UNIVERSITI PUTRA MALAYSIA

MOLECULAR DETECTION OF VIRUSES FROM HAND, FOOT, AND MOUTH DISEASE PATIENTS IN SERI KEMBANGAN, SELANGOR, MALAYSIA

BEH POAY LING

FPSK(M) 2014 19

MOLECULAR DETECTION OF VIRUSES FROM HAND, FOOT, AND MOUTH DISEASE PATIENTS IN SERI KEMBANGAN, SELANGOR, MALAYSIA

By

BEH POAY LING

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

July 2014

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

MOLECULAR DETECTION OF VIRUSES FROM HAND, FOOT, AND MOUTH DISEASE PATIENTS IN SERI KEMBANGAN, SELANGOR, MALAYSIA

By

BEH POAY LING

July 2014

Chairman: Professor Zamberi Sekawi, MD, MPath

Faculty: Medicine and Health Sciences

Hand, foot, and mouth disease (HFMD) is a common acute viral infection that affects infants and young children. The major causative agents of HFMD are enterovirus 71 (EV71) and coxsackievirus A16 (CVA16). Recently, HFMD cases that are caused by coxsackievirus A6 (CVA6) were reported in neighboring countries. The signs and symptoms of the infection are fever, mouth/throat ulcers, rashes and vesicles in palms and soles. Patients that were infected by EV71 might have serious and fatal complications, such as meningitis, encephalitis and acute flaccid paralysis. EV71 outbreaks that caused fatalities were reported in Sarawak and Peninsular Malaysia since 1997. Fatal cases caused by EV71 were reported in Selangor during the outbreaks. Although fatalities reported in Selangor, the data of HFMD in this area was limited. The purposes of this study were; to detect and identify the HFMD viruses from the patients below 12 years old who presenting clinical case definitions of HFMD in Seri Kembangan, Selangor; and to describe the demographic data and clinical presentations of the patients with the viral agents.

A total of 28 specimens were collected from patients who presented with clinical case definitions of HFMD. The specimen collection period was from December 2012 until July 2013. The HFMD viruses were directly detected from specimens by using seminested reverse transcription-polymerase chain reaction (snRT-PCR). The positive snRT-PCR products were sequenced and phylogenetic trees were constructed. Virus isolation were performed on positive snRT-PCR specimens and reconfirmed by snRT-PCR. Among 28 specimens, 12 were positive in snRT-PCR. Based on sequencing results, seven of them were CVA6, two CVA16, and three EV71. According to phylogenetic analysis, EV71 strains were classified as sub-genotypes B5; CVA16 strains were identified sub-genotypes B2b and B2c; CVA6 strains were closely related to strains in Taiwan and Japan. In virus isolation, only one of 12 positive snRT-PCR specimens was successfully isolated from the cell culture, which was CVA16. The relationship between demographic data and clinical presentations with viral agents were studied. In conclusion, HFMD in Seri Kembangan, Selangor were caused by different genotypes of viruses, which were CVA6, CVA16 and EV71.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGESANAN VIRUS DARIPADA PESAKIT PENYAKIT TANGAN, KAKI, DAN MULUT SECARA MOLEKUL DALAM SERI KEMBANGAN, SELANGOR, MALAYSIA

By

BEH POAY LING

Julai 2014

Pngerusi: Profesor Zamberi Sekawi, MD, Mpath

Fakulti: Perubatan dan Sains Kesihatan

Penyakit tangan, kaki, dan mulut (HFMD), adalah satu jangkitan virus akut yang biasa dihadapi di kalangan bayi dan kanak-kanak. Agen penyebab utama HFMD ialah enterovirus 71 (EV71) dan coxsackievirus A16 (CVA16). Sejak kebelakangan ini, kes HFMD yang disebabkan oleh coxsackievirus A6 (CVA6) telah dilaporkan di negara jiran. Tanda-tanda dan gejala penyakit ini adalah deman, ulser mulut/tekak, ruam dan vesikel di tapak tangan dan kaki. Pesakit yang dijangkiti EV71 mungkin akan menghadapi komplikasi yang serius dan mengakibatkan maut, seperti meningitis, ensefalitis, dan kelumpuhan layuh akut. Wabak EV71 yang mengakibatkan maut telah dilaporkan di Sarawak dan Semenanjung Malaysia sejak tahun 1997. Kes maut yang disebabkan oleh EV71 juga dilaporkan di Selangor ketika wabak tersebut berlaku. Walaupun kes maut dilaporkan di Selangor, data mengenai HFMD di kawasan ini masih terhad. Tujuan kajian ini adalah; untuk mengesan dan mengenalpasti virus HFMD daripada pesakit bawah 12 tahun yang menunjukkan definisi kes klinikal HFMD di Seri Kembangan, Selangor; dan menghuraikan data demografi dan presembahan klinikal daripada pesakit dengan ejen virus..

Sebanyak 28 spesimen telah dikumpulkan daripada pesakit yang menunjukkan definisi kes klinikal HFMD. Tempoh pengumpulan spesimen bermula pada Desember 2012 sehingga Julai 2013. Virus HFMD dikesan daripada spesimen dengan menggunakan *seminested reverse transcription-polymerase chain reaction* (snRT-PCR). Produk yang snRT-PCR positif telah dijujuk dan pokok filogenetik dibina. Pengasingan virus telah dijalankan dengan menggunakan spesimen positif dalam snRT-PCR dan dikenalpastikan dengan menggunakan snRT-PCR. Antara 28 spesimen, terdapat 12 positif dalam snRT-PCR. Berdasarkan keputusan penjujukan, tujuh daripada mereka adalah CVA6, dua CVA16 dan tiga EV71. Merujuk kepada analisi pilogenetik, strain EV71 telah diklasifikasikan sebagai sub-genotip B5; strain CVA16 adalah dikenalpastikan sebagai sub-genotip B2b dan B2c; strain CVA6 berkait rapat dengan strain dari Taiwan dan Japan. Dalam pengasingan virus, hanya satu daripada 12 snRT-PCR positif spesimen telah berjaya diasingkan dari kultur sel, iaitu CVA16. Hubungan antara data demografi dan persembahan dengan ejen virus

telah dikaji. Dalam kesimpulannya, HFMD dalam Seri Kembangan, Selangor adalah disebabkan oleh genotip virus yang berbeza, seperti CVA6, CVA16 dan EV71.

ACKNOWLEDGEMENTS

First of all, I am heartily thankful to my supervisor, Professor Dr. Zamberi Sekawi for his guidance, support and advice for my study. I appreciate his encouragement, efforts, scientific knowledge and understanding. With his excellent guidance and supervision, I am able to carry out the study smoothly. Again, million thanks to Professor Dr. Zamberi Sekawi.

Secondly, I wish to show my gratitude to my co-supervisors, Dr. Putri Yubbu and Dr. Farid Azizi Jalilian. My study would not be complete without their crucial contribution, guidance and advice. I really appreciate their patience and encouragement to guide me throughout the study.

Besides that, I would like to thank the technical staff from Department of Medical Microbiology and Parasitology for their guidance, patience and assistance. Thank you for those who willing to lend a hand to me when I carried out my work.

Nevertheless, I wish to express my acknowledgement to Klinik Y.F. Chew, Klinik Pakar Kanak-Kanak Dr. Chia, Klinik Hope, Poliklinik Penawar, Poliklinik Taman Impiyan and CT Women and Child Specialist Clinic for their participation in this study.

Lastly, I owe my deepest gratitude to my family and friends for their understanding and support during the completion of my study. Without their encouragement and support, this study would not have been completed.

This study was funded by Research Universiti Grant Scheme (RUGS), sponsored by Universiti Putra Malaysia (04-02-12-1800RU).

I certify that a Thesis Examination Commitee has met on 18 July 2014 to conduct the final examination of Beh Poay Ling on her thesis entitled "Molecular Detection of Viruses from Hand, Foot, and Mouth Disease Patients in Seri Kembangan, Selangor, Malaysia" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

Vasantha Kumari Neela, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Rukman Awang Hamat, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Syafinaz Amin Nordin, PhD
Senior Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Jamal I-Ching Sam, PhD
Associate Professor
Faculty of Medicine
University of Malaya
Malaysia
(External Examiner)

_____
NORITAH OMAR, PhD
Associate Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 18 August 2014

The thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Zamberi Sekawi, MD, MPath
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Putri Yubbu, MBBS, MPaeds
Medical Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Farid Azizi Jalilian, PhD
Assistant Professor
Faculty of Medicine
Ilam University of Medical Sciences
(Member)

_____

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

DECLARATION

Declaration by Graduate Student

I hereby confirm that:

- this thesis is my original work;
- quotation, illustrations and citations have been duly referenced;
- this thesis has not been submitted previously or concurrently for any other degree at any other institutions;
- intellectual property from the thesis and copyright of thesis are fully-owned by Universiti Putra Malaysia (Research) Rules 2012;
- written permission must be obtained from supervisor and the office of Deputy Vice-Chancellor (Research and Innovation) before thesis is published (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writings, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- there is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software.

Signature: _____ Date: _____

Name and Matric No.: Beh Poay Ling, GS 30370

Declaration by Menbers of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to

Signature: _____
Name of Chairman of Supervisory Committee: _____

Signature: _____
Name of Member of Supervisory Committee: _____

Signature: _____
Name of Member of Supervisory Committee: _____

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BGMK	Buffalo green monkey kidney
bp	Base pair
CNS	Central nervous system
CPE	Cytopathic effects
CVA16	Coxsackievirus A16
CVA6	Coxsackievirus A6
ELISA	Enzyme-linked immunosorbent assay
EV71	Enterovirus 71
EV76	Enterovirus 76
EV89	Enterovirus 89
EV92	Enterovirus 92
FBS	Fetal bovine serum
HeLa	Cervical adenocarcinoma
HEV	Human enterovirus
HEV-A	Human enterovirus A
HEV-B	Human enterovirus B
HEV-C	Human enterovirus C
HEV-D	Human enterovirus D
HFMD	Hand, foot, and mouth disease
IgM	Immunoglobulin M
IRES	Internal ribosome entry site
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RD	Human rhabdomyosarcoma

RNase	Ribonuclease
RT-PCR	Reverse transcription-polymerase chain reaction
siRNA	Small-interfering RNA
snRT-PCR	Seminested reverse transcription-polymerase chain reaction
TAE	Tris-acetate-EDTA
UK	United Kingdom
USA	United State of America
UTRs	Untranslated regions
VP	Viral protein
VPg	Viral protein genome-linked
VTM	Viral transport medium

CHAPTER 1

INTRODUCTION

Hand, foot, and mouth disease (HFMD) is a common viral infection that affects infants and children. Most of the HFMD is a self-limiting infection. The major causative agents of HFMD are coxsackievirus A16 (CVA16) and enterovirus 71 (EV71) from genus enterovirus in family *Picornaviridae* (Chen *et al*., 2007). Coxsackievirus A4 to A10, A24, coxsackievirus B2 to B5 and echovirus can cause HFMD as well (Mandel *et al*., 2010).

The HFMD viruses are transmitted through fecal-oral and respiratory transmission. The clinical case definitions of the disease are fever, throat or mouth ulcers, rashes and vesicles on palms and soles (Podin *et al*., 2006). Majority of the infected patients recovered in seven to ten days without any medical treatment (Wong *et al*., 2012). However, EV71 causes severe infections, such as encephalitis, myocarditis, herpangina, aseptic meningitis, pulmonary oedema or haemorrhage, and acute flaccid paralysis (Li *et al*., 2011).

In 1969, the first HFMD caused by EV71 was reported in California (Schmidt *et al*., 1974), and the epidemics continued to be seen in Asia and European countries. First HFMD outbreak caused by EV71 was reported in April 1997 in Sarawak, Malaysia (Chan *et al*., 2000) and 31 fatalities were reported in the same year (AbuBakar *et al*., 1999). In June 1997, HFMD was reported in Peninsular Malaysia and 11 deaths were encountered (Shekhar *et al*., 2005). The outbreaks of HFMD were reported continuously in Sarawak in 2003, 2006, and 2008/2009 (Ooi *et al*., 2009); in Peninsular Malaysia in 2000 and 2005 (Chua *et al*., 2007).

HFMD epidemics have become significant public concern due to fatalities caused by EV71 among the infants and young children and reoccurrence of the outbreaks in Malaysia. Death cases were reported in Selangor in 1997 due to HFMD caused by EV71 (Shekhar *et al*., 2005). The data and viral agents caused HFMD in Selangor were limited as well. Besides that, each HFMD outbreaks can be caused by different genotype of viruses such as EV71 and CVA16. Therefore, the purpose of this study was to detect, identify and classify the HFMD viruses from the patients who below 12 years old presenting clinical case definitions of HFMD in Seri Kembangan, Selangor.

General objective:

To detect and identify viruses from patients presenting with clinical case definitions of HFMD in Seri Kembangan, Selangor and to describe the demographic data and clinical presentations of the patients.

Specific objectives:

1. To detect and identify the HFMD viruses (EV71, CVA16 and CVA6) by using seminested reverse transcription-polymerase chain reaction (snRT-PCR).

2. To classify the HFMD viruses (EV71, CVA16 and CVA6) by using phylogenetic analysis.

3. To describe the demographic data and clinical presentations of the patients with positive snRT-PCR specimens.

REFERENCES

AbuBakar, S., Chee, H.Y., Al-Kobaisi, M.F., Jiang, X., Chua, K.B. and Lam, S.K. (1999). Identification of enterovirus 71 isolates from an outbreak of hand, foot and mouth disease (HFMD) with fatal cases of encephalomyelitis in Malaysia. *Virus Research* 61(1):1-9.

Ang, L.W., Koh, B.K., Chan, K.P., Chua, L.T., James, L. and Goh, K.T. (2009). Epidemiology and control of hand, foot, and mouth disease in Singapore, 2001-2007. *Annals Academic of Medicine Singapore* 38:106-112.

Bible, J.M., Iturriza-Gomara, M., Megson, B., Brown, D., Pantelidis, P., Earl, P., Bendig, J. and Tong, C.W. (2008). Molecular epidemiology of human enterovirus 71 in the United Kingdom from 1998 to 2006. *Journal of Clinical Microbiology* 46(10):3192-3200.

Bruu, A.L. (2003). Enteroviruses: polioviruses, coxsackieviruses, echoviruses and newer enteroviruses. In: "A Practical Guide to Clinical Virology Second Edition", Eds Haaheim, L.R., Pattison, J.R. and Whitley, R.J. *John Wiley and Sons*, UK 44-45.

Cabrerizo, M., Tarrago, D., Munoz-Almagro, C., Del Amo, E., Dominguez-Gil, M., Eiros, J.M., Lopez-Miragaya, I., Perez, C., Reina, J., Otero, A., Gonzalez, I., Echevarria, J.E. and Trallero, G. (2013). Molecular epidemiology of enterovirus 71, coxsackievirus A16 and A6 associated with hand, foot, and mouth disease in Spain. *Clinical Microbiology and Infection* 24(10):1469-0691.

Chan, L.G, Parashar, U.D., Lye, M.S., Ong, F.G.L., Zaki, S.R., Alexander, J.P., Ho, K.K., Han, L.L., Pallansch, M.A., Suleiman, A.B., Jegathesan, M. and Anderson, L.J. (2000). Deaths of children during an outbreak of hand, foot, and mouth disease in Sarawak, Malaysia: clinical and pathological characteristics of the disease. *Clinical Infectious Diseases* 31(3):678-683.

Chan, Y., Wee, K., Chiam, C., Khor, C., Chan, S., Wan Nor Amalina, W. and Sam, I. (2012). Comparative genetic analysis of VP4, VP1 and 3D gene regions of enterovirus 71 and coxsackievirus A16 circulating in Malaysia between 1997-2008. *Tropical Biomedicine* 29(3):451-466.

Chan, Y.F., Sam, I. and AbuBakar, S. (2010). Phylogenetic designation of enterovirus 71 genotypes and subgenotypes using complete genome sequences. *Infection, Genetics and Evolution* 10(3):404-412.

Chan, Y.F., Sam, I., Wee, K.L. and Abubakar, S. (2011). Enterovirus 71 in Malaysia: a decade later. *Neurology Asia* 16(1):1-15.

Chang, L.Y., King, C.C., Hsu, K.H., Ning, H.C., Tsao, K.C., Li, C.C., Huang, Y.C., Shih, S.R., Chiou, S.T., Chen, P.Y., Chang, H.J. and Lin, T.Y. (2002). Risk factors of enterovirus 71 infection and associated hand, foot, and mouth disease/herpangina in children during an epidemic in Taiwan. *Pediatrics* 109(6):e88.

Chang, L.Y., Hsia, S.H., Wu, C.T., Huang, Y.C., Lin, K.L., Fang, T.Y. and Lin, T.Y. (2004). Outcome of enterovirus 71 infections with or without stage-based management: 1998 to 2002. *Pediatric Infectious Disease Journal* 23(4):327-332.

Chang, L.Y., Lin, T.Y., Huang, Y.C., Tsao, K.C., Shih, S.R., Kuo, M.L., Ning, H.C., Chung, P.W. and Kang, C.M. (1999). Comparison of enterovirus 71 and coxsackievirus A16 clinical illnesses during the Taiwan enterovirus epidemic, 1998. *Pediatric Infectious Disease Journal* 18(12):1092-1096.

Chen, H.F., Chang, M.H., Chiang, B.L. and Jeng, S.T. (2006a). Oral immunization of mice using transgenic tomato fruit expressing VP1 protein from enterovirus 71. *Vaccine* 24(15):2944-2951.

Chen, K.T., Chang, H.L., Wang, S.T., Cheng, Y.T. and Yang, J.Y. (2007). Epidemiologic features of hand-foot-mouth disease and herpangina caused by enterovirus 71 in Taiwan, 1998–2005. *Pediatrics* 120(2): e244-e252.

Chen, T.C., Chen, G.W., Hsiung, C.A., Yang, J.Y., Shih, S.R., Lai, Y.K. and Juang, J.L. (2006b). Combining multiplex reverse transcription-PCR and a diagnostic microarray to detect and differentiate enterovirus 71 and coxsackievirus A16. *Journal of Clinical Microbiology* 44(6):2212-2219.

Chen, Y.J., Chang, S.C., Tsao, K.C., Shih, S.R., Yang, S.L., Lin, T.Y. and Huang, Y. C. (2012). Comparative genomic analysis of coxsackievirus A6 strains of different clinical disease entities. *PloS one* 7(12): e52432.

Chiu, C.H., Chu, C., He, C.C. and Lin, T.Y. (2006). Protection of neonatal mice from lethal enterovirus 71 infection by maternal immunization with attenuated *Salmonella enterica* serovar Typhimurium expressing VP1 of enterovirus 71. *Microbes and Infection* 8(7):1671-1678.

Chua, K., Chua, B., Lee, C., Ismail, N., Kiyu, A. and Kumarasamy, V. (2007). Genetic diversity of enterovirus 71 isolated from cases of hand, foot, and mouth disease in the 1997, 2000 and 2005 outbreaks, Peninsular Malaysia. *Malaysian Journal of Pathology* 29(2):69-78.

Chumakov, M., Voroshilova, M., Shindarov, L., Lavrova, I., Gracheva, L., Koroleva, G., Vasilenko, S., Brodvarova, I., Nikolova, M., Gyurova, S., Gacheva, M., Mitov, G., Ninov, N., Tsylka, E., Robinson, I., Frolova, M., Bashkirtsev, V., Martiyanova, L. and Rodin, V. (1979). Enterovirus 71 isolated from cases of epidemic poliomyelitis-like disease in Bulgaria. *Archives of Virology* 60(3-4):329-340.

Clementz, G.C. and Mancini, A.J. (2000). Nail matrix arrest following hand-foot-mouth disease: a report of five children. *Pediatric Dermatology* 17(1):7-11.

Daniel, W.W. 1999. Biostatistics: A foundation for analysis in the health sciences. 7th edition. *John Wiley & Sons*, New York.

Foo, D.G.W., Alonso, S., Chow, V.T.K. and Poh, C.L. (2007). Passive protection against lethal enterovirus 71 infection in newborn mice by neutralizing antibodies elicited by a synthetic peptide. *Microbes and Infection* 9(11):1299-1306.

Fujimoto, T., Iizuka, S., Enomoto, M., Abe, K., Yamashita, K., Hanaoka, N., Okabe, N., Yoshida, H., Yasui, Y., Kobayashi, M., Fujii, Y., Tanaka, H., Yamamoto, M. and Shimizu, H. (2012). Hand, foot, and mouth disease caused by coxsackievirus A6, Japan, 2011. *Emerging Infectious Diseases* 18(2):337.

Gau, S.F., Chang, L.Y., Huang, L.M., Fan, T.Y., Wu, Y.Y. and Lin, T.Y. (2008). Attention-deficit/hyperactivity–related symptoms among children with enterovirus 71 infection of the central nervous system. *Pediatrics* 122(2):e452-e458.

Goksugur, N. and Goksugur, S. (2010). Hand, foot, and mouth disease. *New England Journal of Medicine* 362(14):e49.

Gopalkrishna, V., Patil, P.R., Patil, G.P. and Chitambar, S.D. (2012). Circulation of multiple enterovirus serotypes causing hand, foot and mouth disease in India. *Journal of Medical Microbiology* 61(3):420-425.

Hagiwara, A., Tagaya, I. and Yoneyama, T. (1978). Epidemic of hand, foot and mouth disease associated with enterovirus 71 infection. *Intervirology* 9(1):60-63.

He, Y.Q., Chen, L., Xu, W.B., Yang, H., Wang, H.Z., Zong, W.P., Xian, H.X., Chen, H.L., Yao, X.J., Hu, Z.L., Luo, M., Zhang, H.L., Ma, H.W., Cheng, J.Q., Feng, Q.J. and Zhao, D.J. (2013). Emergence, circulation, and spatiotemporal phylogenetic analysis of coxsackievirus a6-and coxsackievirus a10-associated hand, foot, and mouth disease infections from 2008 to 2012 in Shenzhen, China. *Journal of Clinical Microbiology* 51(11):3560-3566.

Hosoya, M., Kawasaki, Y., Sato, M., Honzumi, K., Hayashi, A., Hiroshima, T., Ishiko, H., Kato, K. and Suzuki, H. (2007). Genetic diversity of coxsackievirus A16 associated with hand, foot, and mouth disease epidemics in Japan from 1983 to 2003. *Journal of Clinical Microbiology* 45(1):112-120.

Huang, C.C., Liu, C.C., Chang, Y.C., Chen, C.Y., Wang, S.T. and Yeh, T.F. (1999). Neurologic complications in children with enterovirus 71 infection. *New England Journal of Medicine* 341(13):936-942.

Ishiko, H., Shimada, Y., Yonaha, M., Hashimoto, O., Hayashi, A., Sakae, K. and Takeda, N. (2002). Molecular diagnosis of human enteroviruses by phylogeny-based classification by use of the VP4 sequence. *Journal of Infectious Diseases* 185(6):744-754.

Jee, Y., Cheon, D.S., Kim, K., Cho, J., Chung, Y.S., Lee, J., Lee, S.H., Parl, K.S., Lee, J.H., Kim, E.C., Chung, H.J., Kim, D.S., Yoon, J.D. and Cho, H.W. (2003). Genetic analysis of the VP1 region of human enterovirus 71 strains isolated in Korea during 2000. *Archives of Virology* 148(9):1735-1746.

Kehle, J., Roth, B., Metzger, C., Pfitzner, A. and Enders, G. (2003). Molecular characterization of an enterovirus 71 causing neurological disease in Germany. *Journal of Neurovirology* 9(1):126-128.

Li, L., He, Y., Yang, H., Zhu, J., Xu, X., Dong, J., Zhu, Y. and Jin, Q. (2005). Genetic characteristics of human enterovirus 71 and coxsackievirus A16 circulating from 1999 to 2004 in Shenzhen, People's Republic of China. *Journal of Clinical Microbiology* 43(8):3835-3839.

Li, Y., Zhu, R., Qian, Y., Deng, J., Sun, Y., Liu, L., Wang, F. and Zhao, L. (2011). Comparing enterovirus 71 with coxsackievirus a16 by analyzing nucleotide sequences and antigenicity of recombinant proteins of VP1s and VP4s. *BMC Microbiology* 11(1):246.

Li, Z.H., Li, C.M., Ling, P., Shen, F.H., Chen, S.H., Liu, C.C., Yu, C.K. and Chen, S.H. (2008). Ribavirin reduces mortality in enterovirus 71–infected mice by decreasing viral replication. *Journal of Infectious Diseases* 197(6):854-857.

Liu, F., Liu, Q., Cai, Y., Leng, Q. and Huang, Z. (2011). Construction and characterization of an infectious clone of coxsackievirus A16. *Virology Journal* 8(1):1-8.

Lu, Q.B., Zhang, X.A., Wo, Y., Xu, H.M., Li, X.J., Wang, X.J., Ding, S.J., Chen, X.D., He, C., Liu, L.J., Li, H., Yang, H., Li, T.Y., Liu, W. and Cao, W.C. (2012). Circulation of coxsackievirus A10 and A6 in hand-foot-mouth disease in China, 2009–2011. *PLoS ONE* 7(12):e52073.

Lum, L., Wong, K., Lam, S., Chua, K., Goh, A., Lim, W., Ong, B., Paul, G., AbuBakar, S. and Lambert, M. (1998). Fatal enterovirus 71 encephalomyelitis. *Journal of Pediatrics* 133(6):795-798.

Mandell, G.L., Bennett, J.E. and Dolin, R. (2010). In: "Mandell, Douglas, and Bennett's principles and practice of infectious diseases", *Churchill Livingstone/Elsevier*, Philadelphia, PA 172.

McMinn, P., Lindsay, K., Perera, D., Chan, H. M., Chan, K. P., and Cardosa, M. J. (2001a). Phylogenetic analysis of enterovirus 71 strains isolated during linked epidemics in Malaysia, Singapore, and Western Australia. *Journal of Virology* 75(16):7732-7738.

McMinn, P., Stratov, I., Nagarajan, L. and Davis, S. (2001b). Neurological manifestations of enterovirus 71 infection in children during an outbreak of hand, foot, and mouth disease in Western Australia. *Clinical Infectious Diseases* 32(2):236-242.

Melnick, J.L., Proctor, R.O., Ocampo, A.R., Diwan, A.R. and Ben-Porath, E. (1966). Free and bound virus in serum after administration of oral poliovirus vaccine. *American Journal of Epidemiology* 84(2):329-342.

Mirand, A., Schuffenecker, I., Henquell, C., Billaud, G., Jugie, G., Falcon, D., Mahul, A., Archmbaud, C., Terletskaia-ladwig, E., Diedrich, S., Huemer, H.P., Enders, M., Lina, B., Peigue-Lafeuille, H. and Bailly, J.L. (2010). Phylogenetic evidence for a recent spread of two populations of human enterovirus 71 in European countries. *Journal of General Virology* 91(9):2263-2277.

Nagy, G., Takátsy, S., Kukán, E., Mihály, I. and Dömök, I. (1982). Virological diagnosis of enterovirus type 71 infections: experiences gained during an epidemic of acute CNS diseases in Hungary in 1978. *Archives of Virology* 71(3):217-227.

Nishikii, Y., Nakatomi, A., Doi, T., Oka, S. and Moriuchi, H. (2002). Favorable outcome in a case of perinatal enterovirus 71 infection. *Pediatric Infectious Disease Journal* 21(9):886-887.

Nix, W.A., Oberste, M.S. and Pallansch, M.A. (2006). Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. *Journal of Clinical Microbiology* 44(8):2698-2704.

Oberste, M.S., Maher, K. and Pallansch, M.A. (2004). Evidence for frequent recombination within species human enterovirus B based on complete genomic sequences of all thirty-seven serotypes. *Journal of Virology* 78(2):855-867.

Oberste, M.S., Maher, K., Kilpatrick, D.R., Flemister, M.R., Brown, B.A. and Pallansch, M.A. (1999). Typing of human enteroviruses by partial sequencing of VP1. *Journal of Clinical Microbiology* 37(5):1288-1293.

Oberste, M.S., Maher, K., Michele, S.M., Belliot, G., Uddin, M. and Pallansch, M.A. (2005). Enteroviruses 76, 89, 90 and 91 represent a novel group within the species human enterovirus A. *Journal of General Virology* 86(2):445-451.

Ooi, M.H., Solomon, T., Podin, Y., Mohan, A., Akin, W., Yusuf, M.A., de Sel, S., Kontol, K.M., Lai, B.F., Clear, D., Chieng, C.H., Blake, E., Perera, D., Wong, S.C. and Cardosa, J. (2007). Evaluation of different clinical sample types in diagnosis of human enterovirus 71-associated hand-foot-and-mouth disease. *Journal of Clinical Microbiology* 45(6):1858-1866.

Ooi, M.H., Wong, S.C., Mohan, A., Podin, Y., Perera, D., Clear, D., del Sel, S., Chieng, C.H., Tio, P.H., Cardosa, M.J. and Solomon, T. (2009). Identification and validation of clinical predictors for the risk of neurological involvement in children with hand, foot, and mouth disease in Sarawak. *BMC Infectious Diseases* 9(1):3.

Österback, R., Vuorinen, T., Linna, M., Susi, P., Hyypiä, T. and Waris, M. (2009). Coxsackievirus A6 and hand, foot, and mouth disease, Finland. *Emerging Infectious Diseases* 15(9):1485.

Perera, D., Shimizu, H., Yoshida, H., Van Tu, P., Ishiko, H., McMinn, P.C. and Cardosa, M.J. (2010). A comparison of the VP1, VP2, and VP4 regions for molecular typing of human enteroviruses. *Journal of Medical Virology* 82(4):649-657.

Perera, D., Yusof, M., Podin, Y., Ooi, M., Thao, N., Wong, K., Zaki, A., Chua, K., Malik, Y., Tu, P., Tien, N., Puthavathana, P., McMinn, P. and Cardosa, M. (2007). Molecular phylogeny of modern coxsackievirus A16. *Archives of Virology* 152(6):1201-1208.

Podin, Y., Gias, E. L., Ong, F., Leong, Y.W., Yee, S.F., Yusof, M. A., Perera, D., Teo, B., Wee, T.Y., Yao, S. C., Yao, S.K., Kiyu, A., Arif, M.T. and Cardosa, M.J. (2006). Sentinel surveillance for human enterovirus 71 in Sarawak, Malaysia: lessons from the first 7 years. *BMC Public Health* 6(1):180.

Pöyry, T., Hyypiä, T., Horsnell, C., Kinnunen, L., Hovi, T. and Stanway, G. (1994). Molecular analysis of coxsackievirus A16 reveals a new genetic group of enteroviruses. *Virology* 202(2):982-987.

Puenpa, J., Theamboonlers, A., Korkong, S., Linsuwanon, P., Thongmee, C., Chatproedprai, S. and Poovorawan, Y. (2011). Molecular characterization and complete genome analysis of human enterovirus 71 and coxsackievirus A16 from children with hand, foot and mouth disease in Thailand during 2008-2011. *Archives of Virology* 156(11):2007-2013.

Romero, J.R. and Rotbart H.A. (1993). PCR detection of the human enterovirus. In: "Diagnostic molecular microbiology: principles and applications". Eds. Persing, D.H., Smith, T.F., Tenover, F.C. and White, T.J. *American Society for Microbiology*, Washington 401-406.

Rotbart, H.A. (2002). Treatment of picornavirus infections. *Antiviral Research* 53(2):83-98.

Rueckert, R.R. (1996). In: "Picornaviridae: the viruses and their replication", Eds. Fields, B.N., Knipe, D.M. and Howley, P.M. *Fields Virology*, Philadelphia 1:609-654.

Schmidt, N.J., Lennette, E.H. and Ho, H.H. (1974). An apparently new enterovirus isolated from patients with disease of the central nervous system. *Journal of Infectious Diseases* 129(3):304-309.

Shekhar, K., Lye, M.S., Norlijah, O., Ong, F., Looi, L.M., Khuzaiah, R., Marzuki, I., Hussien, I., Wong, S.L., Mohan, J., Sinniah, M. and AbuBakar, S. (2005). Deaths in children during an outbreak of hand, foot, and mouth disease in Peninsular Malaysia--clinical and pathological characteristics. *Medical Journal of Malaysia* 60(3):297-304.

Singh, S., Chow, V. T., Phoon, M. C., Chan, K. P. and Poh, C. L. (2002). Direct detection of enterovirus 71 (EV71) in clinical specimens from a hand, foot, and mouth disease outbreak in Singapore by reverse transcription-PCR with universal enterovirus and EV71-specific primers. *Journal of Clinical Microbiology* 40(8):2823-2827.

Solomon, T., Lewthwaite, P., Perera, D., Cardosa, M. J., McMinn, P., and Ooi, M. H. (2010). Virology, epidemiology, pathogenesis, and control of enterovirus 71. *Lancet Infectious Diseases* 10(11):778-790.

Stanway, G. (2005) Family Picornaviridae. In: "Virus Taxonomy Eighth Report of the International Committee on Taxonomy of Viruses", Eds. Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A. *Elsevier/Academic Press*, London 757–778.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28(10):2731-2739.

Tan, E.L., Chow, V.T.K., Kumarasinghe, G., Lin, R.T.P., MacKay, I.M., Sloots, T.P. and Poh, C.L. (2006). Specific detection of enterovirus 71 directly from clinical specimens using real-time RT-PCR hybridization probe assay. *Molecular and Cellular Probes* 20(2):135-140.

Tan, E.L., Tan, T.M.C., Chow, V.T.K. and Poh, C. L. (2007). Inhibition of enterovirus 71 in virus-infected mice by RNA interference. *Molecular Therapy* 15(11):1931-1938.

Tyler, K.L., McPhee, D.A. and Fields, B.N. (1986). Distinct pathways of viral spread in the host determined by reovirus S1 gene segment. *Science* 233(4765):770-774.

Van der Sanden, S., Koopmans, M., Uslu, G. and van der Avoort, H. (2009). Epidemiology of enterovirus 71 in the Netherlands, 1963 to 2008. *Journal of Clinical Microbiology* 47(9):2826-2833.

Van Loon, A. M., Cleator, G. C. and Ras, A. (1999). External quality assessment of enterovirus detection and typing. European Union Concerted Action on virus meningitis and encephalitis. *Bulletin of the World Health Organization* 77(3):217.

Van Tu, P., Thao, N. T.T., Perera, D., Truong, K.H., Tien, N.T.K., Thuong, T.C., Ooi, M.H., Cardosa, M.J. and McMinn, P. C. (2007). Epidemiologic and virologic investigation of hand, foot, and mouth disease, southern Vietnam, 2005. *Emerging Infectious Diseases* 13(11):1733.

Wang, S.M., Ho, T.S., Shen, C.F. and Liu, C.C. (2008). Enterovirus 71, one virus and many stories. *Pediatrics and Neonatology* 49(4):113-115.

Wang, X., Zhu, C., Bao, W., Zhao, K., Niu, J., Yu, X.F. and Zhang, W. (2012). Characterization of full-length enterovirus 71 strains from severe and mild disease patients in northeastern China. *PLoS ONE* 7(3):e32405.

Wei, S.H., Huang, Y.P., Liu, M.C., Tsou, T.P., Lin, H.C., Lin, T.L., Tsai, C.Y., Chao, Y.N., Chang, L.Y. and Hsu, C.M. (2011). An outbreak of coxsackievirus A6 hand, foot, and mouth disease associated with onychomadesis in Taiwan, 2010. *BMC Infectious Diseases* 11(1):346.

Welch, J., Maclaran, K., Jordan, T. and Simmonds, P. (2003). Frequency, viral loads, and serotype identification of enterovirus infections in Scottish blood donors. *Transfusion* 43(8):1060-1066.

Weng, T.Y., Chen, L.C., Shyu, H.W., Chen, S.H., Wang, J.R., Yu, C.K., Lei, H.Y. and Yeh, T.M. (2005). Lactoferrin inhibits enterovirus 71 infection by binding to VP1 protein and host cells. *Antiviral Research* 67(1):31-37.

Whitton, J.L., Cornell, C.T. and Feuer, R. (2005). Host and virus determinants of picornavirus pathogenesis and tropism. *Nature Reviews Microbiology* 3(10):765-776.

WHO. (2011). A guide to clinical management and public health response for hand, foot, and mouth disease (HFMD). *Geneva: WHO*.

Witsø, E., Palacios, G., Rønningen, K. S., Cinek, O., Janowitz, D., Rewers, M., Grinde, Bjørn. and Lipkin, W.I. (2007). Asymptomatic circulation of HEV71 in Norway. *Virus Research* 123(1):19-29.

Wong, K.K., Malik, Y.A. and Rahman, M. (2012). Molecular detection of human enterovirus 71 causing hand, foot, and mouth disease in Klang Valley, Malaysia. *Current Research Journal of Biological Sciences* 4(3): 323-328.

Wu, C.N., Lin, Y.C., Fann, C., Liao, N.S., Shih, S.R. and Ho, M.S. (2001). Protection against lethal enterovirus 71 infection in newborn mice by passive immunization with subunit VP1 vaccines and inactivated virus. *Vaccine* 20(5):895-904.

Wu, T.C., Wang, Y.F., Lee, Y.P., Wang, J.R., Liu, C.C., Wang, S.M., Lei, H.Y., Su, I.J. and Yu, C.K. (2007). Immunity to avirulent enterovirus 71 and coxsackie A16 virus protects against enterovirus 71 infection in mice. *Journal of Virology* 81(19):10310-10315.

Wu, T.N., Tsai, S.F., Li, S.F., Lee, T.F., Huang, T.M., Wang, M.L., Hsu, K.H. and Shen, C.Y. (1999). Sentinel surveillance for enterovirus 71, Taiwan, 1998. *Emerging Infectious Diseases* 5(3):458-460.

Wu, Y., Yeo, A., Phoon, M., Tan, E., Poh, C., Quak, S. and Chow, V.T. (2010). The largest outbreak of hand; foot and mouth disease in Singapore in 2008: the role of enterovirus 71 and coxsackievirus A strains. *International Journal of Infectious Diseases* 14(12):e1076-e1081.

Xu, F., Yan, Q., Wang, H., Niu, J., Li, L., Zhu, F., He, S., Zhang, S., Weng, Z., Cheng, T., Cai, Y., He, D., Chen, Y., Ge, S., Yeo, A.E.T., Zhang, J., Ng, M.H. and Xia, N. (2010). Performance of detecting IgM antibodies against enterovirus 71 for early diagnosis. *PLoS ONE* 5(6):e11388.

Yu, H., Chen, W., Chang, H., Tang, R., Zhao, J., Gan, L., Liu, B., Chen, J. and Wang, M. (2010). Genetic analysis of the VP1 region of enterovirus 71 reveals the emergence of genotype A in central China in 2008. *Virus Genes* 41(1):1-4.

Zhang, J., Sun, J., Chang, Z., Zhang, W., Wang, Z. and Feng, Z. (2011). Characterization of hand, foot, and mouth disease in China between 2008 and 2009. *Biomedical and Environmental Sciences* 24(3):214-221.

Zhang, X., Wang, H., Ding, S., Wang, X., Chen, X., Wo, Y., Wang, L., Huang, D., Liu, W. and Cao, W. (2013). Prevalence of enteroviruses in children with and without hand, foot, and mouth disease in China. *BMC Infectious Diseases* 13: 606.

Zheng, Z.M., He, P.J., Caueffield, D., Neumann, M., Specter, S., Baker, C.C. and Bankowski, M.J. (1995). Enterovirus 71 isolated from China is serologically similar to the prototype E71 BrCr strain but differs in the 5′-noncoding region. *Journal of Medical Virology* 47(2):161-167.

Zong, W., He, Y., Yu, S., Yang, H., Xian, H., Liao, Y. and Hu, G. (2011). Molecular phylogeny of coxsackievirus A16 in Shenzhen, China, from 2005 to 2009. *Journal of Clinical Microbiology* 49(4):1659-1661.