行政院國家科學委員會專題研究計畫　成果報告

腸病毒 71 型宿主基因分析-找尋易感染或抗感染之基因（子計畫五）

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（計畫名稱）

腸病毒 71 型宿主基因分析--找尋易感染或抗感染之基因

Host Genetic Factors of EV71 Infection: Identification of Host Susceptible or Resistant Genes

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（一）計畫中文摘要。

關鍵詞：腸病毒 71 型，宿主因素，基因變異，敏感基因，症狀嚴重程度

1998 年台灣腸病毒 71 型流行造成 78 位孩童先有手足口病繼而快速死於肺水腫或肺出血。而 2000 年及 2001 年則分別有 41 位及 47 位孩童因感染腸病毒而死亡。但感染相同腸病毒 71 型後引起之症狀差異很大，從毫無症狀(約有 71%) 至死亡(0.05%)。造成腸病毒 71 型嚴重程度之差異，其真正機轉仍未明。雖然我們已經作了部分腸病毒 71 型之基因分析，但並未找到有關腸病毒 71 型之毒性基因，而不同基因型之腸病毒 71 型之嚴重度亦無差別。因此是否因宿主本身之基因差異，因而造成感染相同腸病毒 71 型後症狀嚴重程度之差異是很值得探究的。故本計劃希望能找到易感染腸病毒 71 型之敏感基因及與嚴重程度相關之基因變異型。

我們將收集嚴重程度不同之腸病毒 71 型個案，及其父母和兄弟姐妹及正常之小孩當對照組(case-parental trio design, sibship design, case-control design)。粹取其 DNA，利用聚合酵素鏈反應法 (PCR)、 限制脢 (restriction enzymes)及 DNA 序列分析等，將研究兩組基因變異型(polymorphism)及其與腸病毒 71 型之感染及與嚴重程度之相關性。因為我們發現腸病毒 71 型死亡個案之發炎細胞激素 (TNF-α, IL-1 β, and IL-6)明顯升高，故第壹組基因變異型分析將包括免疫相關之基因，如 TNF-α promoter, IL-1 β promoter, IL-6 promoter, CD40-ligand promoter, CTLA-4 and HLA genotyping。第貳組基因變異型分析將包含受腸病毒 71 型感染後有明顯表現不同之基因、其調控基因及腸病毒 71 型受體基因。最後將利用基因統計分析，以個案對照研究法、父母對照(TDT, transmission distortion test)及兄弟姐妹對照研究法(STDT)，找出易感染腸病毒 71 型之敏感基因及與嚴重程度相關之基因變異型。

我們已收集了 150 個 EV71 的個案，及 100 個正常孩子當對照組，這 150 EV71 個案，有 38%(57/150)是輕症；44%(66/150)在中樞神經併發症，如無菌性腦膜炎、腦炎、類小兒麻痺症候群等；有 18%(27/150)在中樞神經受侵犯後發生心肺衰竭(肺水腫)。經過治療，最後有 7%(11/150)死亡，有 24%(36/150)有後遺症。

經過 PCR， 及 限 制 ， 自 動 化 基 因 定 序 等 方 法[redacted]性與臨床嚴重度或會不會感染 EV71 作相關的研究統計。結果發現，感染 EV71 的個案其 TNF-α promoter type II 的比率為 26%，而正常孩童 TNF-α promoter type II 的比率為 14%(P=0.045)，顯示 TNF-α promoter type II 可能與容不容易感染 EV71 有正相關性。然而 TNF-α promoter type II 與 EV71 感染後之嚴重程度及預後並無相關。另一個基因，IL-6 promoter 對容不容易感染 EV71，或感染 EV71 後之臨床嚴重度及預後並不影響。

未來我們將持續收集 EV71 的個案，並繼續研究宿主基因與 EV71 感受性的關係，及與臨床嚴重度。

（二）計畫英文摘要。

Key words: EV71, host factor, genetic variants, clinical outcome

EV71 has caused large epidemics with lots of fatal cases and cases with sequelae. However, the clinical syndromes and severity of the same EV71 strain are very diverse, ranging from asymptomatic (71%) to fatal (0.05%) diseases. To date, no clear viral virulence factor has been found and there is no association between EV71 genotypes and clinical outcomes. Therefore, host factors may be important to the clinical outcomes of EV71 infections. By investigating host genetic background, we may find some genetic variants that influence susceptibility and disease severity to EV71 infection.

A total of 150 EV71 cases were collected and about 100 control cases had been collected. The clinical diagnosis and outcome of the 150 EV71 cases are as the following: 38% (57/150) of the cases were uncomplicated, 44% (66/150) of our EV71 cases had central nervous system involvement, such as meningitis, myoclonic jerk, encephalitis, polio-like syndrome, etc and 18% (27/150) developed cardiopulmonary failure soon after CNS involvement. After intensive resuscitation and medical care, eleven (7%) children died and 36 (24%) children had sequelae of dysphagia, central hypoventilation, cranial nerve palsy and limb weakness/atrophy.

With PCR, SNP detection with appropriate restriction enzyme and automatic gene sequencing, polymorphisms of immune-related genes are being studied and correlated with susceptibility and clinical outcomes of EV71 infection. In allelic association study for the candidate genes with case-control study, EV71 cases had higher percentage of type II TNF-α promoter polymorphism in comparison with normal control children (26% vs. 14%, p=0.045). However, there was no significant difference of IL6 promoter polymorphism between EV71 cases and normal control children. TNF-α promoter polymorphism did not correlate with clinical severity and clinical outcome. IL6 promoter polymorphism did not correlate with clinical severity and clinical outcome, either. From our preliminary results, TNF-α promoter polymorphism seem related to susceptibility of EV71 infection and whether the TNF-α promoter regulates or enhances the susceptibility of EV71 needs further investigation.

Further case collection, and sequencing of other immune-related genes and the second group of candidate genes such as EV71 receptor and their regulatory genes will be done in the future. Thereafter, we will compare genetic variants among EV71 cases with different severity and normal children to find susceptible or resistant genes and genetic polymorphism related to clinical outcomes, to find the strength of disease associations with different combinations of polymorphism.

二、研究計劃內容

Background and Significance.

The importance of EV71

Enterovirus 71 (EV71) has been associated with outbreaks in the United States, Europe, Australia, Japan, Brazil and Malaysia (1-10), since it was originally recognized in California in l969 (1). Before 1998, three large outbreaks with dozens of fatal cases occurred in Bulgaria, Hungary, and Malaysia in l975, l978 and 1997, respectively (3, 5, 10).

Unfortunately, the largest and most severe EV71 epidemic exploded in Taiwan in 1998 (11-16). A total of 129,106 cases of hand-foot-and-mouth disease and herpangina (HFMD/HA) were reported, 405 cases had severe neurologic complications and/or pulmonary edema, and 78 children died (15). Retrospective review found that sporadic cases of EV71 had occurred in Taiwan in 1980 and 1986 (15). In addition, sequences of some EV71 isolates in 1998 showed high degree of identity at VP-1 region with those of the EV71 strain isolated in 1986 (17). Apparently, EV71 has circulated in Taiwan for at least 18 years. However, factors underlying the widespread emergence of EV71 infection in 1998 remained unknown. As poliovirus is nearly eradicated, non-polio enteroviruses becomes more important as a possible way for non-polio enteroviruses to reemerge in the absence of readily available vaccines and effective antiviral therapy. Furthermore, since EV71 has caused two large-scaled epidemics with many fatal cases in Malaysia and Taiwan recently (10, 15), EV71 may become the most important enterovirus related to the fatal and morbid cases.

Diverse manifestations of EV71 with high transmissibility

EV71 can cause fatal disease and most of the fatal cases died of fulminant pulmonary edema (15). However, EV71 infection causes very diverse manifestations, ranging from asymptomatic (about 71%) to fatal disease (about 0.05%) (18). It remains unknown that the reason why different hosts of the same EV71 infection have different clinical outcomes and the pathogenesis of pulmonary edema is still unclear. **The diverse clinical manifestations may be related to virus virulence, virus load, or host factors. However, up to now no relationship between EV71 genotypes and clinical outcomes has been found (17, 19).** Since EV71 caused the largest epidemic of the world in Taiwan in 1998 and continues to circulate and cause fatal and severe cases in Taiwan recently (78 fatal cases in 1998, 41 fatal cases in 2000 and 47 fatal cases in 2001, according to the data of CDC, Taiwan), it is very important to delineate the pathogenesis and genetic virulence factor, host susceptible or resistant genes of EV71.

Genetic variants related to infections

The causes of host different susceptibility and diverse severity of the same pathogens remain mystery. In addition to environmental factors and pathogen-related virulence factors, host genetics should play some role on the different ways that we respond to the same infectious agent. As advances in genomic medicine, there is compelling evidence for a genetic component, including twin studies of tuberculosis, leprosy, malaria, and *Helicobacter pylori* infection and a large survey that found that individuals adopted in childhood had a markedly increased risk of death from infection if a biological parent had died prematurely of infection (20-24).

Genetic variations in the immune system are related to susceptibility and clinical outcomes of various infections. For example, the highly polymorphic human leukocyte antigen (HLA) genes are increasingly recognized as a correlate of susceptibility or severity to some infections including malaria (25), tuberculosis (26), HIV infection (27), and hepatitis B (28). The functional role of HLA is to present antigens to the immune system, and the extraordinary genetic diversity of HLA is postulated to have arisen as a host strategy to counter antigenic diversity in infectious organisms. Polymorphism of another immune regulatory gene, tumor necrosis factor-α (TNF- α) promoter, a key mediator of fever and the inflammatory response to infection, is associated with higher mortality rates and morbidity rates of bacterial sepsis (29). TNF- α promoter polymorphism was also associated with severity of malaria, leishmania, and leprosy (30-32).

Another group of genetic variants related to infectious susceptibility is receptor/co-receptor or binding molecule of pathogens. A beautiful example is the relation between the Duffy blood group antigen (DARC) and susceptibility to *Plasmodium vivax* malaria (33). The single nucleotide polymorphism in the DARC promoter region, that prevent binding of the erythroid transcription factor GATA-1, suppresses DARC express in erythrocyte and completely protects infection with P. vivax (34). Another example is the genetic variant of co-receptor of HIV (CCR5), which is related to resistance of HIV infection (35, 36). The variant of CCR5 was also found to be associated with disease progression of AIDS (37).

Choice of candidate genes related to EV71 infections

The choice of candidate genes in this study will be similar to the above studies, and that will be immune-related genes and receptor/adhesion molecule-related genes. We have demonstrated **prominent inflammatory cytokine elevation such as TNF-~, IL-1 , and Il-6 in fatal EV71 cases but not in non-fatal cases** (38). Such cytokine responses were similar to those in bacterial sepsis. These evidence suggest that host genetic factors and immune response may play important roles on susceptibility and clinical outcome of EV71 infection. Therefore, the first group of candidate genes in this study will be immune-related genes with identified polymorphisms and associated with clinical susceptibility and outcomes. They consist of TNF-α promoter, IL-1 promoter, IL-6 promoter, CD40-ligand promoter, CTLA-4 and HLA.

The second group of candidate genes for this study will include genes of differential expression and significant protein interaction in EV71 infected cells (Projects 2, 3 & 4), their regulatory genes and EV71 receptor gene. The second group of candidate genes will be the new unique genes, whose genetic variants may be related to prognosis or potential target genes for the treatment of EV71.

Specific Aims

(1). Comparison of gene variants in EV71 cases with different severity and normal children to find susceptible or resistant genes and genetic polymorphism related to the clinical outcomes

(2). To find the strength of disease associations with different combinations of polymorphism

(3). Pathogenesis of EV71-related fatal pulmonary edema

(4). Provide therapeutic guidelines for EV71 infections: to discover genes encoding novel molecules that fight EV71 infection and to pinpoint critical pathways in immune regulation,

leading to new therapeutic strategies for EV71 infection, especially fatal pulmonary edema.

Methods and Materials

1. Design of genetic studies

(1). EV71 cases. There would be usually 100 to 200 EV71 cases and 300 to 500 non-71 EV cases each year at Chang Gung Children Hospital. EV71 cases will be selected based on our previous experience and study. At least 200 EV71 cases will be collected. Informed consents will be obtained from all the cases or their guardians.

Their clinical data including demographic data, clinical manifestations, course, laboratory data, outcome and treatment were recorded. Clinical outcomes including recovery without sequel, with sequel or death were also recorded.

(2). Case-control design: the normal children and asymptomatic children. Blood from 100 normal children in our vaccine clinical trial study had been collected.

(3). Case-parental trio design: DNA of 180 parents of about 100 EV71 cases has been collected up to now and will be collected continuously. DNA of parents of more than 200 EV71 cases will be collected.

(4). Sibship design: DNA of 150 siblings of about 100 EV71 cases has been collected up to now and will be collected continuously. DNA of siblings of more than 200 EV71 cases will be collected.

(5). Sample size consideration:

The sample size consideration is based on the case-parental trio design in which TDT test will be employed. It is estimated that a sample comprising 194 TDT families (mother-father-affected child) will be needed to attain a type I error rate of 5% and power of 80% under the assumption that prevalence of 0.016 and that a relative risk of 2.9 for disease in disequilibrium with an allele at a marker locus that demonstrates 0% recombination; 211 families are needed if recombination fraction is assumed to be 0.02..

2. Laboratory methods

A. Virological diagnosis and cytokine response

(1)Virus isolation and serotyping were done with traditional cell culture system and fluorescent monoclonal antibody staining.

(2). EV71 neutralization Ab was done with the standard protocol of neutralization test in microtiter plates

(3). EV71 IgM was measured with μ-capture ELISA, whose sensitivity and specificity was 91.5% and 93.1% respectively

(4). Cytokines: Concentrations of IL-1β, IL-6, and TNF-α will be measured using enzyme-linked immunosorbent assay kits following the manufacturer's instructions (Quantikine, R&D Systems, Minneapolis, USA).

B. Genotyping

(1). **Genomic DNA extraction** will be extracted from 5 ml whole blood of EV71 cases and the control with Genomic DNA Purification Kit (Bioman Scientific Co.).

(2). **PCR of the candidate genes**. One group of candidate genes are immune-related genes including the pro-inflammatory cytokine-related genes (TNF-α promoter, IL-1 β promoter, IL-6

promoter), CD40-ligand promoter, CTLA-4 and HLA typing. The other group of candidate genes will be the genes of differential expression and significant protein interaction in EV71 infected cells (Project 2, 3 &4), their regulatory genes and EV71 receptor gene.

i).**TNF-~:promoter**: primers for TNF-α promoter include: sense 5'-CAAACACAGGCCTCAGGACT-3' and anti-sense5'-CCCTATTGCCTCCTCCATTTCTTTTG-3' (nucleotide -501 to –205) ; sense 5'-CAAACACAGGCCTCAGGACT-3' and anti-sense 5'-CGTCTGCTGGCTGGGTGTGC-3' (nucleotide –501 to +11). A mixture of DNA, 250uM dNTP, 1uM primer, 2U Tag polymerase, 10mM Tris HCl buffer, 2 mM MgCl2 and 50 mM KCl will be run at the DNA thermal cycler (GeneAmp PCR System 9600, Perkin-Elmer). PCR conditions are 95^{o}C for 5 min, then 30 cycles of (95^{o}C for 1 min; 66^{o}C for 1 min; 72^{o}C for 1 min) and finally 72^{o}C for 7 min.

ii). **IL-6 promoter**: Sequences of NF-κB binding site of IL-6 promoter will be included. Primers include sense 5'-ATATTAGAGTCTCAACCCCC-3' and anti-sense 5'-ATTTGATAAATCTTTGTTGG-3'; 5'-GGAGACGCCTTGAAGTAACTG-3' and anti-sense 5'-GAGTTCCTCTGACTCCATCGCAG-3'. PCR will be performed with an initial denaturation of 94 ^{o}C for 4 min, followed by 35 cycles of 95 ^{o}C for 40 sec, 55 ^{o}C for 40 sec, and 72 ^{o}C for 1 min, and finally 72 ^{o}C for 7 min.

(3). **Purification of PCR product was** done with the purification kit (Roche Molecular Biochemicals) before SNP detection and automatic DNA sequencing.

(4). **Single nucleotide polymorphism** (SNP) detection: PCR product will be digested with appropriate restriction enzymes to detect SNP. Restriction enzyme for TNF-α promoter is **NcoI**, for IL-1 β **Tag1**, for IL-6 promoter **NlaIII, FokI, MbI1**; for CD40-ligand promoter **Hind III**, **XholI;** for CTLA-4 **Fnu 4H1** (New England Biolabs). The size of the digestion products will be determined by using microtiter array diagonal gel electrophoresis. If SNP is detected, further gene sequencing will be performed as the following. If there are no appropriate restriction enzymes, direct automated DNA sequencing will be performed with the original primers.

(6) **HLA genotyping**: PCR utilising sequence-specific primers (SSP) methodology will be used for genotyping HLA antigens (HLA-A, HLA-B, HLA-C and HLA-DR) (Innogenetics, Gent, Belgium).

3. Statistical genetic analysis

All the clinical data, the results of microarray and gene sequencing will be forwarded to the Division of Biostatistics and Bioinformatics of NHRI under the supervision of Dr. C.A. Hsiung together with Component Project 1 for processing, management and analysis.

The first statistical genetic analysis we will perform is allelic association study for the candidate genes listed in the part 2 of B4d. Later on, we will perform the study of other candidate genes suggested by other component projects (Projects 2, 3 and 4).

There are three types of association studies in this proposal depending on the types of the pedigree; namely, case-control design, case-parental trio design, and sibship design. There are basically two aspects of the problem deserve special attentions. One is about "identifying" the important phenotypes and the other regards the choice of haplotypes. A careful evaluation of the significance level is needed and the issue of sample size is also considered. The popular

likelihood methods, haplotype relative risk method (HRR, HHRR), transmission distortion test (TDT), STDT and their ramification will be applied to our studies.

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Results

1. Case collection for case-control design, case-parental trio design, and sibship design:

A total of 150 EV71 cases were collected. About 100 control cases had been collected. Nearly 100 families with 100 case-parental trio and 100 sibship pairs had been collected.

The clinical diagnosis and outcome of the 150 EV71 cases are as the following tables:

Sixty-two percents (93/150) of our EV71 cases had central nervous system involvement, such as meningitis, myoclonic jerk, encephalitis, polio-like syndrome, etc and 27 (18%) cases developed cardiopulmonary failure soon after CNS involvement. After intensive resuscitation and medical care, eleven (7%) children still died and 36 (24%) children had sequelae of dysphagia, central hypoventilation, cranial nerve palsy and limb weakness/atrophy. Some of them are now being cared in chronic respiratory care center.

Table 1. Clinical Diagnosis of EV71 Cases

Clinical Diagnosis	N (%)
Asymptomatic	10 (7%)
Uncomplicated HFMD	47 (31%)
Complicated HFMD	
CNS involvement (meningitis/encephalitis/polio-like)	66 (44%)
CNS involvement plus cardiopulmonary failure	27 (18%)

Table 2 Outcome of EV71 Cases

Clinical Outcome	N (%)
Complete recovery	103 (67%)
With sequelae	36 (24%)
Expired	11 (7%)

2. Allelic association study for the candidate genes with case-control study

EV71 cases had higher percentage of type II TNF-α promoter polymorphism in comparison with normal control children (26% vs. 14%, p=0.045, Table 3). However, there was no significant difference of IL6 promoter polymorphism between EV71 cases and normal control children (Table 4).

Table 3 TNF-~ promoter polymorphism of EV71 cases and the control children

TNF-α promoter polymorphism	EV71 cases	Control Children
Type I (-308GG)	97 (74%)	84 (86%)
Type II (-308GA or AA)	34 (26%)	14 (14%)

P=0.047 with $\chi 2$ test

Table 4 IL6 promoter polymorphism of EV71 cases and the control children

IL6 promoter polymorphism (-572 region)	EV71 cases	Control Children
CC	74 (66%)	53 (60%)
GC/GG	38 (34%)	36 (40%)

P=0.42 with $\chi 2$ test

A. HLA typing: ongoing

3. TNF-a promoter polymorphism and IL6 promoter polymorphism vs clinical syndrome/severity and clinical outcome

As Table 5 and Table 6 show, TNF-α promoter polymorphism did not correlate with clinical severity and clinical outcome. IL6 promoter polymorphism did not correlate with clinical severity and clinical outcome, either (Table 7 and Table 8).

Table 5 TNF-~ promoter polymorphism and clinical severity of EV71 cases

Genotype	Clinical Severity		
TNF-α promoter	Uncomplicated	CNS involvement	CNS plus cardiopulmonary failure
TypeI(-308GG)	27(62%)	52 (82%)	19 (76%)
TypeII(-308GAor AA)	16 (38%)	12 (18%)	6 (24%)

P=0.099 with $\chi 2$ test

Table 6 TNF-~ promoter polymorphism and clinical outcome of EV71 cases

Genotype	Clinical Outcome		
TNF-α promoter	Complete Recovery	With sequelae	Expired
Type I (-308GG)	63 (72%)	25 (74%)	9 (90%)
TypeII(-308GAorAA)	25 (28%)	9 (26%)	1 (10%)

P=0.46 with ¡2 test

Table 7 IL-6 promoter polymorphism and clinical severity of EV71 cases

Genotype	Clinical Severity		
IL6 promoter	Uncomplicated	CNS involvement	CNS plus cardiopulmonary failure
(-572 region)			
CC	22 (56%)	36 (73%)	14 (64%)
GC/GG	17(44%)	13 (27%)	8 (36%)

P=0.24 with χ2 test

Table 8 IL-6 promoter polymorphism and clinical outcome of EV71 cases

Genotype	Clinical Outcome		
IL6 promoter	Complete Recovery	With sequelae	Expired
(-572 region)			
CC	49 (65%)	17 (61%)	8 (89%)
GC/GG	26(35%)	11(39%)	1(11%)

P=0.29 with χ2 test

Future works

1. Continue case collection
2. Genotyping of 1st and 2nd groups and candidate genes
3. Analysis of allelic association with case-control, case-parental trio and sibship designs
4. Continue case collection
5. Genotyping of 1st and 2nd groups and candidate genes
6. Analysis of allelic association with case-control, case-parental trio and sibship designs

Discussion and Conclusion

We found that about half (93/150, 62%) of our EV71 cases had central nervous system involvement and 27 (18%) cases developed cardiopulmonary failure plus fulminant pulmonary edema. After intensive resuscitation and medical care, eleven (7%) children still died and 36 (24%) children had sequelae of dysphagia, central hypoventilation, cranial nerve palsy and limb weakness/atrophy. Some of them are now being cared in chronic respiratory care center.

From our preliminary results, TNF-α promoter polymorphism seem related to susceptibility

ofEV71 infection because the percentage of type II TNF-α promoter in EV71 cases is significantly higher than the percentage in normal control children. Whether the TNF-α promoter regulates or enhances the susceptibility of EV71 needs further investigation.

IL6 promoter polymorphism is not related to the susceptibility of EV71 infection because the percentages of IL6 promoter polymorphism are not significantly different between EV71 cases and normal children.

TNF-α promoter polymorphism and IL6 promoter polymorphism are not correlated with clinical severity and outcome of EV71 cases. However, cases who died rapidly after cardiopulmonary edema had high percentage (90%) of CC of the IL6 promoter at –572 region. Because the limited number of such fatal cases, there was no statistically significance. We will collect more cases to define the role of IL6 promoter polymorphism on clinical severity and outcome.

Publications

Conference proceedings

1. **Chang LY**, Lu HK, Hsia SH, et al. TNF-α promoter polymorphism is not related to the severity of EV71 Infection. Acta Paediatrica Sinica 2002;43 (suppl A):115-6.

Manuscripts submitted

2. Luan-Yin Chang,[1] Shao-Hsuan Hsia,[2] Chang-Teng Wu,[2] Yhu-Chering Huang,[1] Kuang-Lin Lin,[3] Tsui-Yen Fang,[1] Tzou-Yien Lin[1] . Outcome of EV71 Infections with or without Stage-based Management, 1998 – 2002

3. Luan-Yin Chang, M.D., Kou-Chien Tsao, BS,* Shao-Hsuan Hsia, M.D.,† Shin-Ru Shih, Ph.D.,‡ Chuang-Guei Huang, M.S.,* Tsui-Yen Fang, R.N., Yhu-Chering Huang, M.D., Tzou-Yien Lin, M.D. Transmission and Clinical Features of EV71 Infections in Household Contacts

Manuscripts under preparation

4. TNF-~ promoter polymorphism is related to susceptibility of EV71 infection but not related to clinical severity and outcome of EV71 Infection

Manuscripts submitted

Outcome of EV71 Iinfections with or without Stage-based Mmanagement, 1998_-_2002

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Running Title: Outcome of EV71 Infection

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Abstract

Enterovirus 71 (EV71) infection may progress through four stages, one of which is cardiopulmonary failure. In Taiwan in 1998, almost all the EV71 patients with cardiopulmonary failure died. To improve clinical outcome of EV71 patients, we developed a stage-based management program in 2000. Of the patients who did not receive stage-based management, 83% (15/18) of cases with central nervous system involvement plus cardiopulmonary failure died during the acute stage of the infection, two died at convalescence and one had sequelae of dysphagia and limb weakness. Of the patients who received stage-based management, 33% (12/36) died during the acute stage, 8% (3/36) died at convalescence, 14% (5/36) recovered completely and 43% (16/36) had severe sequelae ($p<0.001$). After adjusting for age and sex, stage-based management was significantly associated with a reduction in acute mortality (95% CI 0.008 to 0.34; $p=0.0019$). Significant reduction in fatality rate suggested that stage-based management was superior to conventional management for EV71-related cardiopulmonary failure.

Key word: enterovirus 71; pulmonary edema; management; outcome; fatality

Introduction

There were large outbreaks of e~~E~~nterovirus 71 (EV71) infection resulting in dozens of deaths~~caused several epidemics and large outbreaks with dozens of fatal cases occurred~~ in Bulgaria in 1975 (1), Hungary in 1978 (2), and Malaysia in 1997 (3), and the largest and most severe EV71 epidemic, to date, in Taiwan in 1998 (4). During the 1998 Taiwan EV71 epidemic**,** ~~it was catastrophic that~~ almost all the patients with cardiopulmonary failure died (4, 5). Thi~~s e cause of the~~ catastrophic outcome may have been due to our lack of ~~be due to no~~ previous experience of ~~of how to~~ managing such~~e the~~ patients ~~of such condition~~ or delayed parental or medical recognition of how critical the patients were~~awareness of such critical condition either by parents or physicians~~ at that time. This lack of knowledge is understandable, as no articles or reports on this disease and its management were published until 1998.~~there was no published article of such cases and on management of such cases before 1998.~~

According to our clinical studies, enterovirus 71 (EV71) infection may progress through ~~follow~~ four stages: ~~and almost all the patients with cardiopulmonary failure died in 1998 in Taiwan (5). The four stages include~~ hand, foot, and mouth disease (HFMD)/herpangina (Stage 1~~I~~), CNS involvement (Stage 2~~II~~), cardiopulmonary failure (Stage 3~~III~~), and convalescence (Stage 4~~IV~~). In 1998 in Taiwan almost all the patients with cardiopulmonary failure died (5). In 2000, t~~T~~o improve the survival, we developed a disease~~stage-based~~ management program that varied according to the ~~for~~ different stages of EV71 infection ~~in 2000~~ (6, 7). This~~, and this~~ study was conducted to compare case-fatality rate and sequelae of EV71 infection before and after we developed stage-based management.

Patients and Methods

Definition of Clinical Syndromes and Stages

From 1998 to 2002, we identified all the EV71 patients~~cases~~ at Chang Gung Children's Hospital~~were collected~~ and followed up their cases to find out what their current states of health were.~~for their current health status.~~ These patients were~~were those who~~ clinically diagnosed~~is~~ as having the disease if their ~~by means of~~ viral cultures showed~~ing~~ EV71 or positive EV71 IgM or if a four-~~-~~fold rise in EV71 neutralizing antibody serotiters was found between the acute and convalescent sera.

With regard to case definition and clinical syndromes,~~For case definition of EV71 clinical syndromes,~~ Stage 1, HFMD, ~~-~~were found to have~~cases shows~~ oral ulcers~~,~~ and vesicular rash appearing on the hands, feet, knees, and/or the buttocks; herpangina includinged oral ulceration over anterior tonsillar pillars, the soft palate, buccal mucosa or uvula. Stage 2, CNS involvement: aseptic meningitis with~~shows~~ headache, irritability or myoclonic jerk and CSF pleocytosis (>5x10^6 leukocytes/L) but without altered level of consciousness and focal signs; encephalitis with~~shows~~ altered level of consciousness plus CSF pleocytosis; poliomyelitis-like syndrome with~~shows~~ acute limb weakness and decreased reflex and muscle strength; and encephalomyelitis with the~~shows~~ occurrence of both encephalitis and poliomyelitis-like syndrome. Stage 3, c~~C~~ardiopulmonary failure, was defined as pulmonary edema or hemorrhage with or without decreased ejection fraction of left ventricle necessitating inotropic agent support. Pulmonary edema was defined as alveolar congestion on a chest radiography and pink frothy fluid from the endotracheal tube; pulmonary hemorrhage as evidenced by alveolar congestion on a chest radiography or~~and~~ fresh blood from the endotracheal tube.

Stage-based Therapeutic Strategy

Between~~In~~ 1998 and 1999, cases of EV71 were managed with various symptom-control treatments and with no~~out~~ special strategy. In 2000, with the approval of Taiwan's Enterovirus Ad Hoc Committee and the Center for Disease Control, we~~We~~ developed a treatment program based on the four stages of ~~stage-based management for different stages of~~ EV71 infection ~~in 2000, which was approved by the enterovirus ad-hoc-committee and Center for Disease Control in Taiwan~~. Since that time~~2000~~, all ~~the~~ EV71 cases at Chang Gung Children's Hospital have~~d~~ received this~~the~~ stage-based management.

The clinical stages and the disease management of these stages have been published previously~~Clinical staging and management were published on the previous articles~~ (6, 7) and are summarized in~~on~~ Table 1. Patients with S~~s~~tage 1 uncomplicated ~~——~~HFMD/herpangina required treatment of symptom~~satic treatment~~ only. Patients identified as having Stage 2 CNS involvement were hospitalized and their treatment included~~-~~

~~We identified and hospitalized children with stage 2 CNS involvement. Management included~~ fluid restriction and~~,~~ administration of osmotic diuretics for those with~~to patients presenting~~ signs of increased intracranial pressure (IICP)~~,~~ and furosemide for those~~to patients who are~~ suspected having~~to be~~ fluid overload~~. ed, In~~intravenous immunoglobulin (IVIG) was administered and ~~closely monitor~~ blood pressure, oximeter, coma scale and blood sugar were closely monitored. Patients were admitted to our ~~Admit patient to~~ ICU if they were found to have ~~patient presents~~ tachypnea/apnea, hypertension/hypotension, signs of IICP ~~signs~~, or~~and~~ hyperglycemia.

~~For the children with~~ Patients identified as having Stage 3~~,~~ C~~c~~ardiopulmonary F~~f~~ailure were identified as such when they required ~~was defined as patients with cardiopulmonary failure requiring~~ ventilator support and inotropic agents. We subdivided Stage 3 into~~, we divided this stage into two substages,~~ Stage 3A Hypertension/pulmonary E~~e~~dema S~~s~~tage and Stage 3B H~~h~~ypotension S~~s~~tage, and treated them accordingly. ~~, based on therapeutic requirements. The landmark of~~ Stage 3A patients experienced~~is~~ hypertension, cold sweating, hyperglycemia and pulmonary edema. Their intensive care included~~s~~ continuous fluid restriction, administration of milrinone to control severe hypertension and to increase cardiac output, early intubation with positive pressure mechanical ventilation with increased positive end expiratory pressure for pulmonary edema. High frequency oscillatory ventilator was~~should be~~ considered if pulmonary edema or~~/~~ hemorrhage persisted~~s~~ or if they developed severe hypoxemia ~~develops~~.

When a patient's blood pressure drops below the normal range for his or her age~~the patient's age~~, the disease has entered~~s~~ stage 3B. In some cases, blood pressure is very unstable and oscillates between 3A and 3B, requiring fine adjustments of cardiovascular drugs. ~~However, we observed that blood pressure of some patients was very unstable and oscillate to and fro between 3A and 3B, and need very fine adjustments of cardiovascular drugs~~In 3B, while p~~P~~ulmonary edema may improve,~~but~~ neurologic and cardiovascular conditions deteriorate. Inotropic agents such as dopamine and epinephrine become necessary to maintain~~keep~~ sufficient perfusion pressure.

Patients who are found to have a totally recovered cardiac output are considered to have entered the Stage 4 Convalescence. At Stage 4, ~~stage, characterized by totally recovered cardiac output,~~rehabilitation begins for~~was started for~~ limb weakness/atrophy, dysphagia, diaphragm dysfunction, apnea~~/~~ or central hypoventilation. In order to prevent pneumonia, sufficient chest care is also necessary.~~,~~

and sufficient chest care necessary to avoid recurrent pneumonia. Patients with apnea/ or central hypoventilation and dysphagia needed tracheostomiesy and with ventilator support and some of them need to be transferredal to respiratory care centers.

Outcome Assessment

A patient was considered to be an early fatality if death occurred within 8 weeks of onset of the disease and a convalescent stage fatality if death occurred more than 8 weeks after onset of the disease.The early fatality rate was assessed eight weeks after admission and death at convalescence was defined that patients died more than 8 weeks after the onset of disease. Sequelae includedwere defined as having residual neurologic deficits, seizure, need for tracheostomy with or without ventilator support, need for nasogastric tube or other disabilitiesy after 6 months of follow-up.

Laboratory Methods for Vvirus Iisolation, EV71 IgM, and Neutralizing antibody

Throat swabs, rectal swabs, cerebrospinal fluid, vesicular fluid or autopsied tissue were submitted for virus isolation. Samples were inoculated into human embryonic fibroblast (MRC-5), LLC-MK2, HEp-2, RD cell cultures. Once enteroviral cytopathic effect involved more than 50% of the cell monolayer, the cells were scraped. I and indirect fluorescent antibody staining with panenteroviral antibody (Chemicon International, Inc., Temecula, CA) was performed to identify the enterovirus. These isolates were subsequently identified as EV71 by immunofluorescence with EV71 monoclonal antibody (Chemicon International, Inc., Temecula, CA).

To test EV71 IgM, an EV71 isolate, TW/2086/98, was amplified and purified as an antigen for use in μ-capture enzyme-linked immunosorbent assay (ELISA), whose diagnostic sensitivity and specificity for acute EV71 infection have been reported to bewere 91.5% and 93.1% respectively when compared withas compared to conventional virus culture methods [8].

The measurement ofLaboratory methods for measuring EV71 neutralizing antibody was done in microtiter plates followinged standard protocol of neutralization test in microtiter plates. Serum and 50ul of EV71, which contained one hundred 50% tissue culture infective doses ($TCID_{50}$) of EV71 strain TW/2272/98 (GenBank accession number AF119795), were mixed and incubated onto the microtiter plates with RD cells at 35℃ in a 5% CO_2 incubator. Cytopathic effect (CPE) was observed under an inverted microscope after an incubation period of 2 to 7 days, and serotiter was determined when CPE was observed in 1 $TCID_{50}$ of the virus back titration. Seropositivity was defined as serotiter equal to or greater than 8.

Statistical Analysis

Data is expressed as mean ±standard deviation. Chi-square test was used for to analysis ofze categorical data, and; student t-test forwas used to analyze continuous

variables. A multiple logistic regression analysis was performed for the~~to~~ calculation of~~e the~~ multivariate-adjusted odds ratios for risk factors. P values less than 0.05 were considered significant.

Results

Clinical Syndromes

There were a total of 527 EV71 cases. Of these 527 EV71 cases, 509 cases were diagnosed by~~based on~~ positive EV71 isolation plus positive serologic test;~~ and~~ the other 18 cases were diagnosed by~~based on~~ positive EV71 IgM and positive EV71 neutralization antibody.

Clinical syndromes associated with~~of~~ EV71 are shown in~~on~~ Table 2. The number of 1998 ~~–~~ 1999 pre-stage-based managed EV71 cases came to 196; the number of 2000 – 2002 stage-based managed EV71 cases came to 331.~~, and 331 EV71 cases after stage-based management, 2000-2002.~~ The distribution of ~~clinical~~associated syndromes among the patients who did not receive stage-based management was similar to that of those who did~~was similar before and after stage-based management~~ (p = 0.29). Overall, 69% (365/527) had no complications, 20% (108/527) had CNS complications and 10% (54/527) had CNS involvement plus cardiopulmonary failure. The incidence of CNS involvement plus cardiopulmonary failure was 9.1% (18/196) in those who did not receive~~before~~ stage-based management and 11% (36/331) in those who did~~after stage management~~ (p = 0.64).

Clinical O~~o~~utcomes of EV71 CNS I~~i~~nvolvement

Forty-seven~~There were 47~~ EV71 who did not receive stage-based management and 61 who did had~~cases of~~ CNS involvement. Its manifestations included~~ without stage-based management and 61 EV71 cases of CNS involvement with stage-based management. Manifestations of CNS involvement included~~ limb weakness, limb hypesthesia, myoclonic jerk or seizure attacks~~/~~, or conscious disturbance.

The~~ir~~ clinical outcomes of two groups were not ~~were not~~ significantly different~~with or without stage-based management as~~ (Table 3)~~ shows~~. Most (79%, 85/108) of those with~~the~~ CNS involvement ~~cases~~ recovered; 18% (20/108) suffered~~had sequelae of~~ polio-like syndrome of limb weakness or cranial nerve palsy. Three cases, who did not receive~~ without~~ stage-based management, died during ~~the~~ convalescence: two, who were left in a~~: two of~~ vegetative state,~~us~~ died of pneumonia 2 and 4 months after onset of the disease onset; one, who suffered subsequent~~, and one with sequelae of~~ dysphagia and limb weakness, died of suffocation at home 3 months after onset of disease.~~ onset.~~

~~Further analysis reveals that~~ Aage, sex and stage management were not found to affect~~ did not affect~~ the occurrence of sequelae in CNS cases, though those with sequelae were more likely to be found to have ~~but only CSF WBC. CNS cases with sequelae had~~ higher CSF WBC than those without~~ than CNS cases without sequelae~~ (299±305/uL vs. 141±122/uL, p=0.05).

Clinical Outcomes of Enterovirus 71-related Cardiopulmonary Failure

All ~~the~~ patients with~~of~~ EV71-related cardiopulmonary failure had fever and 95% (51/54) had either HFMD or herpangina. All of the cardiopulmonary failure patients had been found to have had~~They also had preceding~~ prior CNS involvement, including~~such~~ limb weakness, limb hypesthesia, myoclonic jerk or seizure attacks, ~~/~~upward gaze, ~~/~~and nystagmus. From s~~S~~everal hours to 2 days after CNS involvement, these patients suddenly~~y d~~ developed ~~sudden appearances of~~ tachypnea, tachycardia (range 135-250 beats per minute), cyanosis and coma.

After intubation, ~~All the~~ chest X-ray films of these children ~~after intubation~~ showed~~demonstrated~~ alveolar density with~~and~~ no cardiomegaly, EKG did not show arrthymia, and cardiac echography showed normal or mildly decreased ejection fractions. From the endotracheal tube came~~After intubation, children had~~ a white frothy secretion and then a~~, then~~ pink frothy fluid, which ~~and~~ sometimes contained~~also had~~ fresh blood. ~~from endotracheal tube.~~

Eighteen EV71 cases of CNS involvement plus cardiopulmonary failure occurred in 1998. ~~and did not receive stage-based management.~~ Most (83%, 15/18) of these patients, who did not receive stage-based management, ~~m~~ died soon after cardiopulmonary resuscitation. Three~~; only~~ survived the cardiopulmonary failure during the acute stages of the EV71-~~-~~related illness. ~~However,~~ One of three survivors ~~one of these three~~ died of ventilator disconnection~~during sleep~~ 6 months after onset of the disease and another~~after disease onset; the other one~~ died of multi-resistant *Acinetobacter baumanii* pneumonia 4 years after onset.~~disease onset.~~ The only one who has survived~~survivor~~ still has tracheostomy, dysphagia with occasional~~ly~~ aspiration pneumonia, left facial palsy and right hemiparesis.

Thirty-six EV71 cases with CNS plus cardiopulmonary failure received stage-based management in the ~~in~~ 2000 ~~–~~ 2002 time period. Twelve (33%) died at the acute stage despite ~~of~~ stage management. Five (14%) cases recovered completely, and the other 19 (53%) had sequelae. Of the 19 survivors with sequelae, 2~~two~~ died from~~of~~ unknown sudden cardiac arrest at a chronic respiratory care center or~~and~~ at home ~~respectively~~, and 1~~one~~ died of pneumothorax at a chronic respiratory care center.

Factors A~~a~~ssociated with F~~f~~atality in E~~e~~nterovirus 71-related C~~c~~ardiopulmonary F~~f~~ailure

To analyze factors associated with fatality in EV71-related cardiopulmonary failure during the acute stage, we compared the data of those who survived EV71-related cardiopulmonary failure and those who did not.~~the data between alive and fatal cases of EV71-related cardiopulmonary failure.~~ As Table 5 shows, patients over 2 years old and those with CSF WBC over 100/uL were at higher risk of dying of cardiopulmonary failure, though who received stage

management had less of a risk than those who did not. ~~cases with age over 2 years, and CSF WBC over 100 /uL had higher risk of fatality but receiving stage management decreased the risk of fatality.~~ Although the younger children ~~tend to~~ developed cardiopulmonary failure more often than the older ones~~children~~, ~~it is much more difficult to~~ rescuinge older children was much more difficult. ~~with EV71-related cardiopulmonary failure. After~~ M~~m~~ultivariate analysis found ~~,~~ ~~receiving~~ stage management to be the most~~is the most~~ significant factor affecting fatality (OR 0.1, 95% CI 0.024-0.414, p=0.0015).

Discussion

~~Our~~ The stage-based management program we developed was found to significantly decrease~~d~~ the case-fatality rate (33%) in patients with~~of~~ EV71-related ~~cases with~~ cardiopulmonary failure when we compared the fatality rates of those who underwent~~in comparison with historical~~ conventional management (83%). The decrease may be ~~causes of decreasing mortality may be~~ due to earlier admission of high-risk groups, usage of IVIG, and more advanced intensive care they received. ~~Up to now, there is no standard therapy for EV71-related cardiopulmonary failure and we think that setup of the standard therapy for EV71 infection is necessary.~~

The~~First of all,~~ pathogenesis of the disease is very important in determining ~~for~~ appropriate management.

~~–~~ During the onset of disease, ~~(~~Stage I~~)~~, ~~there is~~ viremia occurs~~status~~. For common acute viral infections such as chickenpox, herpes simplex or influenza viral infections, it is suggested that antiviral therapy ~~is usually supposed to~~ be used as soon as possible to stop further spread, as~~, and~~ only early usage of an~~the~~ antiviral agent can~~could~~ have impact on disease outcome (9-11). However, since no~~However, up to now, there is no~~ effective anti-EV71 drug has been made available, our only management choice is ~~in the market. Therefore, only~~ supportive therapy with~~is given and~~ close observation of the symptoms and signs of advanced disease. ~~is necessary.~~

CNS involvement, the main clinical feature of ~~During~~ Stage 2~~II, CNS involvement~~,~~the main clinical feature~~ may be caused by viral invasion of the CNS combined with the resulting immune response there~~in the CNS~~. The manifestations may be myoclonic jerk, limb weakness, cranial nerve palsy, seizure and conscious disturbance (5, 12). Increased intracranial pressure should be prevented and treated, so we recommend fluid restriction~~ed~~ and osmotic diuretics. IVIG is given in all cases beyond S~~s~~tage 2 because of the~~II. IVIG may have two effects: one is the~~ resulting antiviral activity, which may~~to~~ prevent further spread of the virus, and because of the resulting~~the other is~~ immunomodulation. ~~However,~~ IVIG did not, however, affect the incidence of sequelae ~~n~~or prevent progress to cardiopulmonary failure. ~~Because~~ The patients with CNS involvement that received ~~cases with~~ stage-based management, ~~(~~including those receiving IVIG treatment, almost the same~~) had similar~~ incidence of sequelae as those do did not~~than CNS cases without IVIG treatment~~. Both groups had almost the same proportion of ~~and the proportion of~~ cardiopulmonary failures.~~cases were almost the same with (in 2000-2002) or without stage management (in 1998-1999).~~ Some cases with CNS involvement ~~did~~ develop~~ed~~ cardiopulmonary failure during the administration of IVIG ~~administration~~.

~~Higher CSF WBC was found in~~ CNS cases with sequelae were found to have higher CSF WBC, possibly indicating that the more severe the CNS involvement, the higher the incidence of sequelae.. ~~Higher CSF WBC may indicate more severe involvement of the CNS, so the incidence of sequelae on such a condition was higher.~~

The pathogenesis of EV71-related pulmonary edema (Stage 3~~III~~) is still controversial. Some have~~We had~~ hypothesized that EV71 involvement of the brainstem starts~~made~~ autonomic nervous system dysregulation (ANS dysregulation), tachycardia, rapid change of the vascular tone and resistance, left ventricular dysfunction, and then neurogenic pulmonary edema [6, 13, 14]. A study by~~In another report by~~ Wu JM et al. found~~, they found that~~ the mechanism of EV71-related pulmonary edema not to be~~is not~~ directly caused by viral myocarditis but possibly related to~~and may be related to~~ increased pulmonary vascular permeability caused by brainstem lesions and/or systemic inflammatory response [15]. In our studies, patients with cardiopulmonary failure were found to have significantly elevated levels of seral proinflammatory cytokines, white blood cell counts, and glucose levels and the best predictor for this complicated condition was found to be the level of serum IL-6 (16,17). CNS proinflammatory cytokines significantly elevated on the first two days of CNS involvement regardless of whether there was~~no matter~~ cardiopulmonary failure ~~occurred or not~~ (17). At this~~In this~~ stage, when there is mounting of the immune response ~~is mounted~~, viral clearance can be accompanied by severe inflammatory damage of the system and CNS.

Putting all the clinical evidence~~s~~ together, we think that the combination of CNS, particularly the ~~(especial~~ brainstem,~~)~~ and systemic inflammatory response may trigger EV71-related cardiopulmonary collapse (17). Therefore, usage of IVIG, immunomodulator, to decrease both CNS and systemic inflammatory response, appropriate inotropic agents and fluid therapy are all important in treatment.

This study show~~s~~ed that immune modulators such as intravenous immunoglobulin (IVIG) plus advanced cardiopulmonary failure could ~~could~~ save~~rescue~~ EV71 patients from fulminant cardiopulmonary failure; ~~,~~however, most of the survivors suffered from sequelae related to brainstem or spinal cord dysfunction, including dysphagia, hypoventilation, facial palsy, and ~~also~~ polio-like syndrome. The sequelae are most related to the degrees and the location of neuron damage, and rarely related to hypoperfusion. Since their sequelae may sometimes cause life-threatening events, chronic care is also very important during the convalescence stage.

In conclusion, to date no standardized therapy for EV71-related cardiopulmonary failure has been recommended. We think that at least a preliminary one should be established. We suggest that management guidelines for EV71 infection include CNS management, cardiopulmonary support and care for convalescence based on different clinical stages; the

guidelines, though not perfect, are quite comprehensive, and the mortality rate of EV71-related cardiopulmonary collapse did decrease after 1999. The possible reasons of decrease in mortality rate may be due to earlier hospitalization of the high-risk cases and thus earlier administration of intravenous immunoglobulin and appropriate cardiopulmonary support.

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Table 1. Clinical Sstaging and Mmanagement

Stages	Clinical Manifestations	Management
1	HFMD/herpangina	Symptomatic treatment only
2	CNS involvement	Fluid restriction, osmotic diuretics for increased intracranial pressure (IICP), and furosemide for fluid overloaded (CVP>8 cmH2O), intravenous immunoglobulin (IVIG) for encephalitis and/or polio-like syndrome and close monitoring of heart rate, blood pressure, oxygenation, coma scale and blood glucose
3	Cardiopulmonary failure	
3A	Hypertension/pulmonary Edema	Phosphodiesterase inhibitor, milrinone, to increase cardiac output, early intubation with positive pressure mechanical ventilation with increased positive end expiratory pressure for pulmonary edema, and high frequency oscillatory ventilator if pulmonary edema/ hemorrhage persists or severe hypoxemia develops
3B	Hypotension	Adding inotropic agents such as dopamine and epinephrine
4	Convalescence	Rehabilitation for limb weakness, dysphagia, apnea/ or central hypoventilation, and sufficient chest care to avoid recurrent pneumonia

NOTE. HFMD, hand, foot, and mouth disease; CVP, central venous pressure; CNS, central nervous system.

Table 2. Clinical Syndromes of Enterovirus 71 before and after Stage-based Management

Year	Before, 1998-1999 (N=196)	After, 2000-2002 (N=331)	Total (N=527)
Uncomplicated cases	131 (67%)	234 (71%)	365 (69%)
HFMD	102	209	311
Herpangina	18	14	32
Febrile illness	7	10	17
AGE	1		1
Viral exanthema	3	1	4
CNS cases	47 (24%)	61 (18%)	108 (20%)
Meningitis	12	21	33
Encephalitis	18	20	38
Polio-like syndrome	8	15	23
Encephalomyelitis	9	5	14
CNS involvement plus cardiopulmonary failure	18 (9.1%)	36 (11%)	54 (10%)

k l qb. $\chi 2=2.46$, P=0.29. HFMD, hand, foot, and mouth disease; AGE, acute gastroenteritis; CNS, central nervous system.

Table 3. Clinical Outcomes of Enterovirus 71 CNS Involvement Without Cardiopulmonary Failure Before and After Stage-based Management

Year	Before, 1998-1999 (N=47)	After, 2000-2002 (N=61)
Recovery	35 (74%)	50 (82%)
Sequelae	9 (19%)	11 (18%)
Limb weakness or cranial nerve palsy	8	11
Seizure	1	
Fatality at convalescence	3 (6%)*	0

NOTE. $\chi 2=4.1$, P=0.13 *One with sequelae of dysphagia and limb weakness died of suffocation at home 3 month after EV71 encephalomyelitis. Two with sequelae of vegetative status died of pneumonia 2 and 4 months respectively after EV71 encephalitis.

Table 4. Clinical Outcomes of Enterovirus 71-related Cardiopulmonary Failure-Before and After Sstage-based Management

Year	Before	After
	(N=19)	(N=36)
Recovery	0	5 (14%)
Sequelae	1 (6%)	16 (43%)
Limb weakness or cranial nerve Palsy		2
Dysphagia		1
Hypoventilation + dysphagia		1
Dysphagia + limb weakness	1	
Combined (Hypoventilation + Dysphagia + limb weakness)		12
Fatality	17 (94%)	15 (42%)
Death at acute stage	15 (83%)	12 (33%)
Death at convalescence	2* (11%)	3**(8%)

NOTE. P<0.001, $\chi 2=13.3$; + denotes plus. *One died of ventilator disconnection-related hypoxia 6 months after disease onset, and the other died of

pneumonia 4 years after disease onset. ** One died of unknown sudden cardiac arrest at chronic care center, one died of unknown cause at home and one died of pneumothorax.

Table 5. Factors associated with fatality in enterovirus 71-related cardiopulmonary failure

Factors	Survival (N=27)	Fatal (N=27)	Unadjusted OR (95% CI)	p
Age>=2	7% (2/27)	44% (12/27)	10 (1.96-50.94)	0.0056
Sex (Male/female)	20/7	16/11	1.964 (0.62-6.22)	0.25
Receiving stage management	89% (24/27)	44% (12/27)	0.1 (0.024-0.414)	0.0015
CSF WBC>=100	7% (2/27)	41% (11/27)	8.59 (1.68-43.95)	0.0098

NOTE. OR, odds ratio; CSF, cerebrospinal fluid; WBC, white blood cell count.

Manuscript submitted

Transmission and Clinical Features of EV71 Infections ~~Transmission of EV71~~ in Household Contacts ~~and Clinical Features of Infected Children and Adults~~

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Key words: enterovirus 71;~~,~~ hand, foot~~,~~ and mouth disease;~~,~~ herpangina; household transmission;~~,~~ ~~family, household, asymptomatic, complication,~~ risk factors

Short title: Household Transmission of Enterovirus 71

Word count: 2524

Abstract

Context Although eEnterovirus 71 ~~may~~ has ~~cause large~~caused epidemics associated with ~~lots of~~significant morbidity and mortality, its ~~fatality but~~ transmission ~~pattern~~ has~~is~~ not been thoroughly investigated~~well-investigated~~.

Objective T~~T~~o ~~study~~ evaluate e~~E~~nterovirus 71 transmission within households and outcome of e~~E~~nterovirus 71 infection ~~the~~ in children and adults.~~, we did a prospective family cohort study.~~

Design, Setting, and Subjects From February 2001 to August 2002, w~~W~~e performed a prospective family cohort study to ~~enrolled~~ investigate patients and family members of patients who presented with signs and symptoms suggestive of e~~E~~nterovirus 71. Household members underwent c~~173 possible EV71 patients at Chang Gung Children's Hospital and their 611 family members from February 2001 to August 2002. They received c~~linical evaluations, virologic studies and questionnaire~~-~~based interviews. ~~**investigation. If any family members had positive EV71 isolation, their data were analyzed. re between acute and convalescent sera.**~~

Main Outcome Measures Enterovirus 71 infection was confirmed by isolating e~~E~~nterovirus 71, detecting e~~E~~nterovirus 71 IgM or demonstrating a four-fold change in neutralizing antibody titers. Clinical syndromes at presentation included asymptomatic, uncomplicated and complicated illness such as central nervous system involvement or cardiopulmonary failure. Unfavorable outcome was defined as death or sequelae.

Results ~~A total of~~We studied 433 family members ~~in~~ from 94 families with positive e~~E~~nterovirus 71 isolation~~. were further analyzed.~~ In addition to the 94 index patients, 52% (176/339) of household contacts ~~of all the family members~~ were infected~~. In addition to 94 index patients:,~~ 84% (68/81) ~~of their~~ siblings~~, and~~ 84% (21/25) ~~of their~~ cousins~~, were infected whereas~~ 41% (72/175) ~~of their~~ parents, 28% (10/36) ~~of their~~ grandparents and 26% (5/19) ~~of their~~ uncles/aunts were infected~~got EV71 infection~~. Of ~~Of the~~ 183 infected children, 6% (11/183) were asymptomatic,~~,~~ 73% (133/183) suffered no~~were un~~complicated~~ed~~ions and 21% (39/183) had complications; ~~cases, and were complicated cases among whom~~ 10 ~~cases were fatal~~died and 13 had sequelae. Age younger than 3 years was the most significant factor associated with an unfavorable outcome ~~of~~ in children. ~~Of~~ Among ~~the~~ 87 infected adults, 53% (46/87) were asymptomatic~~, ,~~ 39% (34/87) had nonspecific illnesses of fever, sore throat or gastrointestinal discomfort and ~~only~~ 8% (7/87) had ~~HFMD~~hand, foot and mouth disease.~~, and 39% (34/87) had nonspecific illness.~~

Conclusions E~~E~~nterovirus 71 infections in young children are associated with serious complications, long-term sequelae and death. We found the high ratio of

asymptomatic adults and high household transmission rate, which make controlling transmission of enterovirus 71 infections difficult.

~~Household EV71 transmission was very high, and both infected children and adults could be the source of familial or extra-familial transmission. EV71-infected children tend to have severe disease than adults.~~

INTRODUCTION

Outbreaks of enterovirus 71 (~~Enterovirus 71 (~~EV71)~~)~~ ~~has caused outbreaks in some parts of the world,~~have been documented ~~–~~since it was ~~originally~~ recognized in California in l969.[1] Forty-four fatalities have been reported in Bulgaria (1975), 47 in Hungary (1978) and 30 in Malaysia (1997)~~Before 1998, three large outbreaks occurred in Bulgaria with 44 deaths in 1975, Hungary with 47 deaths in1978, and Malaysia with 30 deaths in 1997.~~, respectively.[2-4] ~~Unfortunately,~~T ~~t~~he largest and most severe EV71 epidemic ~~exploded~~ occurred in Taiwan in 1998.[5-10] ~~A total~~Out of over hundred thousands cases of hand, foot, and mouth disease and herpangina (HFMD/HA), ~~were reported,~~ 405 ~~cases~~ had severe neurologic complications and/or pulmonary edema; seventy-eight~~, and 78~~ children died.[9]

~~We initiated~~In a ~~a~~ sero~~e~~pidemiologic~~al~~ study before and after the 1998 outbreak, we ~~and~~ found that ~~the~~ pre-epidemic and post-epidemic EV71 seroprevalence rates in adults and children older than 6 years ~~of age were~~ranged from 57 to 67%.[11] Lu CY *et al.* examined serial serum antibody titers to EV71 in blood samples from 81 children born in 1988.[12] Samples were obtained yearly from 1989 to 1994 and from 1997 and 1999. ~~in 81 children who were born in 1988 and had yearly blood samples saved from 1989 to 1994 as well as in 1997 and 1999.[12] The investigators discovered that~~ EV71 seroconversion occurred with a yearly incidence of 3% to 11% between 1989 and 1997. By 1997, ~~and that~~ 68% ~~of these children~~ had serologic~~al~~ evidence of EV71 infection~~s by 1997~~.[12] A s~~S~~ereoepidemiologic~~al~~ study in Singapore ~~also showed~~ demonstrated that the EV71 seroprevalence rate ~~of~~ in the general population was ~~also~~ as high~~, up~~ as ~~to~~ 60 to 70%.[13] It is evident that~~These indicated that~~ EV71 infection is not un~~so un~~common and that ~~reports or identificati~~documented~~on of the~~ cases do not accurately ~~may just~~ reflect ~~the tip of iceberg~~the actual number of infections.

~~It remains unclear~~ W~~w~~hy the 1998 EV71 outbreak in Taiwan was so large ~~and extensive~~is not clear. ~~in 1998 in Taiwan, and EV71 transmission was not defined, either.~~[9,10] We know that ~~Previous seroepidemiological study showed that intra-~~familial transmission ~~is~~ is as~~one of major factor to get~~a major route of EV71 infection,~~.~~[11] and that secondary household transmission rates of enteroviruses vary, including those for poliovirus, enterovirus 70 and coxsackievirus A24.[14-16] ~~In addition, few reports studied the manifestations and outcome of EV71 infection in adults~~The two adult fatalities that have been reported in ~~although two fatal adult EV71 cases had been reported in~~ Bulgaria and Singapore indicate that adults can suffer serious complications from EV71 infections.~~, respectively.~~[2,17] Severity of EV71 infections in adults and disease transmission from adults to other family members, including children, needs further investigation.~~It needs investigations whether enterovirus 71 infection in adults is less severe and whether adults may introduce the virus into the~~

~~family and transmit it to their children.~~

~~There were studies on the household transmission of some enteroviruses such as poliovirus, EV70 and CA 24.[15-17]~~ Since vaccination programs have controlled the spread of poliovirus, we feel that EV71 infections are now the leading enteroviral disease associated with mortalities and disabling sequelae. Data regarding household EV71 transmission are necessary to control, manage and prevent EV71 infections. ~~The extent of secondary transmission may vary a lot with different enteroviruses.[17]~~ To date, EV71 transmission within households has not been studied.~~However, there are no studies on EV71 transmission within households. After the eradication of poliovirus, we think that EV71 have become the most important enterovirus that causes fatal and disabled disease. The data of household EV71 transmission will be very helpful and important for further control, management and prevention of EV71.~~ Therefore, we investigated ~~the pattern and degree of~~ EV71 transmission within families~~,~~ and ~~the~~ clinical ~~manifestations and outcomes of both~~aspects of infection~~sed~~ in children and adults.

METHODS

Patient SSelection ~~of possible EV71 cases~~ and Fthe family Ssurveillance

At Chang Gung Children's Hospital in Taiwan, we studied patients and the families of patients who were suspected of having EV71 infections from April 2001 to August 2002. Informed consent was obtained from all subjects.

~~The~~ Study flowchart ~~of this study~~ is shown in Figure 1. ~~According to our previous studies,[11,18] in comparison with coxsackievirus A16 infection, children with EV71-related HFMD/HA were more likely to have fever higher than 39°C and for longer than 3 days and complications, such as encephalitis, polio-like syndrome, meningitis, pulmonary edema, shock.~~ If patients at emergent department, outpatient clinics or inpatient wards had clinical syndromes suggestive of EV71 infection~~any of the above clinical charaters at Chang Gung Children's Hospital~~, they and their family members were asked to participate in the ~~family~~ study. ~~.~~ Throat and rectal swabs for virus isolation were obtained from i~~The i~~ndex patients. Study subjects were screened for virus isolation of throat swab, ~~received throat and rectal swabs for virus isolation,~~ EV71 IgM and neutralizing antibodies~~y~~. ~~Their~~ Cclinical manifestations, courses and outcomes were recorded~~.~~

~~After informed consents were given, the family members received clinical evaluation, questionnaire investigation, throat swabs for virus isolation, and first blood sampling for EV71 antibody~~. ~~The~~ Qquestionnaire-based interviews collected~~included~~ information regarding demographic data ~~data~~, contact time, pattern and ~~the~~ presence of current~~/ or~~ recent signs and symptoms (~~symptoms and signs of~~ ulcers, sore throat, rash, fever, abdominal pain~~,~~ and diarrhea) and preceding contact history with extra-familial people who had clinical syndromes suggestive of EV71 infection. Follow-up t~~T~~elephone interviews ~~of the above symptoms~~repeated questions about signs and symptoms ~~was done~~ at 2, 4 and 8 weeks ~~later~~intervals. If ~~any other~~a family members ~~had discomfort~~experienced signs or symptoms of illness, a physician or nurse examined the patient and obtained laboratory samples to ~~would check the cases and did necessary exam to~~ confirm or exclude enterovirus infection.

If the index case or any family member ~~had~~ tested positive for EV71 isolation, a second blood sample was obtained from all ~~the~~ family members ~~received second blood sampling~~ 4 weeks after the first blood sample. ~~ing,~~ Additional c~~and c~~linical ~~outcome~~ assessments and ~~further~~ data analysesis ~~would be~~were then performed~~, too~~.~~.~~

Definitions of EV71 Iinfection, Cclinical Ssyndromes, Ooutcomes and Identified Source of Infection

Laboratory evidence of EV71 infection was defined as ~~positive~~ EV71 isolation, the presence of EV71 IgM~~,~~ or a four-fold change~~change~~ in EV71 neutralizing antibody serotiters between acute and convalescent sera~~.,~~ ~~or positive EV71 IgM.~~

EV71 seropositivity was defined as a serotiter ~~equal to or over~~ 8.

~~For~~ In uncomplicated cases, evidence of HFMD infections ~~patients had~~included oral ulcers on the tongue~~on the tongue~~ and buccal mucosa~~,~~ and a ~~plus~~ vesicular rash on the ~~on the~~ hands, ~~the~~ feet, ~~the~~ knees~~,~~ or ~~the~~ buttocks. Evidence of herpangina ~~Herpangina~~ included oral ulcerations on anterior tonsillar pillars, ~~the~~ soft palate, buccal mucosa or ~~the~~ uvula. Nonspecific febrile illness was defined as rectal temperature greater than 38^{o}C without other symptoms. ~~and~~ Eenteritis was defined as diarrhea with or without abdominal pain. Upper respiratory tract infection was defined as sore throat, coryza~~,~~ or cough ~~but no~~without herpangina ~~and no~~or rash.

~~As for~~In complicated cases, aseptic meningitis was defined as a clinically compatible illness with cerebrospinal fluid (CSF) pleocytosis (>5 leukocytes/mm^3 ~~if the~~in patients ~~was~~ older than one month ~~of age,~~ or >25 leukocytes/mm^3 ~~if the patient was a~~in ~~newborn~~neonates) ~~plus~~ and negative bacterial cultures. Encephalitis was characterized by an altered level of consciousness ~~plus~~ accompanied by CSF pleocytosis.~~;~~ Evidence of a ppoliomyelitis-like syndrome ~~with~~ included acute limb weakness ~~plus~~ with ~~decreased~~ diminished reflexes and muscular~~le~~ ~~power~~strength. A diagnosis of e~~E~~ncephalomyelitis ~~by definition included~~ was made when there was evidence of ~~both~~ encephalitis and poliomyelitis-like syndrome. Cardiopulmonary failure was defined as pulmonary edema/~~ or~~ hemorrhage with or without decreased left ventricle ejection fraction ~~of left ventricle necessitating~~severe enough to require inotropic ~~agent~~ support. Pulmonary edema was defined as radiographic alveolar congestion ~~on a chest radiography~~ and pink frothy fluid from the endotracheal tube.~~;~~ P~~p~~ulmonary hemorrhage was defined as radiographic alveolar congestion ~~on a chest radiography~~ and fresh blood from the endotracheal tube. Unfavorable outcome was defined as death or ~~having~~ sequelae after 6 months of follow-up.~~,~~ ~~and~~ F~~f~~avorable outcome was defined as complete recovery.

Identified source of EV71 infection in the household was defined as the first case in the family had clear contact history with people who had illness such as HFMD or herpangina suggestive of EV71 infection. Unknown source of infection was defined as the first case in the family did not have contact history with people who had illness suggestive of EV71 infection. Case to case interval was ~~defined as~~ the time ~~interval of disease onset~~ between onsets of disease for ~~first~~primary ~~case~~ and secondary cases in the family~~the other cases in the family~~.

Laboratory M~~m~~ethods

Virus I~~i~~solation and S~~s~~erotyping

Throat swabs, rectal swabs~~,~~ or stool samples were submitted for virus isolation. Samples were inoculated into human embryonic fibroblast, LLC-MK2, HEp-2 and

rhabdomyosarcoma (RD) cell cultures. ~~Once~~ When enteroviral cytopathic effect involved more than 50% of the cell monolayer, ~~the~~ cells ~~will~~ were ~~be~~ scraped and subjected to indirect fluorescent antibody staining with panenteroviral antibodies~~y~~ (Chemicon International, Inc., Temecula, CA). ~~was performed to identify the enterovirus. These~~I ~~i~~solates were ~~subsequently~~ identified as EV71 by immunofluorescence with EV71 monoclonal antibodies~~y~~ (Chemicon International, Inc., Temecula, CA).

EV71 N~~n~~eutralizing~~ation~~ A~~a~~ntibodies~~y~~

~~The~~ Laboratory methods for measuring EV71 neutralizing antibody followed standard protocol of neutralization test in microtiter plates.[18,19] Serum and 50ul of EV71, containing one hundred 50% tissue culture infective dose ($TCID_{50}$) of EV71 strain TW/2272/98 (GenBank accession number AF119795), were mixed and incubated onto the microtiter plates with RD cells at 35℃ in a 5% CO_2 incubator. Cytopathic effect (CPE) was observed under an inverted microscope after an incubation period of 2 to 7 days, and serotiter was determined when CPE was observed in 1 $TCID_{50}$ of the virus back titration. Seropositivity was defined as a serotiter 8.

EV71 IgM D~~d~~etection

EV71 isolate TW/2086/98 was amplified and purified as an antigen for ~~use in~~ μ-capture ELISA. In comparison with standard method of c~~With the c~~onventional virus culture, ~~as a standard method, the~~ s~~s~~ensitivity and specificity ~~of this~~for μ-capture ELISA were 91.5% and 93.1%, respectively.[20]

Statistical Analysis~~s~~

~~The~~ D~~d~~ata ~~was~~ were analyzed with the SAS S~~s~~tatistical P~~p~~ackage ~~SAS system~~ (V~~v~~ersion 8.2, SAS Institute, Cary, North Carolina). We used ~~the~~ S~~s~~tudent's *t* test for continuous variables~~;~~ and ~~used~~ χ^2 test~~s~~ for categorical data. Univariate analysis ~~was done to~~ screened for statistically significant variables. ~~Then, a~~S~~ s~~tep-wise multiple logistic regression analysis was performed to adjust confounders simultaneously and to calculate ~~the~~ multivariate-adjusted odds ratios for risk factors of EV71 infection and an unfavorable outcome. The α level of model selection was set ~~to be~~at 0.15 for in-and-out models. A ~~*P*~~ *p*-value ~~value will be considered significant if it was~~ less than 0.05 was considered significant.

RESULTS

Demography and Iinfection Rrates

A total 173 families (including 343 children and 441 adults) were surveyed from April 2001 to August 2002; EV71 was isolated from 54% (94/173) families, Of these 173 families, 94 (54%) no viruses were isolated from 50/173families had positive EV71 isolation, 50 (29%), and did not have positive virus isolation, non-71 enteroviruses were isolated from 29/173 (17%) had positive non-71 enterovirus isolation. The 94 families with positive for EV71 isolation were further analyzed (Fig. 1).

Table 1 shows the demographic data and the rates for isolating EV71, detecting y, the positive EV71 isolation, positive EV71 IgM and demonstrating seropositive neutralizing antibodiesy seropositive rates among index cases, siblings, cousins, parents, grandparents, uncles/aunts and babysitters. The Ooverall infection rate was 52% (176/339) after EV71 was had been introduced into the family by the first cases. Excluding the index cases, the infection rate in child household contacts was 84% (89/106), and the infection rate in adult household contacts was 37% (87/233) ($p<0.001$). with $\chi 2$ test) The 60% (109/183) In addition, EV71 isolation rate (60%, 109/183) of from infected children was also significantly higher than the 11% (10/87) EV71 isolation that rate (11%, 10/87) offrom infected adults ($p<0.001$) with $\chi 2$ test).

EV71 infection rates declined as age increased (Table 2). Furthermore, 100% (71/71) of children younger than 2 years of age gotwere infected with EV71 infections. Among children, there was no significant differences of in the infection rate existed between siblings and cousins. Of Among adults, parents had a higher infection ratethe infected rate of parents (41%, 72/175) was higher than that other adults (26%, 15/58) of other adults ($p=0.05$). The Infectioninfected rate for mothers (43%, 40/92) of the mothers was similar to that thatrate for fathers (39%, 32/83) of the fathers ($p=0.61$). The EV71 seropositive rates of for all the family members were as high up asto 93% (401/429) during the convalescence.

2.Factors Aassociated with EV71 Iinfection in Cchildren

Table 3 shows factors associated with EV71 infection in children. Male sex and age less than 6 years of age were associated with increased risk of EV71 infection. Children attending kindergarten or school had a lower incidence of EV71 infection. Larger family size and more children in the family did not significantly increase the risk of infection. Stepwise multiple logistic regression analysis indicated that the most significant factors associated with infection in children were age less than 6 years (adjusted OR=9.11, 95% confidence interval=2.90-28.65, p=0.0002) and male gender (adjusted OR=4.11, 95% confidence interval=1.19-14.15, p=0.025).

3.Sources of Ttransmission for to Pthe primary Ccases in the family

Source of transmission was identified inOnly 47% (44/94) of the primary primary infection cases in childrenchildren cases had clear contact history of identified source of EV71 infection, which is shown in(Fig. 1). Among the 44 families in which the source of the original EV71 infection was identified, 40% (46/114) of adults became infected. Among these 46 infected adults, 20 became symptomatic and developed symptoms after their children had become ill. Children with primary infections transmitted EV71 to 79% (34/43) of the other children in their families.

Among the 53% (50/94) 50 families in which the original source of EV71 transmission was not identified, adults in 28% (26/94) with EV71-infected children of unknown source of infection, at least 26 (28%) families had adults with had asymptomatic EV71 infections. in their families. At least two individuals became ill on the same day in 19% (18/94) families. In these cases, an asymptomatic EV71-infected adult family member could have been the source of transmission to the children. In addition, there were 18 families with at least two cases who became ill on the same day, so it indicated presence of asymptomatic EV71-infected family member to transmit the virus.

Among the 44 families whose first EV71-infected children had known source of transmission, 40% (46/114) adults in the families also got EV71 infection. Among these 46 infected adults, 20 had symptoms and all their disease developed later than their first EV71-infected children's illness. The first EV71-infected children also transmitted EV71 to 79% (34/43) of the other children in their families.

Therefore, theH household secondary attack infection rates was were estimated to be 79% (34/43) infor the children and 40% (46/114) infor the adults (Fig. 1).

TheI intervals between primary case and secondary cases in the family ranged from 0 to 15 days., the Mmedian interval was 3 days and the mean interval was 3.7 (SD 2.6) days.

There were 18 families with at least two cases who became ill on the same day, and all these 18 families did not have the identified source of EV71 infection.

4.Clinical Ssyndromes and Ooutcomes of EV71 Infected children and adults.

The Cclinical syndromes and outcomes of for all the infected children and adults are shown in Table 4. EV71-infectedC cchildren had significantly higher rates than adults of for complications, long-term sequelae and fatalitiesy than adults.

Of 183 infections ined children, 6% (11/183) were asymptomatic, and –73% (133/183) were uncomplicated. Complications such as meningitis, encephalitis, polio-like syndrome and cardiopulmonary failure occurred inand 21% (39/183)had complications such as meningitis, encephalitis, polio-like syndrome or

cardiopulmonary failure;. 7% (Thirteen of these13/183) had suffered from long-term sequelae of limbthat included muscular weakness/atrophy, dys swallowing dysfunction, cranial nerve palsiesy or central hypoventilation. and 5%Five percent-((10/183) died.

Table 5 shows factors associated with an unfavorable outcome in infected children based on univariate analysis. Age was the most significant factor. Secondary cases, contact history with HFMD/HA, larger family size and more children in the family were not associated with a significantly higher rate of an unfavorable outcome. Children in kindergarten and school had lower rates of an unfavorable outcome. Stepwise multiple logistic regression analysis indicated that the most significant factor associated with an unfavorable outcome in infected children was age less than 3 years (adjusted OR=6.19, 95% confidence interval=1.77-21.6, p=0.0044).

Of the 87 EV71-infected adults, 53% (46/87) were asymptomatic.; All symptomatic adults recovered completely from and the other had uncomplicated illnesses that included such as HFMD/HA, herpangina, febrile illnessfever, upper respiratory tract infection or and viral exanthema.

;
all recovered.

Table 5 shows factors associated with unfavorable outcome in infected children in univariate analysis. Age was the most significant factor associated with clinical outcome of children. The secondary children cases in the family, contact history with HFMD/HA, larger family size and more children in the family did not have significantly higher rate of unfavorable outcome than the primary children cases. Kindergarten or school children had lower rate of unfavorable outcome possibly due to older age.

After stepwise multiple logistic regression analysis, the most significant factors associated with unfavorable outcome in infected children was younger than 3 years of age (adjusted OR=6.19, 95% confidence interval=1.77-21.6, p=0.0044).

COMMENT

~~New York Virus Watch data showed that secondary coxsackievirus infections were more frequent in mothers (78%) than in fathers (47%).[16] However, we observed similar incidence of EV71 infection between mothers (43%, 40/92) and fathers (39%, 40/92). The EV71 infection rates of parents (41%, 72/175) were higher than other adults (26%, 15/58), and this indicated that closer contact or longer contact made higher incidence of EV71 transmission.~~

In this prospective family cohort study, we found that EV71~~E~~ infections in young children are associated with serious diseases; we also found the high ratio of asymptomatic adults and high household transmission rate, which make controlling transmission of EV71 infections difficult. Long periods of viral shedding may account for widespread transmission of enteroviral diseases. This is certainly the case for polio and coxsackievirus infections.~~The greater spread of polio and coxsackievirus may derive from longer periods of viral shedding.~~[14] ~~However, the period of EV71 viral shedding has not been well defined.~~ In a previous ~~Our previous~~ study, we found that EV71 ~~cases might shed the virus~~was present in ~~the~~ the stool of infected patients for up to 5 weeks.[21]~~The other,~~ Previous research has demonstrated a higher~~which could not be stopped by hand washing only. Our previous study showed that higher~~ rate of EV71 isolation ~~rate~~ from throat swabs than ~~the rate~~ from rectal swabs or ~~or~~ stool: 90% vs. 32%, respectively.[22] We speculated that aerosol transmission would explain the high secondary infection rate in Taiwan, despite hand washing precautions in practice since 1998.[23] Consequently, ~~Based on the results of the high EV71 isolation rate from throat swab and the high household secondary attack rate, we speculate that main transmission route during the acute EV71 infection may be aerosol transmission and~~ isolation of EV71 patients may be ~~mandatory~~ necessary to prevent ~~the spread~~aerosol transmission.

New York Virus Watch data indicate that secondary coxsackievirus infections are more frequent in mothers (78%) than fathers (47%).[15] However, we found similar secondary EV71 infection rates in both mothers and fathers: 43% and 39%, respectively (Table 1). EV71 infection rates for parents (41%, 72/175) were higher than for other adults (26%, 15/58), suggesting that close or longer contact facilitated EV71 transmission.

As Figure 1 shows, it is likely that sequence of transmission was not only child-to-adult, but also adult-to-child. Because EV71 seropositive rates for all family members were as high as 93% during convalescence (Table 1), it is likely that almost all the susceptible family members were infected once EV71 had been introduced. The high infectivity of EV71 is similar to that of poliovirus.[14] ~~The aerosol transmission may also explain the reason why EV71 still causes lots of cases after~~

~~hand washing has been highly advocated since 1998 enterovirus epidemic in Taiwan.[23]~~

Among children, ~~Intra~~familial transmission produced a higher rate of clinical symptoms (93%) compared to extra-familial transmission (29%) in our previous EV71 seroepidemiological study~~in children cause significantly higher rate of clinical symptoms (93%) in comparison to social transmission (29%) in our previous study~~.[11] ~~The difference in illness development may be due to V~~higher virulus load, ~~of intrafamilial infection,~~ host genetic factors or ~~virus~~ virulence may account for this difference. Because the rate of asymptomatic infection with EV71 after social contact ~~Since the asymptomatic ratio~~ is high (about 71%),[11] it was difficult to identify the source of primary infections. We were successful in only 47% of the cases. ~~after social contact with EV71, the infection source of the first symptomatic case in the family is difficult to find and we could only identify 47% source of infection in this study. Such observation also indicated that asymptomatic EV71 cases may also transmit the virus to other people without caution and this also makes prevention of EV71 infection more difficult.~~

EV71 infections in adults were less serious than those in children. ~~Adults could be infected by EV71 and their symptoms and clinical outcomes were significantly different from those of children. Although there were only two reported fatal adult EV71 cases in the literature,[2,14]~~ Although EV71 infection is a differential ~~is still a~~ consideration ~~for~~ in cases of adult encephalitis, unexplained pulmonary edema or cardiopulmonary failure and has been reported in the deaths of two adults,[2,17] most infected adults are asymptomatic or suffer from symptoms of a mild upper respiratory tract infection. ~~However, most of infected adults did not have typical syndrome, hand, foot, and mouth disease.~~ Consequently, EV71 transmission by infected adults who are asymptomatic or mildly symptomatic ~~Therefore, infected adults may transmit the virus into the community without warning sign.~~is the likely source of many infections. In those cases (50/94) in which the primary source of infection could not be identified, we estimate that up to 28% (26/94) of EV71 infections were introduced into the family by an asymptomatic adult (Fig. 1).

~~Although T~~the first symptomatic case~~s~~ in a family ~~in the family almost were~~was usually in a child, and m~~children and m~~ost ~~of the~~ symptomatic parents developed symptoms later than their ~~offspring~~children. In these cases, it is likely that EV71 was ~~These observations suggested that EV71 might be~~ introduced into the family by children and secondarily transmitted to adults. ~~Therefore, either way transmission is possible.~~

In conclusion, EV71 infections in children, especially those less

than 3 years old, are associated with serious complications, long-term sequelae and death. H~~The EV71 seropositive rates of all the family members were high up to 93% during the convalescence, and it indicated that almost all the susceptible in the family were infected once EV71 was introduced into the family. The striking high infectivity of EV71 was just similar to that of poliovirus.[15]~~ igh infectivity, the absence of effective antiviral drugs and the high ratio of ~~Regarding the high~~ asymptomatic adults in the~~ratio of~~ general population ~~and high household transmission rate, make it will be very difficult to~~ controlling transmission of ~~the~~ EV71 ~~infection~~ infections difficult. At this time, developing a v~~by using antiviral drugs or hand-washing measurement, not to mention there is no effective antiviral drug against EV71. V~~accine ~~development may be the only~~appears to be the most effective ~~way to~~approach to control the transmission of ~~the~~ EV71 infections.

Author Contributions: Study concept and design: Chang, Lin.

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Analysis and interpretation of data: Chang, Tsao, Hsia, Lin

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Obtained funding: Chang, Lin.

Administrative, technical, or material support: Tsao, Shih, Huang, Lin

Study supervision: Lin.

Funding/Support: ~~In conclusion, we found high household transmission rate of EV71 and there was either child-to-adult or adult-to-child transmission. About one half of EV71-infected adults did not have symptoms and infected children tended to have severe disease than adults.~~ This study was supported by grants from the Chang Gung Memorial Hospital (CMRP1089) and National Science Council (NSC 90-2314-B-002-463 and NSC 91-3112-B-002-029).

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Table 1. Demographicy Data and Rates for Isolating the positive rates ofEV71-, Detecting EV71 EV71 isolation, IgM, Demonstrating Seropositivity for Neutralizing Antibodies y and Infection among family members

Subjects	Number	Age	Sex (Male/ Female)	Positive EV71 Iisolation Rrate	Positive EV71 IgM Rrate	EV71 Nneutralizing Sseropositive Rrate during Convalescence*	Infectioned Rrate†
Children							
Index Ccases	94	2.5±2.0	61/33	87% (82/94)	95% (88/93)	98% (91/93)	100% (94/94)
Siblings	81	5.1±3.5	39/42	21% (17/81)	77% (59/77)	96% (76/79)	84% (68/81)
Cousins	25	4.8±6.6	16/9	40% (10/25)	71% (17/24)	92% (22/24)	84% (21/25)
Adults							
Parents	175	31.5±5.4	83/92	5% (9/175)	17% (29/175)	91% (159/175)	41% (72/175)
Grandparents	36	59.3±10.4	14/22	0% (0/36)	15% (5/34)	94% (34/36)	28% (10/36)
Uncles/Aunts	19	27.4±8.7	10/9	5% (1/19)	16% (3/19)	84% (16/19)	26% (5/39)
Babysitters	3	36.6±7.7	0/3	0% (0/3)	0% (0/3)	100% (3/3)	0% (0/3)

* EV71 neutralizing seropositive rate during the convalescence was defined as an EV71 neutralizing antibody serotiter equal or over 8 four weeks after illness onset. † EV71 infection was defined as either positve positive EV71 isolation, a four-fold changes change inof EV71 neutralizing antibody serotiters, or positive presence of EV71 IgM.

Table 2. EV71 Infection Rates ~~by~~ in Different Age Groups

Age Ggroup (Y~~in~~ ears)	EV71 Iinfection Rrates	Adjusted Oodds Rratio*	95% CI	*P* Value
Age≦6	96% (159/165)	1.0	--	--
6<age≦18	72% (26/36)	0.10	0.033-0.30	<0.001
18<age≦40	39% (71/181)	0.025	0.010-0.059	<0.001
Age>40	27% (14/51)	0.014	0.005-0.040	<0.001

*Adjusted odds ratio was calculated using the age_~~<=~~6 group as the reference group and was adjusted by sex.
CI denotes confidence interval.

Table 3. Factors Associated with EV71 Infection in Children

Factor	Infected (N=183)	Not Infected (N=17)	*P* Value
Male/Female Ratio	1.57 (112/71)	0.31 (4/13)	0.003
Mean Age	3.3±2.4	10.0±7.6	0.002
Age≦6 Years	86% (158/183)	35% (6/17)	0.001
Family Size≧6	52% (95/183)	53% (9/17)	0.94
Number of Children ~~number~~≧3	45% (82/183)	59% (10/17)	0.27
Kindergarten or School Attendance	34% (62/183)	71% (12/17)	0.006

Table 4. Clinical Syndromes and Outcomes of 183 EV71-infected Children and 87 EV71-infected Adults

Syndromes / Outcomes	Number of Children No. (%) (N=183)	Number of Adults No. (%) (N=87)	*P* Value
Syndromes			<0.001*
Asymptomatic	11 (6%)	46 (53%)	
Uncomplicated Cases	133 (73%)	41 (47%)	
HFMD	90 (49%)	7 (8%)	
Herpangina	19 (10%)	8 (9%)	
Nonspecific Febrile Illness	4 (2%)	1 (1%)	
Upper Respiratory Tract Infection	16 (9%)	18 (21%)	
Enteritis	2 (1%)	2 (2%)	
Viral Exanthema	2 (1%)	5 (6%)	
Complicated Cases	39 (21%)	0	
HFMD plus Meningitis	9 (5%)	0	
HFMD plus Encephalitis	11 (6%)	0	
HFMD plus Polio-Like Syndrome	5 (3%)	0	
HFMD plus Encephalomyelitis and Cardiopulmonary Failure	14 (8%)	0	
Outcomes			0.003†
Complete Recovery	160 (87%)	87 (100%)	
With Sequelae	13 (7%)	0	
Death	10 (5%)	0	

HFMD denotes hand, foot, and mouth disease.

* p-V-value was measured with χ^2 test in-to comparcison of the percentages of asymptomatic cases, uncomplicated cases and complicated cases between between infected children and infected adults.

†p-V-value was measured with χ^2 test in-to comparcison of the percentages of recovery, sequelae and death between between infected children and infected adults.

Table 5. Factors Associated with an Unfavorable Outcome in Infected Children

Factors	Children with an Uunfavorable Ooutcome* (N=23)	Children with a Ffavorable Ooutcome (N=160)	*P* Vvalue
Age≦3 3Yyears	87% (20/23)	52% (83/160)	0.002
Males sex	65% (15/23)	61% (97/160)	0.67
Secondary Ccase in the Ffamily	26% (6/23)	46% (68/149) †	0.09
Contact Hhistory with HFMD/HA	52% (12/23)	71% (110/160)	0.11
Family Ssize≧6	48% (11/23)	53% (84/160)	0.68
Number of Children number ≧3	43% (10/23)	45% (72/160)	0.89
Kindergarten or Sschool Aattendance	9% (2/23)	38% (60/160)	0.013

*Unfavorable outcome was defined as death or having log-term sequelae. and Ffavorable outcome was defined as complete recovery.

† Because 11 out of 160 infected children with favorable outcomes did not have symptomswere asymptomatic, the 11 cases could not be defined as primary or secondary case in the family.