sensitivity and specificity were all higher than ELISA.
Conclusion: The most common pathogens responsible for viral encephalitis in Cangzhou, Hebei province, China, were EVs, and the multiplex RT-PCR was a rapid, sensitive, accurate method of testing the viruses responsible for causing these illnesses.

1. Introduction

Viral encephalitis is a diffuse or focal inflammatory process of the brain parenchyma caused by the infection of a variety of viruses (Yang et al., 2013), which is an infectious disorder of the central nervous system much in the child. In China, the main causes of viral encephalitis are Japanese encephalitis virus (JEV), enterovirus (EVs), herpes simplex virus (HSV), mumps virus (MUV), human cytomegalovirus (HCMV), measles virus (MV), rubella virus (RV), varicella zoster virus (VZV), Burkitt's lymphoma virus and (EBV) and so on (Jmor et al., 2008; Shaw et al., 1991). Cangzhou is a prefecture-level city of Hebei province in China, which includes 14 counties and the total population is about 7,200,000. In order to know exactly epidemiological features of viral encephalitis in Cangzhou of China, patients hospitalized in Cangzhou central hospital with the diagnosis of viral encephalitis from January 2010 to December 2012 were retrospectively analyzed and 4014 patients were taken up in this research. At the same time, cerebrospinal fluids (CSFs) collected in the acute phase of the patients were tested with five RNA virus associated with viral encephalitis.

2. Materials and methods

2.1. Patients and samples

Patients hospitalized in Cangzhou central hospital with the diagnosis of viral encephalitis from January 2010 to December 2012 were retrospectively analyzed and 4014 patients were taken up in this research (Table 1). To select patients for evaluation of the cause of viral encephalitis, we used the following criteria: ① acute onset; ② fever (armpit temperature ≥38 °C), headache and vomiting; ③ consciousness barrier of different degrees; ④ meningeal irritation signs. Any patients who accord with ①+②+③ or ①+②+④ can be the selected cases (Yi et al., 2010). On the basis of above criteria, 90 patients were selected for evaluation. CSF samples from the acute phase of these 90 cases were obtained and evaluated for the presence of viral RNA by our multiplex RT-PCR (Yi et al., 2010; Xiangjun et al., 2013a,b). Meanwhile, IgM antibodies of EVs, JEV, MUV, MV and RV in the CSF samples were tested by enzyme linked immunosorbent assay (ELISA) (Kantola et al., 2011).

2.2. Primer design of RT-PCR

According to genome sequences recorded in GenBank database about EVs (including poliovirus type 1–3, enteric cytopathogenic human orphan virus type 2, 6, 7, 9, 19, enterovirus type 71,

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Table 1
The geographical distribution of 4014 cases of viral encephalitis in Cangzhou area.

Area	City	Botou	Cangxian	Dongguang	Haixing	Hejian	Huanghua	Mengcun
Cases	668	440	636	278	94	594	82	132
Percent (%)	16.6	11.0	15.8	6.9	2.3	14.8	2.0	3.3

Area	Nanpi	Renqiu	Qingxian	Suning	Wuqiao	Xianxian	Yanshan	Total
Cases	164	22	90	140	20	428	226	4,014
Percent (%)	4.1	0.6	2.2	3.5	0.5	10.7	5.6	100

coxsackie virus group A type 2, 5, 6, 7, 9 and group B type 1–6), JEV, MUV, MV and RV, five pairs of specific primers were designed using Primer Premier and synthetized by Sunbiotech co., Ltd. (Beijing, China) which were showed in Table 2. Total cellular RNA was extracted from CSF sample by using Virus RNA Kit (Omega Bio-Tek). Reverse transcription was done with random primer of six base and after PCR amplification results were distinguished by 1% agarose gel electrophoresis.

2.3. The PCR conditions

The polymerase chain reaction (PCR) was performed using 25 μl reaction system including 4 μl cDNA template, 1 μl each specific primer (10 mM EVs, JEV, MUV, MV and RV), 2.5 μl PCR buffer, 4 μl dNTP (2.5 mM), 0.5 μl Taq polymerase and the other water. Thirty cycles of amplification were performed with procedures of denaturation at initial denaturation 94 °C for 4 min, then at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 45 s, and extension at 72 °C for 7 min.

2.4. ELISA test

RNA virus antibodies of CSF samples included EV-IgM antibody which was tested by indirect ELISA with Human enterovirus 71 virus IgM ELISA Kit (Measles, German), JEV-IgM antibody which was tested by indirect ELISA with Human Coronaviruses IgG ELISA Kit (Shanghai Beixi Biotechnology Co., Ltd., China), and MV-IgM, MUV-IgM, RV-IgM antibody which were tested by indirect ELISA with ELISA Kit (Human, German), all according to the manufacturer's instructions.

2.5. Statistical analysis

Data were input by software EpiData 3.1 and statistically analyzed by software EpiInfo 2002. And statistical analysis of component ratio was done by χ^2-test with software SPSS 13.0.

Table 2
Primer sequences and length of PCR products.

Virus	Primer sequences (5′-3′)	Length of PCR products (bp)
EVs*	FP: GGTGCGAAGAGTCTATTGAGC RP: GGAAACACGGACACCCAAAGT	152
JEV*	FP: CAAGCACGGCATGGAGAAACA RP: CCAGCACCTTTGAGTTGGAGC	429
MUV*	FP: CAAGAAGGCAAAGGGCGACTC RP: TTGCTGTCTTCCGAACCCTGA	274
MV*	FP: ACAGGGAGTGTCTTCAACGCA RP: ATCCGAAAGACGGGTGATGCT	111
RV*	FP: GCGTCCTATTTCAATCCCGGC RP: ACTGTTGGTTGCCGGTGTAGT	352

* EVs means enterovirus; JEV means Japanese encephalitis virus; MUV means mumps virus; MV mean measles virus; and RV means rubella virus.

3. Results

3.1. Case composition

Patients hospitalized in Cangzhou central hospital with the diagnosis of viral encephalitis from January 2010 to December 2012 were retrospectively analyzed and 4014 patients were taken up in this research. Male were 2493 (62.1%) and female were 1521 (37.9%) which male to female ratio was 1.6:1. The average age was 16.0 years old which range from 0 to 93 years old. The percent of 0–3 year old was 20.7% and 4–16 years old was 32.9%. From the view of regional distribution, city of Cangzhou was the most and accounted for 16.6%, and then was Cangxian (15.8%), Hejian (14.8%), Botou (11.0%), Xianxian (10.7%). Neurology and pediatrics were two main source of viral encephalitis and totally accounted for 97%. Cure means that patients are without symptoms associated with viral encephalitis, and CSF, serum and imaging tests returned to normal. Better means above indexes relieved but not completely back to normal when patients left hospital. What's more, the overall curative and better rate can reach 97.4% (Table 3).

3.2. Testing results by RT-PCR

Of the 90 CSF samples tested by RT-PCR, 58 cases were positive for one of the RNA viruses, with a positive rate of 64.4% (Table 4).

3.3. Testing results by ELISA

The same CSF samples were tested by an ELISA, and 45 (50%) had a positive reaction to one of the five RNA viruses The highest positive rate among the five RNA viruses also appeared in EVs which was 22.2% (20/90), and then MUV 12.2% (11/90), JEV 8.9% (8/90), MV 4.4% (4/90) from and RV 2.2% (2/90), which were shown in Table 5.

Table 3
Department, age distribution and outcome of 4014 cases in Cangzhou area.

	Content	Cases	Percent (%)
Department distribution	Neurology	1,693	42.2
	Pediatrics	2,196	54.7
	Others	125	3.1
Age distribution	0–3	829	20.6
	4–16	1,321	32.9
	17–60	1,388	34.6
	≥61	76	1.9
Outcome	Cure	2,032	50.6
	Better	1,878	46.8
	Unhealed	92	2.3
	Death	12	0.3

Cure means that patients are without symptoms associated vital encephalitis, and cerebrospinal fluid, serum and imaging tests returned to normal. Better means above indexes relieved but not completely back to normal when patients left hospital.

Table 4
Detection of viral RNA by RT-PCR in 90 cases of viral encephalitis in Gangzhou central hospital.

Virus	EVs*	JEV*	MUV*	MV*	RV*
No. positive	25	11	13	6	3
Percent (%)	43.1 (25/58)	19.0 (11/58)	22.4 (13/58)	10.3 (6/58)	5.2 (3/58)

* EVs means enterovirus; JEV means Japanese encephalitis virus; MUV means mumps virus; MV mean measles virus; and RV means rubella virus.

Table 5
ELISA results in 90 cases of viral encephalitis in Gangzhou central hospital.

Virus	EVs*	JEV*	MUV*	MV*	RV*
No. positive	20	8	11	4	2
Percent (%)	44.4 (20/45)	17.8 (8/45)	24.4 (11/45)	8.9 (4/45)	4.4 (2/45)

* EVs means enterovirus; JEV means Japanese encephalitis virus; MUV means mumps virus; MV mean measles virus; and RV means rubella virus.

4. Discussion

Viral encephalitis is the most common infectious disease of central nervous system in pediatrics with global distribution, which high incidence often appears from June to September and sometimes it has a regional outbreak in China. Related studies have shown that viruses which can cause central nervous system infection currently are known approximately 130 species (Handique, 2011). The main pathogens include JEV, coxsackie virus of EVs, enteric cytopathogenic human orphan virus, enterovirus type 71, poliovirus, HSV, MUV, MV, RV, VZV, HCMV, EBV and so on. Clinical statistics showed that only 20% of viral encephalitis was infected by DNA virus and the rest were all caused by RNA virus (Markoulatos et al., 2001) among which EVs, JEV, MUV, MV and RV accounted for more than 80% (Chunyan et al., 2007; Ziqian et al., 2008). Traditional diagnosis of viral encephalitis include isolation and culture of virus in CSF, serological indexes, electroencephalogram, imaging examination and some other biochemical and immune indexes. However, low content of virus in CSF sample, time and labor consuming of viral culture, poor sensitivity and specificity or many other reasons resulted in the diagnosis of the disease was later than clinical treatment leading to higher mortality or serious sequelae.

Professor Sun applied reverse transcription nest polymerase chain reaction (RT-M-nPCR) for testing simultaneously HSV DNA virus and EV RNA virus in 100 CSF samples and obtained good results, which the total detected positive rate reached 35% (Yongmei et al., 2005). Professor Gao found the tested positive rate of HSV, coxsackie virus, ECHO virus, adenovirus (ADV), HCMV and EBV with realtime fluorescence quantitative PCR (RTFQ-PCR) was as high as 67.9%, whereas coxsackie virus and ECHO virus accounted for 49.2%. For the patients who have no typical clinical symptoms or failed to identify pathogens with other auxiliary laboratory technology, PCR technology is a rapid, accurate and good method. RTFQ-PCR can more quickly, accurately definite pathogenic species, and has important significance for the treatment and prognosis (Drago et al., 2004; Kawada et al., 2004). It is a pity that the specific fluorescent primers can only be customized by commercial companies with high cost and one test for a virus. However, multiplex RT-PCR achieved the target that one specimen one test can simultaneously test various viruses or different types of one virus (Quereda et al., 2000), which has the advantages of low cost, simple operation, larger information and so on. Therefore, we used a multiplex RT-PCR method established by our laboratory in this study which can simultaneously test the five RNA viruses. Through validated by viral titer test and gene sequencing analysis, the method has good specificity and sensitivity which can reach 62.5 $CCID_{50}$/ml, 250 PFU/ml, 125 $CCID_{50}$/ml, 125 $CCID_{50}$/ml and 125 $CCID_{50}$/ml. Compared with the results of ELISA, the tested positive rate was 100% by multiplex RT-PCR, but not vice versa. That was to say the relevance ratio of RT-PCR was higher than that of ELISA, whereas distribution of the two RNA virus were the same.

The average annual number of patients of viral encephalitis was 1338 cases in this study and the annual incidence rate was 18.6/10 million, far higher than the epidemiologic results in other areas (Jian et al., 2013; Suna et al., 2012). And the overall curative and better rate of this study was as high as 97.4% which was not consistent with high mortality and serious sequelae of viral encephalitis. This result may be made by the following factors: ① clinical diagnosis of viral encephalitis is too loose; ② whether the virus mutates or the existence of different genetic background needs further studies. There are statistically significant differences among distribution ratio of patients with viral encephalitis in Cangzhou between city and countries ($P < 0.05$), which was the same with distribution ratio of patients who hospitalized in Cangzhou central hospital and also has direct relationship with medical environment and geographical position. Laboratory test results showed pathogen distributions of viral encephalitis in Cangzhou were EVs, MUV, JEV, MV and RV, which means the most common pathogens responsible for viral encephalitis in Cangzhou, Hebei province, China, were EVs and its tested positive rate is as high as 64.4%.

Not only can a better diagnosis improve the situation for the patient (not so much for viral encephalitis, where supportive care is the most common), but more importantly, a rapid diagnosis can be very important in community health/public health. If a communicable disease is identified, an outbreak may be spotted early and appropriate intervention can be made to prevent other people from becoming infected. Likewise, if a mosquito-borne virus (e.g., JEV) is identified, then extra effort can be put into mosquito control to prevent additional cases. How to develop an economic, efficient, fast and reliable diagnostic method has become an important subject for doctors from a public health standpoint. This can allow public health personnel make a difference in the community. The next step will be the development of reverse dot blot hybridized PCR which can obtain more information, be more sensitive and more accurate. Even we hope to establish new methods can simultaneously detect DNA virus and RNA virus of viral encephalitis.

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Conflict of interest

The authors declare no conflict of interest in preparing this article.

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