

## Blood Selenium of Low-Level Associated with Development of Hand-Foot-Mouth-Disease

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**Abstract:** In this study, 207 healthy children groups from 0-6 years old was subjected to analysis of subclinical infection of EV71, 25 subclinical infection blood selenium level of 45 healthy people and 41 HFMD patients were compared. Positive rate of subclinical infection of anti-EV71 IgG antibody was 10.8% (17/157) and 26% (13/50), respectively in two healthy children groups, the 0-3 and 4-6 years old groups that of IgM anti-body were 15.2% (24/157) and 8.0% (4/50), respectively. There was highly significant difference ( $p < 0.01$ ) in the positive rate of anti-EV71 IgG and IgM antibodies in both age groups. The positive rate of EV71 nucleic acid in 28 patients with positive anti-EV71 IgM antibody was 89.2% (25/28). The blood selenium level of 41 HFMD patients, 25 subclinical infection and 45 healthy people were ( $\mu\text{g g}^{-1}$ )  $0.081 \pm 0.01$ ,  $0.132 \pm 0.031$  and  $0.137 \pm 0.024$ , respectively. There was highly significant difference ( $p < 0.01$ ) in blood selenium level between the HFMD group and two other groups compared by Chi-square ( $\chi^2$ ) test. A large number of children under the age of 6 years had EV71 subclinical infection. The patients with EV71 infection and low blood selenium were susceptible to HFMD.

**Key words:** HFMD, latent infection, blood selenium, antibody, children, China

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### INTRODUCTION

Hand, Foot and Mouth Disease (HFMD) is an infectious disease caused by intestinal viruses and mainly occurred in infants and children under the age of 5 years which can cause fever as well as rashes and ulcers on hand, foot, mouth and other body parts and can induce complications such as myocarditis, pulmonary edema and aseptic meningoencephalitis for individual patients.

HFMD were caused by >20 types of Enteroviruses of which the most important pathogens were Enterovirus 71 (EV71) and Coxsackievirus A16 (CA16). Since, 2008, HFMD was outbreak in some areas in China. Only in 2009, there were 115, 618 HFMD cases including 773 severe cases, nationwide indicated by the direct network report system of infectious diseases (Ding *et al.*, 2009; Yao *et al.*, 2009; Zhang *et al.*, 2010). The major epidemiological feature of HFMD was sporadic occurrence with significant distribution in areas, people and time.

The severe and death cases also showed sporadic occurrence and significant regional specificity. Because there were no reliable vaccines and other measures to prevent infection, HFMD was a serious issue affecting children's health (Zhang *et al.*, 2009). The epidemiological

features of sporadic HFMD cases bringing great difficulties for tracing the source of infection and route of transmission, many patients with no clear routes of infection and rare cluster occurrence of HFMD suggested us that there might be a lot of people had latent infection. Over 2 years, the researchers found many HFMD patients did not have typical clinical symptoms in Shenzhen through the onsite epidemiological survey. But they were still the virus carriers.

Therefore, it is of great significance and necessity in controlling the dissemination of HFMD among children to conduct survey on HFMD latent infection for the healthy people, particularly the virus carriers. Some studies showed that the selenium level in the host cells would affect the replication, mutation and virulence of the invaded viruses (Beck *et al.*, 2003).

In the study on the correlation of selenium and virus, the most typical was Keshan Disease (KD) and coxsackievirus infection. China had high incidence of Keshan disease which was shown in studies to be associated with soil and people deficient in selenium. At present, Keshan disease was basically eliminated by long-term utilization of supplemental intake of selenium for people in the disease-stricken areas. Then, researcher found that the pathogens, coxsackievirus strains of

Keshan disease in individuals deficient in selenium mutated from avirulent strains CVB3/0 to virulent strains CVB3/20 (Liu *et al.*, 2002).

The epidemiological survey found that only a few children with EV71 infection under the age of 5 years had developed HFMD and only a few of children with HFMD were severe patients. The researchers discussed the relationship among selenium protein, HFMD pathogen infection and pathogenicity.

**MATERIALS AND METHODS**

**Sample:** The 41 sera of HFMD patients with clinical determination and laboratory diagnosis were collected from Shenzhen Children’s Hospital as the blood selenium samples of HFMD patients. Total 207 sera of healthy children were collected from the pandemic areas in Shenzhen to screen the groups of people who had EV71 latent infection and blood selenium determination. The backup samples were stored in the fridge at -20°C.

**Blood selenium detection:** The blood selenium levels of 41 HFMD patients, 25 patients with EV71 latent infection and 25 healthy people were determined by Inductively Coupled-plasma Mass Spectrometry (ICP-MS, US Model: Agilent 7500 c). The three groups were aged <6 years. The detailed determination process was as follows; take and weigh 400 uL sample serum, add 3 mL thick HNO<sub>3</sub> to each weighed serum for heating, refluxing and digestion for 3 h, maintain a constant water volume after removing nitric acid and test the selenium level in the blood by Inductively Coupled-plasma Mass Spectrometry (ICP-MS).

**Antibody detection:** Anti-EV71 IgG and IgM antibodies were determined by Enzyme-linked Immunosorbent Assay (ELISA) and the test was conducted according to the method described by Parmjeet. EV71 antigen was prepared by Beijing Beier Bioengineering Co., Ltd., serum was collected as the first antibody and labeled goat anti-human IgG and IgM antibodies (Kirkegaard Perry Laboratories, Gaithersburg, Md) as the second antibody. The concentration of antigen coating was 10 ug mL<sup>-1</sup> and 50 uL each well. Antiserum and second anti-body were diluted at 1:100 and 1:3000, respectively. OD values were determined at the wavelength of 405 nm.

**RT-PCR:** Viral RNA in blood sample was extracted with High Pure Viral RNA kit (Roche, Applied Science). The extraction process was in accordance with the instructions on the kit. Viral RNA extracted was stored at -80°C for ready use. EV71 nucleic acid amplification used

the real-time fluorescence PCR Method and the method described in EV71 nucleic acid amplification kit (Daan Gene, Guangzhou).

**Statistical analysis:** The software SPSS 16.0 was used for statistical analysis. The values obtained were shown in mean±SD and were analyzed statistically with analysis of variance among groups.

**RESULTS**

The anti-EV71 IgG and IgM antibodies were determined by ELISA for healthy children at the age of 0-3 and 4-6 years, respectively. The significant difference in anti-EV71 IgG antibody in the age group of 4-6 years was higher than that in the age group of 0-3 years. In contrast, the positive rate of anti-EV71 IgM antibody in the age group of 0-3 years was significantly higher than that in the age group of 4-6 years. The results were shown in Table 1.

EV71 nucleic acid of subclinical infection with positive anti-EV71 IgM antibody who were chosen from 207 healthy children was determined by quantitative RT-PCR. Among 28 subclinical infection with positive IgM anti-body, 25 subclinical infection had positive EV71 nucleic acid. The researchers also used fluorescence quantitative RT-PCR for CA16 to detect if there was cross-positive reaction in the same samples and healthy children samples as negative controls in the trial.

The blood selenium level was determined by ICP-MS for HFMD patients and latent infection with EV7. The highly significant difference in blood selenium level was compared by gender stratification for HFMD patients and latent infection with EV7 and the blood selenium level was shown in Table 2 to be not associated with the gender. The researchers also tested the blood selenium levels for healthy children of two different age groups, the 0-3

Table 1: Positive rate of anti-EV71 IgG and IgM antibodies of healthy children

Anti-body types	Age groups (years)	Positive cases	Proportion (%)	$\chi^2$	p-values
IgG	0-3	17	10.80	7.044	0.008
	4-6	13	26.00		
IgM	0-3	24	15.20	1.721	0.190
	4-6	4	8.00		

Table 2: Comparison of blood selenium level between HFMD patients and patients with latent infection under the age of 6 years

Sex	Groups	$\bar{X}$	SD	t	p-values
Male	HFMD patients	0.081	0.013	6.723	0.000
	Latent infection with EV71	0.135	0.033		
Female	HFMD patients	0.075	0.008	13.111	0.000
	Latent infection with EV71	0.128	0.014		
Total	HFMD patients	0.078	0.011	11.521	0.000
	Latent infection with EV71	0.132	0.026		

Table 3: Comparison of blood selenium level for healthy children under the age of 6 years in different age groups

Sex	Age groups		SD	t	p-values
	(years )	$\bar{X}$			
Male	0~3	0.126	0.007	7.694	0.000
	4~6	0.156	0.011	-	-
Female	0~3	0.121	0.010	6.613	0.000
	4~6	0.155	0.012	-	-
Total	0~3	0.124	0.009	10.319	0.000
	4~6	0.155	0.012	-	-

and 4-6 years old group. And the results showed that the blood selenium level of 4-6 years old group was significantly higher than that of the 0-3 years old group. The gender stratification also indicated the blood selenium level of healthy children was not associated with the gender (Table 3).

HFMD was an endemic disease mainly found in the central and Southeastern regions of China. Since 2008, the incidence rate of the 10 provinces in these regions was >80% among which regions with an incidence rate of 196.83/100,000 were selenium-deficient in the soil and the selenium level in the soil was 0.13-0.19 ppm, however in Qihai, Panzhuhua of Sichuan province, Guizhou, Ensi of Hubei province and Xiangxi of Hunan province, the incidence rate of HFMD was as low as 28/100,000 and the selenium level in the soil was 0.23-2.31 ppm. Selenium-deficient in the soil associated with higher morbidity of HFMD in some areas of China.

**DISCUSSION**

Since 2008, HFMD became pandemic in China. The cumulative determine the number of cases and the deaths were 115525 and 353 cases only in 2009. A few patients had severe clinical symptoms. The major pathogens were EV7 and CA16 (Wu *et al.*, 2009). From previous studies, the researchers found that there were a certain number of latent infections in an epidemic season. And the mechanism of people infected with EV71 growing into HFMD and even severe cases have not been known yet. Selenium is the trace elements essential for human body. Selenium involves in various physical links and plays different biological functions *in vivo* mainly in the form of selenoprotein, thus directly and indirectly being associated with a variety of major diseases. Many chemical intervention trials for diseases have proven that selenoprotein had significant effect on the prevention of some major diseases. Many researchers are interested in the study on the effect of selenoprotein on viruses. Some studies suggested that selenium content in the host cells would affect the replication, mutation and virulence of the invaded viruses. The epidemiological and pathological studies showed that all the selenium-deficient patients

might have more obvious symptoms after being infected viruses (Ryan-Harshman and Aldoori, 2005). China had high incidence of keshan disease after 1950s which was shown to be associated with soil and people deficient in selenium.

At present, keshan disease was basically eliminated by long-term utilization of supplemental intake of selenium for people in the disease-stricken areas. Then, researcher found that the pathogenesis of keshan disease was mainly associated with coxsackie virus infection which had been proven by the rat models. In 1994, experiments also found avirulent strains CVB3/0 and virulent strains CVB3/20 were present in the selenium-deficient cells and rat models *in vivo* and the avirulent strains could mutated to virulent strains. Avirulent strains CVB3/0 in the selenium-deficient cells mutated from C to U at nt 234 in the 5' non-coding region while avirulent strains were easy to become virulent strains in rats with severely damaged immune system. After the genes of Glutathione Peroxidase (GPx1), the key member of the selenoprotein family were removed, the rats were infected with coxsackie virus. Finally, 50% of the tested rats had typical symptoms of keshan disease while the wild rat basically had no clinical symptoms indicating that the virulence of the virus was closely related to the cellular redox state that selenoprotein involved in (Liu *et al.*, 2002). The test also proved that selenium was closely related to influenza virus infection. After being fed with low selenium for 4 weeks, the mice were infected with Bangkok/1/79 influenza virus. Experiments observed the damage conditions of lung tissues at different days of infection and found the lung tissues of the selenium-deficient mice had more significant damage than those of the selenium-fed group from the 2nd week of infection and damaged more severely with the passage of time.

By analyzing the genetic variation of viruses isolated from the above lung tissues which found in the influenza viruses obtained from selenium-deficient and selenium-sufficient mice, the RNA mutations of the virus surface protein Hemagglutinin (HA) and Neuraminidase (NA) were random. However, the mechanism protein was quite the opposite. The selenium-deficient mice had *M* gene mutations at 6 loci while the selenium-sufficient mice were found to have no change of nucleotide in *M* gene. All these suggested that the selenoprotein level in the host body significantly affected the heredity and variance of the influenza virus which was also confirmed in the similar test of the selenium-deficient mice with influenza A (H1N1) virus infection. H1N1 virus in selenium-deficient mice *in vivo* significantly increased the replication rate which aggravated the pathological response in the lungs (Beck *et al.*, 2001; Barnard *et al.*, 2007; Sheridan *et al.*, 2007; Liu *et al.*, 2002). Kupka R used supplemental intake of selenium to treat HIV-1 infected women in Tanzanian and significantly reduced the

diarrhea symptoms for these AIDS patients. After Passwater found the slow progress of HIV carriers growing into AIDS patients in regions rich in selenium like Senegal, people attached greater importance to the relationship of selenium and AIDS. It was reported that the blood selenium level of AIDS patients was closely related to CD<sup>4+</sup> and their GPx levels tended to decrease with the development of AIDS (Kupka *et al.*, 2009). Another report followed 949 pregnant Tanzania women infected with HIV for 5.7 years. When the blood selenium increased by 0.1  $\mu\text{mol L}^{-1}$ , the risk of mortality would be reduced by 5%. There were some reports of the clinical study on oral selenium yeast and sodium selenite for AIDS patients. Total 13 stage II AIDS patients and 2 stage IV AIDS patients were chosen to take 100-300  $\mu\text{g}$  oral sodium selenite daily for 3-8 months and many of them were seen to have alleviated symptoms and increased CD<sup>4+</sup>/CD<sup>8+</sup> ratio (Kupka *et al.*, 2004).

Molin *et al.* (2009) used coxsackie virus-B3-infected Balb/C mice to prove that selenium was directly associated with the replication of CVB3. The study proved that the blood selenium level of HFMD patients was significantly lower than that of the patients with EV71 latent infection.

Such a result suggested the blood selenium level might be associated with the development of HFMD, deficiency in selenium might accelerate the development of HFMD and EV71 infected hosts growing into HFMD patients might be associated with the viral replication and mutation as well as the immune state of the host. The above reported proved the correlation of the blood selenium level of the host and the viral replication and mutation. The mechanism of HFMD associated with selenium deficiency should be further studied yet. This test also showed that the blood selenium level of healthy children under the age of 6 years was not associated with gender but age. As compared with the 4-6 years old children, EV71 infection rate of the 0-3 years old children was significantly higher and their blood selenium level was significantly lower which indicated the blood selenium level was associated with EV71 infection. HFMD was mostly found in children under the age of 5 years for their relatively lower blood selenium than that of the adults.

Some reported proved that selenoprotein was directly involved in the formation of T lymphocytes and deficiency in selenium would directly affect the immune response for children under the age of 5 years. The preliminary tests confirmed that people with low blood selenium were also susceptible to EV71.

## CONCLUSION

The researchers reported that regions of rich selenium in the soil in China had rarely incidence of

HFMD and 95% of HFMD cases were found in selenium-deficient regions. The report was consistent with the report on avian influenza in China. No avian influenza cases were found in regions of rich selenium in 2008. Because the selenium level in the soil would directly affect the selenium level of the crops of the local areas, thus affecting people's intake of selenium. The blood selenium levels had associated with selenium levels of the soil in regions.

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