THE EXPRESSION OF RECOMBINANT BACILLE CALMETTE-GUÉRIN (rBCG) CONTAINING VP1 GENE OF ENTEROVIRUS 71 (EV71)

BY

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LIST OF SYMBOLS AND ABBREVATIONS

AFP	: Acute flaccid paralysis
BCG	: Bacille Calmette-Guérin
CFU	: Colony forming unit
CNS	: Central nervous system
DNA	: Deoxyribonucleic acid
ELISA	: Enzyme-linked immunosorbent assay
EV71	: Enterovirus 71
GBS	: Guillain-Barré syndrome
HEPES	: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFMD	: Hand-Foot-And-Mouth disease
HRP	: Horseradish peroxidise
IQ	: Intelligence quotient
MAPc	: Mycolic acid-arabinogalactan-peptidoglycan complex
MHC	: Major histocompatibility complex
MycoORI	: Mycobacterial origin of replication
NCP	: Nitrocellulose paper
OADC	: Oleic Acid Albumin Dextrose Complex
PBS	: Phosphate-buffered Saline
PCR	: Polymerase chain reaction
rBCG	: Recombinant BCG
RNA	: Ribonucleic acid
RT-PCR	: Reverse transcription-polymerase chain reaction
SDS	: Sodium dodecyl sulphate

SDS-PAGE	: Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
TEMED	: N,N,N',N'-Tetramethylethylenediamine
UbGR	: Ubiquitin
UTR	: Untranslated region

ABSTRACT

Enterovirus 71 (EV71) is a positive-sense single-stranded RNA, nonenveloped, icosahedrally symmetrical virus which was first detected in 1969 in United States. EV71 is associated with Hand-Foot-And-Mouth disease (HFMD) and some of EV71 infections may proceed to neurological diseases and even death because of neurogenic pulmonary edema. To date, there is still no vaccine available against this disease. *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) has been suggested to be an attractive vehicle for the delivery of foreign antigens to the immune system. The main purpose of this study is to express a recombinant BCG (rBCG) containing ubiquitin (UbGR)-VP1 fusion gene of EV71. UbGR-VP1 protein was detected in the cell extract but not in the supernatant of the recombinant clone by Western blot analysis using rabbit polyclonal antibody specific to VP1 protein. The immunogenicity of the rBCG is still unknown and can only be confirmed if further study is done.

ABSTRAK

Enterovirus 71 (EV71) ialah virus bebenang RNA tunggal positif, tanpa envelop, simetri ikosahedral yang pertama kali dikesan pada tahun 1969 di Amerika Syarikat. EV71 dikaitkan dengan penyakit tangan-kaki-dan-mulut (HFMD) dan sesetengah jangkitan EV71 akan membawa kepada penyakit saraf dan kematian kerana edema pulmonari saraf. Sehinggga kini, masih belum ada vaksin yang boleh menentang penyakit ini. *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) telah dicadang sebagai satu kenderaan untuk menghantar antigen asing kepada sistem immun. Matlamat utama penyelidikan ini adalah untuk mengekspres Bacille Calmette-Guérin rekombinan (rBCG) yang mengandungi cantuman gen ubiquitin (UbGR)-VP1 EV71. UbGR-VP1 protein telah dikesan di dalam ekstrak sel dan tidak di dalam supernatan klon recombinan melalui pemblotan Western dengan menggunakan antibodi poliklon arnab yang spesifik terhadap protein VP1. Immunogenisiti terhadap rBCG tersebut masih belum dapat diketahui dan hanya boleh dipasti jika penyelidikan yang seterusnya dilakukan.

CHAPTER 1

INTRODUCTION

1.1 Enterovirus 71 (EV71)

1.1.1 Structure

Structurally, EV71 is a small, nonenveloped, icosahedrally symmetrical spherical particle approximately 30 nm in diameter. It has a positive-sense single-stranded ribonucleic acid (RNA). The protein capsid has 60 copies of each structural proteins VP1, VP2, VP3, and VP4 (Brown and Pallansch, 1995 ; Munemura *et al.*, 2003 ; Witsø *et al.*, 2006).

EV71 contains a linear approximately 7.4 kilobase RNA genome comprising a 5' and a 3' untranslated regions (UTR) and a single, long open reading frame (Witsø *et al.*, 2006). The single open reading frame contains P1, P2 and P3 regions which encodes a polypeptide of 2194 amino acids. A variable length poly-A tract is located at the terminus of the 3'UTR. The P1 region encodes 4 viral structural proteins named VP1, VP2, VP3, and VP4. The P2 region encodes non-structural proteins known as 2A, 2B, and 2C while P3 region encodes other non-structural proteins known as 3A, 3B, 3C, and 3D (Brown and Pallansch, 1995 ; Chung *et al.*, 2008). Diagrammatic illustration of EV71 genome is visualized in Figure 1.1.

VP1 encoded in P1 region is the major surface-accessible protein in the mature picornavirus virions. It is arrayed around the fivefold axis of symmetry of the icosahedral virion. VP2 and VP3 comprise the remainder of the virion surface. Each of the capsid proteins contains different amino acid sequences and is composed of

conserved elements that form the β-barrel structural elements of the capsid (Oberste *et al.*, 1999b). There is a canyon on the surface surrounding each of the axes, with antigenic sites on the rim (Webster, 2005). The VP4 protein lies buried in close association with the genomic RNA interacts with the N termini of VP1 (Munemura *et al.*, 2003). The capsid structure is illustrated in Figure 1.2.

P2 and P3 regions encode nonstructural proteins responsible for virus replication and virulence (Chung *et al.*, 2008). The functions of the non-structural proteins encoded in P2 and P3 are not completely known. 2A is a protease in many but evidently not all Picornaviruses. 3C is also a proteinase. 3D is an RNA polymerase. 2B is known to alter host cell membrane permeability, and 2C is important for the formation of viral replication vesicles. 3A plays a role in modulating the host immune response by inhibiting expression of host class I major histocompatibility complex (MHC) molecules (Hughes, 2004).

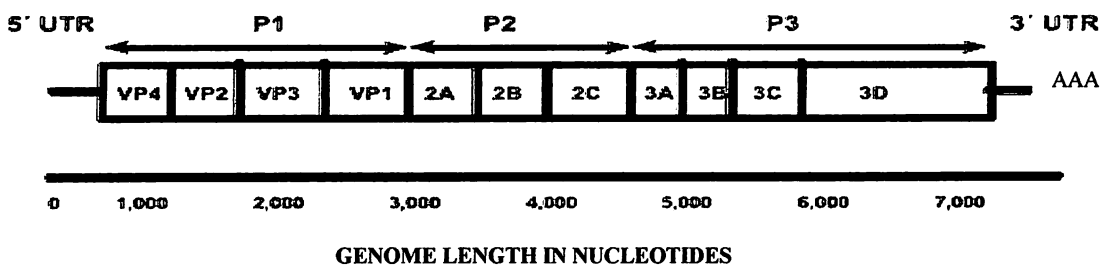

Figure 1.1: EV71 genome structure (adapted from Brown and Pallansch, 1995)

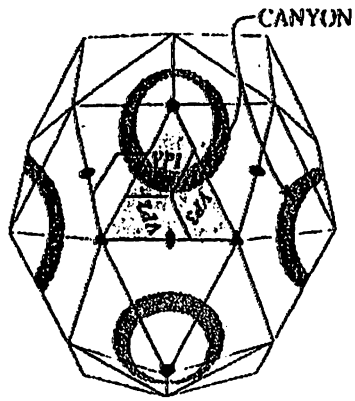

Figure 1.2: EV71 capsid structure (adapted from Webster, 2005)

1.1.2 Virological Classification

As EV71 is a positive-sense single-stranded RNA virus, it belongs to the genus Enterovirus of virus family Picornaviridae (Witsø *et al.*, 2006). Human enteroviruses had traditionally been classified into Echoviruses (type 1 to 7, 9, 11 to 27 and 29 to 34), Coxsackieviruses A (type 1 to 22), Coxsackieviruses B (type 1 to 6), and Polioviruses (type 1 to 3) (Ho, 2000) depending on the infectious properties and pathogenesis of the virus. It was quickly realized that there were significant overlaps in the biological properties of viruses in the different groups (Simmonds and Welch, 2006).

Newly discovered enteroviruses received a numeric designation instead of being assigned to one of the traditional groups. Currently, human enteroviruses are classified into 5 species, Human Enterovirus A, Human Enterovirus B, Human Enterovirus C, Human Enterovirus D, and Poliovirus based on their molecular and biologic characteristics (Saeed *et al.*, 2007 ; Smura *et al.*, 2007).

Using molecular method and neutralization tests with antisera against enteroviruses, human enterovirus can be further classified in serotype level, Human Enterovirus A (CA2 to CA8, CA10, CA12, CA14, CA16, and EV71), Human Enterovirus B (CA9,

CB1 to CB6, E1 to 7, E9, E11 to E21, E24 to E27, E29 to E33, and EV69), Human Enterovirus C (CA1, CA11, CA13, CA15, CA17 to CA22, and CA24), Human Enterovirus D (EV68 and EV70) and Poliovirus (types 1 to 3). Several new serotypes (EV73 to EV78 and EV89 to EV91) were recently described. EV71 belongs to species Human Enterovirus A (Witsø *et al.*, 2006).

Using sequence analysis of the VP1 region and genotyping, EV71 is further classified into genotypes A, B, and C. Genotype B could be further classified into subtypes B1 to B5 and genotype C into subtypes C1 to C4 (McMinn, 2002).

1.1.3 History and Prevalence

EV71 was first described in 1969 after isolating from faeces of a nine-month-old infant suffering from encephalitis in California, United States (Schmidt *et al.*, 1974). The next year, the virus was isolated from the brain of a five-year-old child who died of encephalitis and also from the faeces of four other patients with meningitis or encephalitis. The virus was obtained from 18 additional patients with serious central nervous system (CNS) disease the next three years. EV71 was first isolated outside of California in 1972 in New York. Twenty eight infections were diagnosed by the New York State Department of Health Virus Laboratory. In 19 of these patients, the virus exhibited neurovirulence as manifested by the diagnosis of meningitis, encephalitis, or paralysis (Melnick, 1984). The first isolation of EV71 outside of the United States was made in Australia, during an epidemic of aseptic meningitis in Melbourne between 1972 and 1973 (Kennett *et al.*, 1974). The first linkage of EV71 with Hand-Foot-And-Mouth disease (HFMD) was made during small epidemics in both Sweden and Japan during 1973 (Blomberg *et al.*, 1974 ; Hagiawara *et al.*, 1978). Four epidemic outbreaks with

high mortality rates occurred in Bulgaria in 1975 with 68 deaths (Shindarov *et al.*, 1979), in Hungary in 1978 with 47 deaths (Nagy *et al.*, 1982), in Malaysia in 1997 with at least 31 deaths (Chan *et al.*, 2000), and in Taiwan in 1998 with 78 deaths (Ho *et al.*, 1999).

In Europe, the first reports of large and severe epidemics of encephalitis and acute flaccid paralysis (AFP) due to EV71 came from Bulgaria in 1975 (Chumakov *et al.*, 1979) and Hungary in 1978 (Nagy *et al.*, 1982). In Bulgarian epidemic 705 cases of febrile illness attributable to EV71 infection were recorded (Shindarov *et al.*, 1979). In Hungarian epidemic, a total of 826 cases of aseptic meningitis and 724 cases of encephalitis were reported, with the latter group including cases of cerebellar ataxia and AFP. EV71 infection was confirmed in 323 cases (Nagy *et al.*, 1982). In Asia Pacific region EV71-associated HFMD epidemic was first identified in Japan in 1973 (Hagiawara *et al.*, 1978). Second EV71 epidemic in Japan occurred in 1978 (Ishimaru *et al.*, 1980). A small EV71 outbreak was recorded in Hong Kong in 1985 where several cases of AFP happened (Samuda *et al.*, 1987). The first report of EV71 activity in China was during a HFMD epidemic in Hubei Province in 1987. No cases of AFP or aseptic meningitis were identified during this epidemic (Zheng *et al.*, 1995). EV71 outbreaks also occurred in Singapore (1998) (Singh *et al.*, 2000) and Japan (1998) (Komatsu *et al.*, 1999). A large outbreak of EV71 infection occurred in Perth, Western Australia in 1999 with approximately 6000 cases of HFMD in which 29 cases with severe neurological diseases were reported (McMinn *et al.*, 2001).

In Malaysia, the first epidemic of EV71 occurred in 1997 in Sarawak state (Abu Bakar *et al.*, 1999; Cardosa *et al.*, 1999). Numerous number of HFMD, herpangina and

neurological complications including AFP, cerebellar ataxia and fatal neurogenic pulmonary edema caused at least 31 deaths. According to Podin *et al.* (2006), 2 large outbreaks of HFMD recurred in 2000 and 2003 in Sarawak. The infection of EV71 was detected again in peninsular Malaysia in 1998 at University Hospital Kuala Lumpur. Four fatal cases were reported in peninsular Malaysia, all showing sudden and rapid progression of pulmonary and cardiovascular decompensation leading to death (Lum *et al.*, 1998).

1.1.4 VP1 Gene and Protein

VP1 gene of the human enterovirus genome has been identified as the most informative region to study the evolutionary as well as the virulence determinants of enteroviruses (Brown *et al.*, 1999 ; Caggana *et al.*, 1993 ; Hughes, 2004). VP1 is also the most external and immunodominant of the Picornavirus capsid protein. The VP1 protein is thought to be the major viral neutralization determinant and has high degree of antigenic and genetic diversity (Blomberg *et al.*, 1974 ; Brown *et al.*, 1999).

Human enterovirus VP1 sequences vary in length from 834 to 951 nucleotides (278 to 317 amino acids). The Coxsackie B virus has the shortest VP1 amino acid sequences (278 to 298 amino acids), while EV68 and EV70 has the longest VP1 sequences (312 to 317 amino acids) (Oberste *et al.*, 1999b).

Oberste *et al.* (1999a) reported that the VP1 fragment which directly amplified from the original clinical material through the reverse transcription-polymerase chain reaction (RT-PCR) method showed that VP1 sequence or some portion correlates with serotype and useful in distinguishing within and between serotypes determined by the

conventional neutralization test as compared to other regions like 5' UTR or VP4-VP2 junction. Oberste *et al.* (1999b) also discovered that the nucleotide and amino acid sequences of VP1 of enteroviruses of the same serotype were clearly distinguished from those of heterologous serotypes. Therefore, VP1 sequence is useful in classifying viruses within the Picornaviridae family.

Further more, variable loops between the β-barrel structures are exposed on the virion surface and studies showed that a number of the loops contribute to specific antigenic neutralization sites. VP1 contributes to all three of the major neutralization sites that have been identified on the poliovirus surface. The B-C loop is one of five VP1 loops forming VP1 antigenic site. Replacement of the VP1 B-C loop of Coxsackie B3 by that of Coxsackie B4 through site-directed mutagenesis produced a viable virus with a mixed neutralization phenotype, demonstrating the presence of a serotype specific antigenic neutralization site in the B-C loop of enterovirus (Reimann *et al.*, 1991). Foo *et al.* (2007) reported that the diphtheria toxoid-conjugated synthetic peptides, SP55 and SP70, containing amino acid 163 to 177 and 208 to 222 of VP1, respectively, are capable of eliciting neutralizing antibodies against EV71 in the *in vitro* microneutralization assay with strong IgG1 specific antibody response. These show that the VP1 capsid protein of EV71 able to trigger the immune response by producing the protective neutralizing antibodies and it may contain neutralization epitopes which is one of the most important considerable factors in establishing a candidate vaccine against EV71.

Using the bovine lactoferrin, an iron binding glycoprotein with antiviral property, Weng *et al.* (2005) demonstrated that lactoferrin could interfere with viral attachment by

binding to VP1 as well as host cell and hence blocking the binding of VP1 to host cell. Besides, VP1 protein also plays a role in uncoating process in replication cycle of the enterovirus (Belnap *et al.*, 2000). Deep cleft on virion surface has been identified at the junction of VP1 and VP2 and it is said to be the site of virion attachment to the cellular receptor (Blomberg *et al.*, 1974). Therefore, VP1 is directly involved in the replication cycle of EV71.

Amino acid position-170 is part of a highly conserved region of the enterovirus VP1 protein and has an alanine residue in all of the EV71 consensus sequences. The mutation at codon 170 of VP1 changing from alanine to valine in EV71 genogroup of C2 of the Perth, Western Australia outbreak suggested that the mutation at position 170 may be associated with increased neurovirulence of EV71. It is concluded based on a comparative study of the Perth, Western Australia VP1 deduced amino acid sequences of genogroup C2 viruses with the VP1 consensus amino acid sequences for EV71 (genogroups A, B, C, and CA16). Genogroup C2 isolates obtained from children with severe neurological disease during the Western Australia epidemic had an identical amino acid sequence in VP1, including an alanine to valine substitution at position 170. Alanine to valine substitution at position 170 was not seen in isolate obtained from cases of uncomplicated HFMD (McMinn *et al.*, 2001). This showed that mutation at the VP1 region was associated with the increase of virulence of EV71.

1.1.5 Pathogenesis

Human beings are the only natural hosts of enteroviruses (Chang *et al.*, 1999b). Infection begins with EV71 virus entry into cells by binding to a still unknown receptor (McMinn, 2002). Binding to the receptor induces the 160S-to-135S irreversible

conformational transition to occur (Huang *et al.*, 2000). This conformational change results in the externalization of VP4 and the N-terminus of VP1 components. The externalized N-terminus of VP1 forms an amphipathic helix and allows the 135S or A particle to attach to the cell membrane. Once the N-terminal helix of VP1 has inserted into the membrane, it rearranges to form a pore and permit the RNA to pass through the pore into the cytoplasm. This stage is called the uncoating stage (Belnap *et al.*, 2000).

As 135S particle releases its RNA, it is converted to the 80S or H particle (Belnap *et al.*, 2000). The viral RNA can be used as the translation template to synthesize viral proteins and to amplify viral positive-sense RNA genome. Replication of the genome is carried out by the RNA-dependent RNA polymerase (3D) with the aid of other viral and host factors. Initially, a negative-strand copy is synthesized which is then used as a template for new genomic RNA-strands. The genomic RNA acts as a messenger RNA giving rise to a large polyprotein (Hyypiä *et al.*, 1997).

P1 precursor encoded in P1 region can be cleaved by protease into VP0, VP1 and VP3 which spontaneously assemble into icosahedral procapsid and pack the RNA genome into the provirion. The completed virions consist of 60 copies each of VP1, VP2, VP3, and VP4 and one copy of the viral RNA (Kirkegaard, 1990). Approximately 10^4 to 10^5 of infectious virus particles are produced in one infected cell which is finally destroyed allowing the viruses to be released to infect new target cells where the cycle begins again. The replication of EV71 is completed in approximately 6 hours (Belnap *et al.*, 2000 ; Hyypiä *et al.*, 1997). The replication cycle is summarized in Figure 1.3.

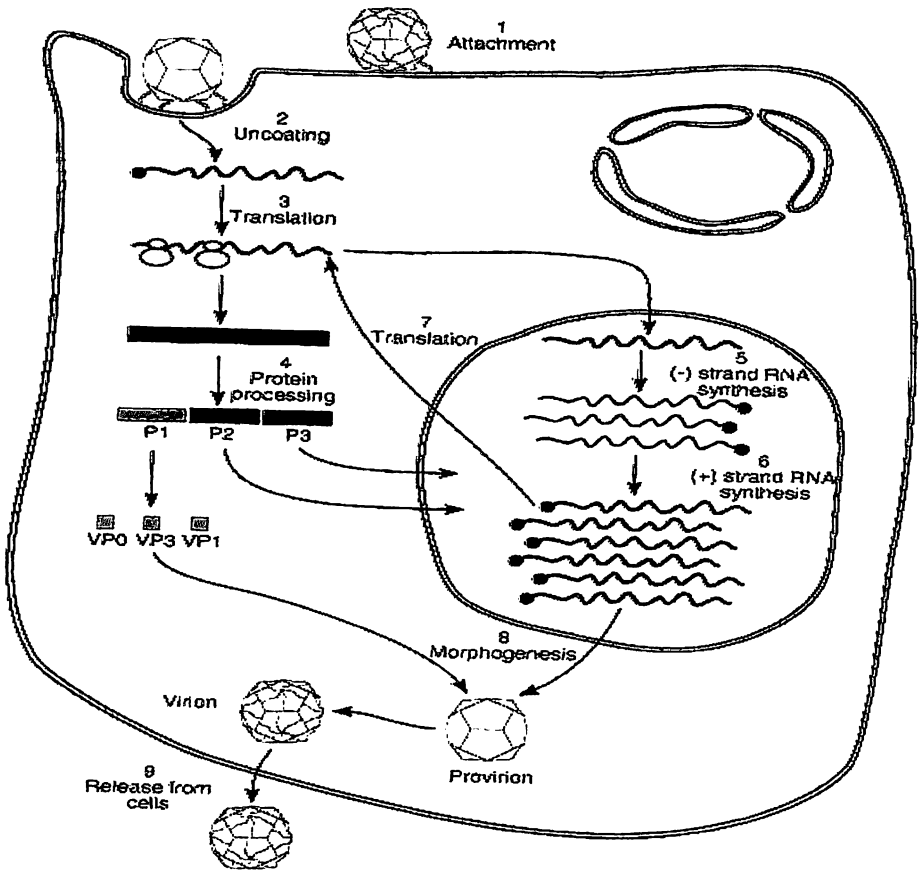

Figure 1.3: Replication cycle of enterovirus (adapted from Racaniello, 2001)

Primary infection with an enterovirus leads to viral replication in the tissue around the gastrointestinal tract, followed by a transient viremia and migration into other tissues (Simmonds and Welch, 2006). The incubation period is usually 3 to 4 days or more. Infection by the virus is often asymptomatic or self-limiting (Abu Bakar *et al.*, 1999).

EV71 is thought to spread mainly by faecal-oral route by contacting with fecal contaminated materials. EV71 can also be transmitted by droplets and secretions of infected persons. EV71 could survive 1 to 2 weeks in the pharynx and its excretion through stool can persist for up to 11 weeks. Therefore, the transmission of EV71 in the acute phase is probably through droplet transmission (Chung *et al.*, 2001). Furthermore,

according to Chang *et al.* (1999b), isolation rate of EV71 was high in throat swabs (90 %) and rectal swabs or feces (32 %) during the Taiwan enterovirus epidemic in 1998.

Enteroviruses may be excreted in human feces and urine, which are present in treated wastewater and which can contaminate rivers, recreational waters, and seawater. These enteroviruses may subsequently travel from water back to humans through many potential routes (Bosch, 1998). According to Hsu *et al.* (2007), occurrence of epidemic EV71 clones in the water sources in the same geographical regions during the outbreak in Taiwan in 2005 suggested water can serve as a potential reservoir and vehicle of infection.

Household transmission also plays an important role in the spread of EV71. According to Chang *et al.* (2004), transmission between siblings or cousins was over 80 %, which was higher than that of parents (40 %) or other adults (20 %). Infected adults would also spread the disease to other members of the family, especially preschool children. It is likely that almost all the susceptible family members were infected once EV71 had been introduced in the household. Chang *et al.* (2002) recorded that 56 % of younger siblings were EV71 seropositive after the epidemic when their elder siblings were EV71 seropositive. The concordance rate of EV71 seropositivity among siblings was 84 %. In addition to sibling transmission, of the 484 preschool children who were EV71 seropositive after the epidemic, 29 % had HFMD or herpangina. Apart from that, it is found that attending child care or kindergarten significantly increased the seropositive rates of anti-EV71 antibody. Contact with symptomatic EV71 cases was found to correlate positively with occurrence of EV71 infection and illness of HFMD or herpangina.

1.1.6 Infection

1.1.6.1 Hand-Foot-And-Mouth disease (HFMD) and Herpangina

EV71 infection can be defined as acute illness with addition of the isolation and typing of EV71 from rectal swab, throat swab, vesicular fluid, cerebrospinal fluid, or necropsy tissue (Chang *et al.*, 1999a). The EV71 infection has a biphasic course with a prodrome of hand-foot-and-mouth disease or herpangina followed by neurologic manifestations (Huang *et al.*, 1999).

HFMD is the most common presentation in EV71 infections, followed by herpangina. The onset of HFMD is characterised by 3 to 4 days of fever with sometimes reaching 39 ^{o}C and the development of a vesicular exanthema on the buccal mucosa, tongue, gums and palate and a papulovesicular exanthema on the hands, feet and buttocks (Komatsu *et al.*, 1999). The formation of rashes due to the other enterovirus infection often larger than those EV71 infections in which the rashes of EV71 frequently popular or petechial with areas of diffuse erythema on the trunk and limbs and is sometimes so tiny that it may be overlooked by parents and even doctors as described in Plate 1.1 (Komatsu *et al.*, 1999 ; McMinn *et al.*, 2001).

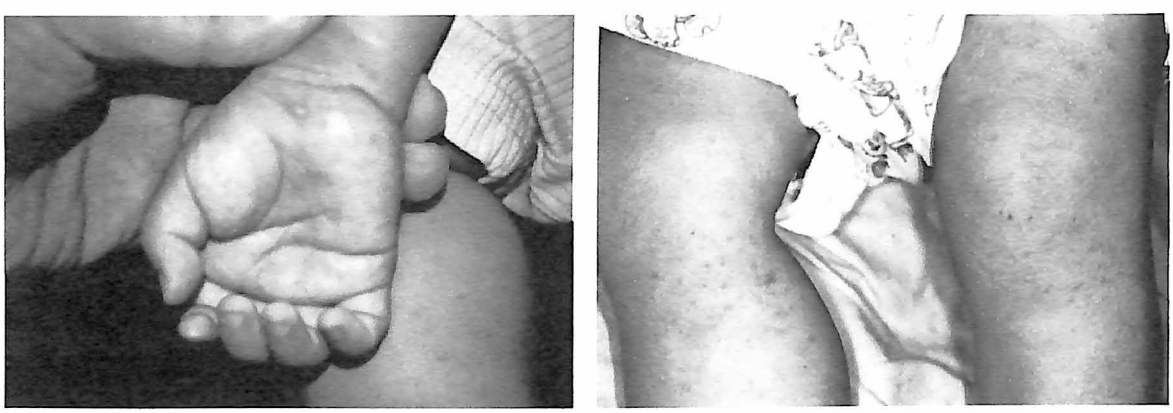

Plate 1.1: Vesicular rashes of EV71 HFMD. Left picture shows lesions of over the hand and right picture shows tiny, easily overlooked vesicular rash over the knees (adapted from Chang *et al.*, 2006)

HFMD and herpangina are uncomplicated illness. Herpangina is an illness characterised by fever and sore throat, associated with the development of raised papular lesions or ulcer on the mucosa of the anterior tonsillar pillars of fauces, soft palate and buccal mucosa or the uvula. Oral ulceration causes pain while eating or drinking, and patients may need intravenous fluid supplement if dehydration occurs (Chang *et al.*, 2006).

1.1.6.2 Neurological Disease (Central Nervous System Involvement)

After suffering the initial HFMD, CNS involvement may occur. EV71 has been recognised as highly neurotropic and associated with neurological diseases like aseptic meningitis, brainstem encephalitis (rhombencephalitis) or cerebellar encephalitis, and AFP. However, fewer will progress to cardiopulmonary failure following CNS invasion. Neurologic disorders begin two to five days after the onset of skin or mucosal lesions or fever (Huang *et al.*, 1999).

Aseptic meningitis can be defined as a clinically compatible illness, with cerebrospinal fluid pleocytosis (leucocytes more than 5/μL if the patient is aged older than 1 month while for newborn is more than 25/μL) with negative bacterial culture or EV71 isolated from cerebrospinal fluid (Chang *et al.*, 1999a). EV71 cases with aseptic meningitis generally present myoclonic jerk during sleep, vomiting, fever or headache. They have mild if any neck stiffness (Huang *et al.*, 1999). Patients may also show signs of changes in consciousness such as lethargy, sleepiness or coma, seizure attacks, ataxia, and cranial nerve palsy. Subtle symptoms of increased sympathetic tone, such as insomnia, profuse sweating, paralytic ileus, neurogenic bladder, and panic or increased startle reflex are often observed. They are usually benign and self limited in nature in which they usually recover 3 to 7 days after hospitalization (Chang *et al.*, 2006 ; Ho, 2000).

EV71 able to induce paralysis through infection and destruction of anterior horn motor neurone of the spinal cord (Alexander *et al.*, 1994). Guillain-Barré syndrome (GBS) is another sign of CNS involvement. GBS is defined as rapid onset of polyneuropathy with characteristic evolution of motor weakness and evidence of delayed nerve conduction. GBS and AFP associated with EV71 infection appears to be milder and associated with higher rates of complete recovery (McMinn *et al.*, 2001). Patient with AFP will have manifestations of brain stem encephalitis before the onset of acute paralysis. The extent of acute paralysis included unilateral weakness involving the upper or lower extremities and bilateral weakness in the lower extremities (Chen *et al.*, 2001).

The most severe neurological manifestation of EV71 infection is brainstem encephalitis (rhombencephalitis). Wang *et al.* (1999) defined brain stem encephalitis as an illness characterized by myoclonus, ataxia, nystagmus, oculomotor palsies, and bulbar palsy, in various combinations, with or without neuroimaging evidence. This disease occurs most frequently as an extension of spinal cord disease (Lum *et al.*, 1998). The most frequently involved organs are medulla oblongata, reticular formation, pons and midbrain (Huang *et al.*, 1999). Brain stem encephalitis is often related to acute inflammation or to the eventual necrosis of nuclei, cranial nerves, and white matter tracts (Wang *et al.*, 1999). AFP and brain stem encephalitis are the manisfestations that strike terror among parents of afflicted children, and cause deaths or disability (Ho, 2000).

Acute cerebellar ataxia has also been linked to EV71 infection (Ishimaru *et al.*, 1980). This disease usually presents as truncal ataxia with or without nystagmus and is

typically accompanied by full recovery acute disease. It is associated with inflammation of grey matter in one or both cerebellar hemispheres and variable degrees of cerebellar cortical atrophy developed upon recovery, despite the complete recovery of function. As the disease progress, cytotoxic edema in the cerebellar hemispher may occur (McMinn *et al.*, 2001).

1.1.6.3 Neurogenic Pulmonary Edema

The most striking clinical characteristic of type III brainstem encephalitis or neurogenic pulmonary edema, is its rapid progression and high mortality (Ng *et al.*, 2001). Early aggressive treatment is required to improve chances of recovery. Wang *et al.* (1999) defined neurogenic pulmonary edema as subsequent development of respiratory distress symptoms and signs (tachycardia, tachypnea, rales, and copious frothy sputum) plus chest roentgenographic evidence of bilateral pulmonary infiltrates without cardiomegaly. Once intubated, children will produce a white frothy secretion, with a pink frothy fluid and then fresh blood from endotracheal tube (Chang *et al.*, 2006). Typically, children develop tachycardia, tachypnea and cyanosis between 1 and 3 days of the onset of fever, HFMD or herpangina (Chang *et al.*, 1999a ; Ho, 2000).

Other symptoms for neurogenic pulmonary edema are hyperglycemia and limb weakness. Hyperglycaemia might be resulted from the loss of blood glucose homoeostasis in which the autonomic nervous system plays an essential part (Chang *et al.*, 1999a). The mortality from this condition is high with most children dying within 12 to 18 hours of the onset of the syndrome (Ng *et al.*, 2001). Limb weakness or hypesthesia may suggest a viral invasion of the corticospinal tract of the brain stem or

the spinal cord. If the virus spreads or ascends, it may invade the reticular formation of the medulla (Chang *et al.*, 1999a).

The pulmonary edema is of neurogenic origin and is secondary to autonomic dysfunction resulting from infection of specific regulatory structures within the brainstem and not cardiac in origin (Wang *et al.*, 1999). Extensive damage to both the dorsal nuclei of the vagus and medial reticular formation (vasomotor) of the medulla may be responsible for pulmonary edema (Chang *et al.*, 1999a ; Lum *et al.*, 1998). The post-mortem studies also showed that brainstem lesions are accompanied by intense neutrophil and mononuclear cell inflammatory infiltrates (Lum *et al.*, 1998).

Lin *et al.* (2002b) suggest that the occurrence of hyperglycaemia or an elevated interleukin-6 level may alert the possibility of this fatal complication in neuroimmunological study of the pathogenesis of fatal EV71 encephalitis with pulmonary edema apart from elevation of white blood cell count, blood glucose, and systemic proinflammatory cytokines such as tumor necrosis factor-α and interleukin-1β.

1.1.6.4 Convalescence and Long Term Sequelae

Some of the patients with EV71 CNS infection still need tracheostomy with ventilator support and nasogastric tube feeding, and some with limb weakness, psychomotor developmental delay, lower intelligence quotient (IQ), or attention deficiency hyperactive disorder. Their sequelae are compatible with magnetic resonance imaging findings, which usually reveal high signal intensity from the pons to cervical spinal cord (Chang *et al.*, 2006 ; Chen *et al.*, 2001).

1.1.7 Immunity

1.1.7.1 Humoral Immunity

As most enterovirus infections occur at mucosal surfaces, so the first line of defence for the host lines is in the mucus. Sensitized B cells may secrete antibody of IgA isotype which is transported through mucosal cells and secreted from the luminal surface. Attempted reinfection with enterovirus with virus strain previously encountered by immune system will result in neutralization of the virus by secreted antibody before it is able to gain access to the epithelial surface. Both B cell and T cells are involved in the generation of an effective antibody response. In the absence of T cell help, only low affinity antibodies can be made. $CD4^+$ T cells involved in providing immunological help for the antibody response are often cross-reactive between virus serotypes, and sometimes between different members of the picornavirus family (Nash and Usherwood, 1998).

Katamura *et al.* (2002) reported that X-linked agammaglobulinemia patients are prone to vaccine-associated paralytic poliomyelitis and to chronic enteroviral meningoencephalitis as defects in immunoglobulin synthesis. The introduction of immunoglobulin therapy had improved the prognosis of CNS enterovirus infection in X-linked agammaglobulinemia. This suggests that humoral immunity also plays a part in immunity of enterovirus infection.

1.1.7.2 Cell-mediated Immunity

Cell-mediated immunity is the main key component involved in host defence against most viruses including enterovirus. T lymphocytes are central for inducing immunological responses and in many instances, for the recognition and destruction of

virus-infected cells. $CD8^+$T cells usually cytotoxic T lymphocytes recognize peptides from endogenously synthesized proteins and $CD4^+$T cells usually helpers, detect peptides derived exogenously. Intracellularly replicate virus will feed antigens into MHC class I pathway. Therefore, immune response to virus will include $CD8^+$T cells in addition to $CD4^+$T cells and antibodies (Whitton and Oldstone, 2001).

With any antigenic challenge, the initial events in the induction of immunity involve the uptake of virus or viral proteins by dendritic cells and processing to peptides for presentation by MHC class II molecules. The production of interleukin-2 and interleukin-2 receptor result in clonal expansion of the interacting $CD4^+$T cell. These events initiate the T helper cell response, which catalyses the induction of $CD8^+$ cytotoxic T cells, activated Natural Killer cells and macrophages. Several cytokines important for the antiviral mechanisms are produced by the $CD4^+$T cell, including interleukin-2, interleukin-4, interleukin-5, and gamma-interferon. Gamma-interferon can function in the intracellular killing even in the absence of $CD8^+$T cell mediated lysis. Meanwhile, interleukin-2 induces the differentiation and proliferation of natural killer cells, $CD4^+$T cells and $CD8^+$T cells. Interleukin-4 and interleukin-5 facilitate production of immunoglobulins made by B cells. B cells are the other major antigen-presenting cells in immune system (Nash and Usherhood, 1998).

$CD8^+$T cells recognize a complex of MHC class I with virus infected peptide on infected cells. The newly synthesized viral proteins are degraded in proteosome and the short fragments of antigen protein bound to MHC class I are transported to the cell surface. This process enable the peptides derived from intracellular pathogens can be processed at an early stage in the infection before the replication or assembly of new

virion has occurred. As MHC class I molecules occur on virtually all nucleated cells, viruses will have difficulty avoiding the attentions of cytotoxic $CD8^{+}$T cells (Nash and Usherhood, 1998). A summary of antiviral response is shown in Figure 1.4.

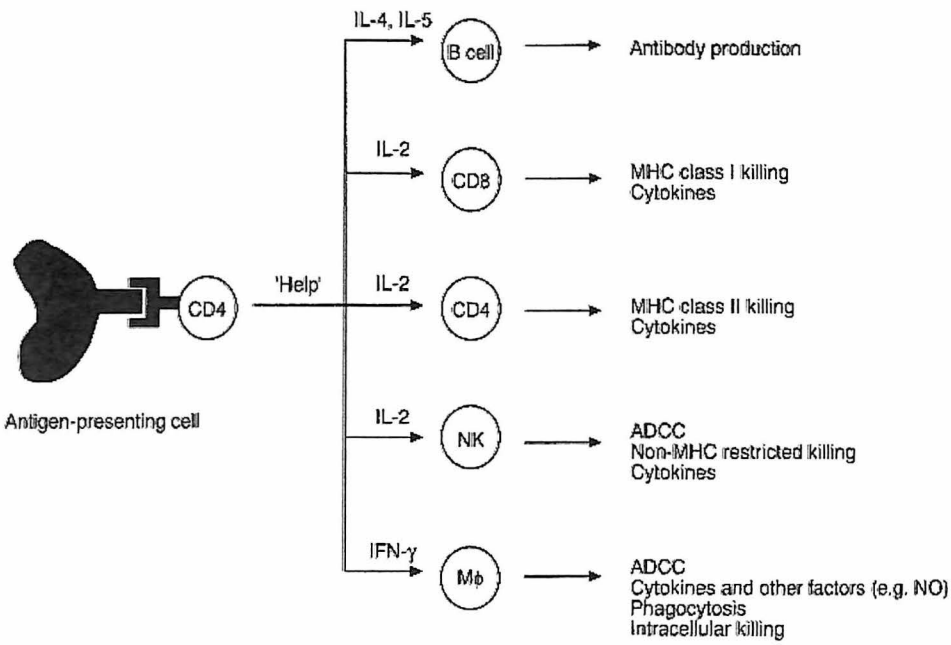

Figure 1.4: Antiviral mechanism (adapted from Nash and Usherwood, 1998)

1.1.8 Diagnosis

Isolation of enteroviruses in cell culture remains the gold standard for laboratory diagnosis. However, no single cell line is optimal for all enterovirus serotypes (Abzug and Rotbart, 1999). EV71 is isolated through the inoculation of patients' samples including cerebrospinal fluid, serum, urine, throat, and rectal specimen onto monkey kidney, HEp-2, Vero, GMK, MRC-5, CaCo-2, RD-18S and human lung fibroblast cells (Abzug *et al.*, 1995 ; Ho, 2000 ; Sato *et al.*, 2006). Growing of enterovirus in cell culture is also slow ranging from 4 to 7 days (Ho, 2000). The isolation is then identified using neutralization test or immunofluorescent assay which mainly relies on the availability of anti-sera or commercial monoclonal antibodies (Abu Bakar *et al.*, 1999; Shih *et al.*, 2003).

Laboratory methods for measuring EV71 neutralizing antibody follow standard protocol for performing the neutralization test on microtiter plates. Serum is heat treated before mixing with tissue culture containing EV71. Cytopathic effect is then observed and the neutralizing antibody titre is determined. The neutralizing antibody titre is defined as the highest dilution of serum that would prevent the occurrence of cytopathic effect. Seropositivity is defined as a neutralizing antibody titer more than 1/8 (Chang *et al.*, 2006).

In finding which clinical sample, or combination of samples, is the most useful for laboratory diagnosis of HFMD, Ooi *et al.* (2007) reported that throat swab and vesicle swab are the most useful samples as compared to others including cerebrospinal fluid and serum. Throat swab is the single specimen most likely to be positive, being positive for 49 % of the patients while vesicle swab gives 48 %. The combination of throat swabs plus vesicle swabs are the most useful for patients with vesicles, identifying virus for 67 % of the patients.

Shih *et al.* (2000) used VP1 capside protein as antigen in an attempt to establish a diagnosis assay. IgM anti-VP1 appeared in sera of patients with a symptomatic EV71 acute infection, whereas IgG anti-VP1 was present in sera of past infection. This finding suggests that detecting IgG and IgM immune responses is an effective means of determining the different phases of EV71 infections.

Wang *et al.* (2004) introduced an IgM-capture enzyme-linked immunosorbent assay (ELISA) which offered an early and rapid detection of EV71 infections. Using virus isolation and neutralization test as standards, the sensitivity and specificity of the

ELISA were 97.7 and 93.3 %, respectively. Most of the IgM positive serum specimens were collected within 7 days after the onset of symptoms, while it appeared detectable up to 94 days after the onset of symptoms. Apart from being highly sensitive, rapid and low in cost, the new IgM-capture ELISA is sufficiently accurate to provide also reliable results for early detection of the virus. EV71 infections can be detected within 4 hours.

Apart from isolation and antibody based diagnostic methods, several diagnostic based on molecular method had been established. Deoxyribonucleic acid (DNA) microchip array developed by Shih *et al.* (2003) may overcome the limitations of clinical application, able to distinguish different genotypes of EV71 and avoiding the isolation of live virus. The whole procedure takes only 6 hours. The novel microchip has a high sensitivity of 89.6 % and specificity of 90.9 %. This novel approach is based on hybridization of amplified DNA specimens with oligonucleotide DNA probes immobilized on a microchip. Two oligonucletides were used as detection probes, the pan-enterovirus sequence located in the 5'UTR and the EV71-specific sequence located in the VP2 region. Singh *et al.* (2002) had developed RT-PCR assay using EV71 type-specific primers for rapid diagnostic test for EV71 during HFMD outbreak in Singapore in year 2000. The specimens were directly subjected to RNA extraction followed by RT-PCR. By using a seminested RT-PCR strategy, specific identification of EV71 sequences directly in clinical specimens was achieved, with a detection rate of 53 %. In contrast, cell culture could isolate EV71 in only 20 % of positive specimens. Seminested PCR is also used by Nix *et al.* (2006) in detection and identification of enterovirus RNA in clinical specimens found that VP1 seminested RT-PCR assay was slightly more sensitive than enterovirus 5'UTR semenisted RT-PCR assay detecting as few as 10 RNA copies per reaction.

1.1.9 Control

1.1.9.1 Treatment

To improve survival, the treatments of the patients are based on the clinical stage of the illness. The stage-based management is as in Table 1.1.

Stages	Clinical Manifestations	Management
1	HFMD/herpangina	Symptomatic treatment only.
2	CNS involvement	Fluid restriction, osmotic diuretics for increased intracranial pressure, and furosemide for fluid overloaded, intravenous immunoglobulin for encephalitis or polio-like syndrome and close monitoring of heart rate, blood pressure, oxygenation, coma scale and blood glucose.
3	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 or hemorrhage persists or severe hypoxemia develops.
	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.

Table 1.1: Clinical staging and management (adapted from Chang *et al.*, 2006)

1.1.9.2 Personal Hygiene

Hand washing is the standard preventive measure targeting the faecal-oral transmission route. Reducing contact between infected and susceptible people during epidemics is another important (Chang *et al.*, 2006 ; McMinn, 2002). Younger infected children have higher virus isolation rates as well as higher transmission ability, and these young children are usually not able to consistently follow adequate hand-washing routines (Chang *et al.*, 2006). Personal hygiene has no any effect if there is little surveillance of EV71 activity is maintained in the community.

1.1.9.3 Antiviral Agents

Although a number of antiviral agents with activity against enteroviruses are currently being developed, however their effect on EV71 is unknown. Therefore, no commercially release of anti-enterovirus at this moment. Pleconaril is a novel compound, orally bioavailable, and systemically acting inhibitor of enteroviruses (Pevear *et al.*, 1999). It integrates into the capsid of Picornaviruses, thus preventing the virus from attaching to cellular receptors and uncoating to release RNA into the cell. This action will prevent the production of viral progeny. Pleconaril is currently undergoing phase III clinical trial in the United States (McMinn, 2002).

Pyridyl imidazolidinone is another novel class of potent EV71 inhibitor. BPR0Z-194, one of the pyridyl imidazolidinones, targets EV71 capsid protein VP1. Experiments revealed that BPR0Z-194 effectively inhibited virus replication in the early stages, implying that the compound can inhibit viral adsorption or viral RNA uncoating (Shih *et al.*, 2004).

Recently, Arita *et al.* (2008) identified two compounds, metrifudil and NF449, with anti-EV71 activity and one compound, GW5074, with both anti-Polio and anti-EV71 activities. N^6-benzyladenosine which is structurally related to metrifudil, is also found to efficiently block the replication of EV71.

1.2 Bacille Calmette-Guérin (BCG)

1.2.1 Background

BCG is the most widely used vaccine against tuberculosis with over 3 billion administered doses and is currently the only vaccine available against tuberculosis

(Grode *et al.*, 2002 ; Ohara and Yamada, 2001). It is prepared from the attenuated *Mycobacterium bovis*. BCG strains can mimic a natural infection without causing disease. As such, recombinant antigens delivered by BCG carrier strains not only target appropriate pathways of MHC antigen processing and presentation, but also stimulate the innate immune system resulting in immune responses similar to the ones elicited in infected individuals (Dietrich *et al.*, 2003).

The parental strain *M. bovis* is closely related to *Mycobacterium tuberculosis* that causes tuberculosis in man. The BCG strain was developed by Albert Calmette and Camille Guérin between 1908 and 1920 from a virulent *M. bovis* strain via 231 serial passages through glycerol-potato-bile medium in which the *M. bovis* became attenuated (Brewer and Colditz, 1995 ; Grode *et al.*, 2002). Initially, the virulent strain was isolated from the udder of a tuberculous cow (Brewer and Colditz, 1995).

At first, cultures of BCG were maintained in Paris. Later, it was subcultured and distributed to several laboratories throughout the world where the vaccine strain called BCG. After years, various strains maintained in their own laboratories were no longer identical to each other. Today, the organism is maintained in several laboratories using a "seed lot" production technique to limit further genetic variation using freeze-dried cell. The BCG vaccines that are currently in use are produced at several sites throughout the world. Each BCG is now known by the location where it is produced, for example, BCG Pasture, BCG Copenhagen, BCG Tice, BCG Japan, and BCG Montreal (World Health Organization, 2008).