

Impact of genetic changes, pathogenicity and antigenicity on Enterovirus-A71 vaccine development

Pinn Tsin Isabel Yee^a and Chit Laa Poh^{a,*}

^aResearch Centre for Biomedical Sciences, Sunway University, Bandar Sunway, Kuala Lumpur, Selangor 47500, Malaysia

*Corresponding Author: Chit Laa Poh, Sunway University; pohcl@sunway.edu.my

Email of first author: isabely@sunway.edu.my

Abstract

Enterovirus-A71 (EV-A71) is an etiological agent of the hand, foot and mouth disease (HFMD). EV-A71 infection produces high fever and ulcers in children. Some EV-A71 strains produce severe infections leading to pulmonary edema and death. Although the protective efficacy of the inactivated vaccine (IV) was $\geq 90\%$ against mild HFMD, there was approximately 80% protection against severe HFMD. The monovalent EV-A71 IV elicits humoral immunity but lacks long-term immunogenicity. Spontaneous mutations of the EV-A71 genome could lead to antigenicity changes and the virus may not be neutralized by antibodies elicited by the IV. A better alternative would be the live attenuated vaccine (LAV) that elicits cellular and humoral immunity. The LAV induces excellent antigenicity and chances of reversion is reduced by presence of multiple mutations which could reduce pathogenicity. Besides CV-A16, outbreaks have been caused by CV-A6 and CV-A10, hence the development of bivalent and trivalent vaccines is required.

Keywords: Enterovirus-A71; Sub-genotype; Pathogenicity; Antigenicity; Live attenuated vaccines; Bivalent; Trivalent; Vaccines.

Introduction

Enteroviruses are associated with a plethora of infections that pose significant health problems in the post-poliovirus eradication era. The clinical symptoms can range from mild seasonal flu-like illness and mild hand, foot and mouth disease (HFMD) to potentially fatal conditions, such as acute flaccid paralysis and pulmonary edema (1). Human enteroviruses are members of the *Enterovirus* genus in the *Picornaviridae* family and they are positive-sense, single-stranded RNA viruses. Enteroviruses are classified into 12 species based on sequence identity and phylogenetic relationships. They are classified into Enterovirus A to H, Enterovirus J and rhinovirus A to C (2). Enterovirus Group A (EV-A) such as Enterovirus-A71 (EV-A71), Coxsackievirus-16 (CV-A16) CV-A6, and CV-A8 have caused more than 7 million infections, including 2457 fatalities in China from 2008-2012. Approximately 82,486 patients developed cardiopulmonary or neurological complications and 1,617 of laboratory confirmed deaths were associated with EV-A71 (3). However, recent large scale HFMD outbreaks in China have been caused by other enteroviruses as well, predominantly CV-A6 and CV-A10 (4-7).

Most of the enteroviruses have no vaccine or antivirals against them. There should be continuous efforts to develop vaccines to accelerate eradication of enteroviral diseases. Of particular interest in this review would be EV-A71 which is considered the most important neurotropic enterovirus after the eradication of Poliovirus (8). The EV-A71 virus consists of a single-stranded, positive sense, ribonucleic acid (RNA) of approximately 7411 base pairs long. The RNA has an open reading frame (ORF) that is flanked by the 5' and 3' Non-Translated Regions (NTRs) (9). The 5'-NTR contains the Internal Ribosome Entry site (IRES) that allows mRNA translation in a cap-independent manner (10). The ORF (P1-P3) is translated into a single polyprotein that undergoes a series of processing events, that eventually is cleaved to structural and non-structural viral proteins (11).

The 4 viral capsid proteins (VP1, VP2, VP3, and VP4) are structural proteins and they are encoded by the P1 region. VP1, VP2 and VP3 are exposed on the external surface of the EV-A71 virus whereas VP4 is located on the internal side of the capsid. The nonstructural proteins involved in genome replication and protein processing (2A-2C, 3A-3D) are encoded by the P2 and P3 regions (12). The 2A and 3C proteinases are vital for virus-host interactions, virus replication and apoptosis of infected cells *in vitro* (13, 14). As the 3D protein is a RNA-dependent RNA polymerase (RdRP) that lacks proofreading mechanism, this led to high mutation rates of 10^{-3} to 10^{-5} mutations per nucleotide copied, per replication cycle (15). Hence, it is known to mis-incorporate 1 or 2 bases for every genome replication (16).

Etiology of HFMD

The EV-A71 virus was first isolated from a child in California, United States in 1969 (17). Based on a 5-year surveillance in China (2008-2014), approximately 43.73%, 22.04%, and 34.22% of HFMD infections were due to EV-A71, CV-A16 and other enteroviruses, respectively. Amongst all etiological agents of fatal HFMD infections, EV-A71 was responsible for 70% severe HFMD cases and 90% HFMD-related deaths (18). Mild EV-71 infection in children are fever ($\geq 39^{\circ}\text{C}$), loss of appetite with rashes (hands, foot and mouth area) and ulcers in the throat, mouth and tongue. However, some sub-genotypes of EV-A71 strains (B3, B4, C4) have been associated with more severe clinical infections which could lead to delayed neurodevelopment, aseptic meningitis, brain stem encephalitis, acute flaccid paralysis, neurogenic pulmonary edema and cardiopulmonary failure (1).

The neurological complications of EV-A71 have led to high fatalities in countries such as China, Malaysia, Taiwan, Vietnam and Cambodia (19). For example, in Taiwan in 1998, there were 129,106 reported cases of HFMD and 405 severe cases with 34 deaths due to EV-A71 (20). In Cambodia alone, there was approximately 69% fatalities out of 78 infections between April and July 2012 (21). Some of the young children died of alveolar pneumonia,

while others developed fatal destructive brain and spinal cord inflammation caused by fatal sub-genotypes of EV-A71 (22). The absence of preventive agents highlights the urgency of developing vaccines to prevent further fatalities caused by EV-A71.

Molecular Epidemiology of EV-A71

The complete cDNA sequences (891bp) of EV-A71 strains (VP1) isolated from various countries over a 30-year period were analyzed and divided into three distinct genotypes (A, B, C) and 11 sub-genotypes. Genotype A consists of the prototype EV-A71 BrCr strain that was identified in 1970 in USA (23). As majority of EV-A71 viruses isolated worldwide were from genotypes B and C, they are sub-classified into sub-genotypes B1 to B5 and C1–C5 (Table 1) (24). The sub-genotype B0 was proposed after analyzing EV-A71 strains from the Netherlands from 1963 to 1967 (25). The B6 strain was the new sub-genotype isolated in Columbia (AF135899), and the sub-genotype B7 was first isolated in Brazil (AY278249) (26).

In recent years, two new genotypes were discovered; one in central Africa (designated as genotype E) and the other from Madagascar (designated as genotype F) by sequencing the VP1 and VP2 regions of isolates (27). Bessaud et al. (2014) used phylogenetic analysis to determine the origin of genotype E and found that it shared common ancestry with genotype D (28). Based on phylogenetic studies, a novel genotype G (V08-5327) was discovered in India by Saxena et al. (2015) (29). However, the new discoveries of isolates belonging to genotypes E, F and G remain questionable as their classifications were based on sequencing of the VP1 (nt. 2442-3332) and VP2 (nt. 954-1715) regions which encompass only 22% of the entire 7.4 kb EV-A71 genome. It was surmised that EV-A71 strains share between 84-97% nucleotide sequence and more than 96% amino acid similarity within the same sub-genotype, while the nucleotide sequence identity between the four major genotypes (A-D) ranged from 78% to 83% (24).

Since 1997, sporadic HFMD epidemics have caused many fatalities among young children in the Asia Pacific region. EV-A71 outbreaks in Malaysia were caused by a mixture of B and C genotypes, C1 and B4, occurring from 1998-2000, C1, B4 and B5 from 2002-2003 and C1 and B5 from 2005-2006. Singapore reported predominantly B sub-genotype infections: B3 (1997-1999), B4 (2000-2003) and B5 (2006 and 2008) (30). Hence, Singapore is currently investing much research efforts into developing an inactivated vaccine (IV) against the B3 sub-genotype. In contrast, there are countries that have a predominant genotype causing HFMD since 1997 and these were the C sub-genotypes (C1, C2, C4) in China, Korea, Holland and United Kingdom (23). The C4 sub-genotype has been known to cause large scale outbreaks and high fatalities in China, hence the IV to be developed was focused on the C4 sub-genotype.

The molecular epidemiology of EV-A71 could be changed by the recombination between circulating EV-A71 genotypes/sub-genotypes or spontaneous mutations of the viral genome. Therefore, there remain challenges in the development of an EV-A71 vaccine especially on the choice of a vaccine immunogen that would elicit broad NtAb titers in an outbreak. The selection of only one sub-genotype or strain may not be sufficient to confer sufficient protection against other EV-A71 sub-genotypes. This is because EV-A71 may undergo a process of antigenic evolution and immune escape mutants can be continuously generated under positive selection. Huang et al. (2011) elucidated that each sub-genotype was continually positively selected by herd immune pressure and hence, this would make it more difficult to select a vaccine strain as strain replacement was an ongoing evolutionary process. In addition, they discussed the intra- and inter-genotypic shifts among different EV-A71 epidemics and that the circulating sub-genotypes had at least 3 different antigenic characteristics. A comparison of the VP1 sequence showed that Glu43, Thr58, Thr184 and Ser240 were signature amino acids for genotype B (B1, B4 and B5) whereas Arg22, Asp31 and Ile249 were present only for sub-genotype C2. Genotype B5 has Asp164 which had a

similar signature amino acid with sub-genotypes C2 and C4 but the Asp164 was absent in B1 or B4 (31). Such genetic changes may contribute to distinct antigenic properties of sub-genotypes, although other capsid protein-coding regions may require further examinations. Genomic changes that have an impact on antigenicity might lead to low levels of protection against different sub-genotype strains. The genomic changes introduced to mutant viruses by site-directed mutagenesis will be useful for the determination of specific effects on virulence and antigenicity.

Vaccine development

Inactivated Vaccines

A number of biopharmaceutical companies are currently at different phases of clinical trials with their inactivated vaccines (IV). There are 3 companies (Sinovac, Vigoo, CAMS) from China, one from Taiwan (NHRI) and another from Singapore (Inviragen) (Table 2). All three companies from China are producing an alum adjuvanted IV against the sub-genotype C4a and have completed Phase III clinical trials. They were able to demonstrate good safety and efficacy profile with their IVs, hence, this had accelerated vaccine licensing in China (32). In fact, China's FDA has recently issued new drug certificates and production licenses for the IV from Sinovac, Vigoo and CAMS. Each company carried out placebo-controlled, double-blinded and randomized trials involving approximately 10,000 infants and young children. Two intramuscular doses of vaccine or placebo was administered to each vaccine candidate with a span of 28 days apart (33).

It was reported that the vaccine efficacy from Sinovac was at 95.1% against mild HFMD and an overall efficacy rate of 94.7% was established over two years. The IV was able to produce high protection against mild EV-A71 infections for a duration of 2 years when 3 doses were given at 6, 12 and 18 months, respectively (34). The IV from Vigoo and CAMS also showed a protective rate of 95%. Nevertheless, although the efficacy of the IVs was more

than 90% against mild HFMD, there was only 80.4% protection against severe HFMD (35). This would mean approximately 19.6% of young children would not be conferred protection against EV-A71-associated serious disease. The IV would be able to effectively reduce the number of mild HFMD cases, but not the mortality rate among infants infected by EV-A71 strains capable of causing fatal HFMD. Hence, viral surveillance studies of emerging and endemic EV-A71 is needed to ensure the efficacy of the newly licensed EV-A71 IVs.

Sinovac and CAMS demonstrated geometric mean titers (GMT) of 165.8 (400 U/two-doses) and 170.6 (100 U/two-doses) which could induce seroconversion in > 90% of subjects (36). The IV from Beijing Vigoo when given at 320 U/two-doses produced 325.3 GMT. All 3 China biopharmaceuticals demonstrated varying NtAb levels although their IVs were developed from the same sub-genotype C4a strain. It could be accounted for by different vaccine dosages given, cell culture and manufacturing conditions utilised by the 3 corporations (37). However, Mao et al. (2012) discovered that after unifying the units of dosages used by the 3 companies, the final IVs containing the alum-adjuvant had similar immunogenicity in mice although there were notable dissimilarities between immunogenicity levels of the wild type and IVs. They concluded that different types of adjuvant and even differing culture systems could influence the varying immunogenicity levels (38).

Due to such variability in the induction of NtAb levels resulting from these different antigenic formats, the WHO Expert Committee on Biological Standardization (ECBS) has recently approved a joint initiative between the National Institute for Food and Drug Control, China (NIFDC) and the National Institutes for Biological Standards and Control, UK (NIBSC) to standardise the levels of EV-A71 NtAb elicited by different antigen dosages (36). This is to ensure that the detection of NtAb and antigen content in clinical trials is as accurate and comparable as possible. It is vital that there are reference standards to measure the efficacy, consistency and potency of the different inactivated EV-A71 vaccines.

Besides C4a, the common sub-genotypes circulating in China after 2000 were the C2, C5, B4 and B5 sub-genotypes, hence, IVs against C4a should be expected to cross-protect against these sub-genotypes (39). Bek et al. (2011) showed that mice vaccinated with the C4 vaccine was able to survive challenges with lethal dosages of the EV-A71 B3 sub-genotype (40). Huang et al. (2013) conducted studies with children to measure NtAb titres that might confer cross-reactive protection against several EV-A71 sub-genotypes. The serology data showed that GMTs of cross-reactive NtAb titers against genotype A was lower (≥ 4 -fold difference) than the sera obtained from children infected with sub-genotypes C2, C4, B4 and B5 (41). Interestingly in another study, the NtAb titers in children induced by the EV-A71 C4a vaccine were low against several sub-genotypes, including the sub-genotype C4a, but it was able to cross-protect against sub-genotypes B4, B5, C1, C2 and C4b. All the NtAb titers induced by C4a were higher than 1:32, which would be deemed protective based on vaccine immune correlation studies (42).

In a Taiwan study, healthy adult volunteers were vaccinated with either 5- μ g and 10- μ g doses of EV-A71 sub-genotype B4 strain and it induced very strong NtAb responses in > 85% of volunteers that did not have pre-existing NtAb against sub-genotypes B1, B5 and C4a. However, the EV-A71 vaccine produced weak cross-neutralizing antibody responses (in ~20% of participants) against sub-genotype C4b and CV-A16. Additionally, more than 90% of the vaccinees did not develop cross-neutralizing antibody responses (Nt < 8) against sub-genotype C2. The researchers discovered that about 45% of the volunteers had < 8 pre-vaccination NtAb level against the sub-genotype B4. After the first EV-A71 immunization, 95% of vaccinees had > 4-fold increase in NtAb, but there was no further increase in NtAb levels after the second booster injection. Nevertheless, this vaccine strain was able to elicit a strong NtAb response (with a NtAb range from 11 - 4019) against sub-genotypes B1, B5 and C4a and could be subjected to more clinical trials for further evaluations (43).

Further studies are needed after vaccination to have a good gauge of the ability of cross-neutralization against all other EV-A71 sub-genotypes, determine the types of immune responses and understand vaccine immune correlates of protection. As CVA-16 co-circulates with EV-A71 in large HFMD outbreaks, it would be vital to produce a bivalent vaccine to reduce epidemics. Unlike the inactivated polio vaccine which comprises 3 serotypes, the EV-A71 IV produced in China only uses the C4a sub-genotype. The C4a elicited immune response was able to confer broad protection against B and C sub-genotypes. However, it also had lower immune protection against EV-A71 genotype A strains (42). In addition, the IV did not undergo clinical trials in other multi-centres and hence, registration and standardisation would be major deterrents for the IV to be utilised in other Asia-Pacific countries (44).

It is also pertinent to monitor cases of antibody dependent enhancement (ADE). ADE refers to sub-neutralizing concentrations of antibodies produced from maternal or previous primary infection which could exacerbate subsequent infection and lead to disease severity. In ADE, sub-neutralizing antibodies can bind to viruses and enhance viral entry via the binding between virus-antibody complexes and Fc receptors (FcR) on FcR-bearing cells such as B lymphocytes, macrophages and natural killer cells (45). ADE responses induced by IVs have been reported for viruses such as EV-A71 (46), poliovirus (47), and Coxsackievirus B (48). Han et al. (2011) described in their studies that pre-existing antibodies worsened EV-A71 infection in animal models. They discovered that a sequential EV-A71 infection in mice led to mortality after a primary infection with a non-lethal EV-A71 strain. This is indicative of the role of ADE in the pathogenesis of infection (46). Therefore, to prevent a disease from being exacerbated by unwanted induction of enhancing antibodies, the development of novel vaccines has to consider the detrimental effects of ADE.

Huang et al. (2015) observed that when Threonine at position 251 within the 3D^{Pol} region was substituted with Isoleucine (T251I), it altered the temperature susceptibility of EV-

A71 sub-genotype C2 from susceptibility to complete resistance at 39.5°C *in vitro*. The strain also had increased viral virulence and showed clinical symptoms *in vivo*. In addition, they confirmed that amino acid residues 98, 145 and 164 within the VP1 capsid region worked cooperatively as antigenic determinants for sub-genotype B4 and B5 strains. A series of recombinant viruses with various mutations (VP1-98K, VP1-145Q and VP1-164E) were constructed and antigenic cartography was carried out. They discovered that the EV-A71 mutant (KQE) carrying mutations at VP1-98K, VP1-145Q and VP1-164E significantly decreased the neutralizing titer by 4-fold against three of the 6 antisera of healthy adults. This was attributed to antigenic changes in EV-A71 and greater viral binding to cells. Interestingly, Huang et al. (2015) indicated that none of the single mutations was responsible for such antigenic changes. As such, viruses that evolved to contain all 3 mutations might become predominant and reduce the efficacy of the IV. Hence, more studies should be carried out to assess if such mutants could arise due to evolutionary pressures on EV-A71 (49).

Molecular characteristics of the live attenuated vaccine

The live attenuated vaccine is currently the focus of intensive development for several viruses as it is economical to manufacture, elicits excellent immunogenicity, confers live long immunity and induces cellular and humoral immunity. The World Health Organization has recommended the use of LAV for diseases caused by the measles virus, rotavirus and yellow fever virus, testifying to the success and potential of LAVs. With increasing studies of virulence determinants for some EV-A71 sub-genotypes, design of a LAV based on mutagenesis of specific nucleotides would allow reduction of virulence and increase in genetic stability. For example, mutations in the 5'-NTR could be introduced to decrease the efficacy of viral replication. As Poliovirus (PV) and EV-A71 have very high nucleotide homology in the 5'-NTR, the virulence determinants of PV reported in previous studies could serve as a reference to produce EV-A71 viruses that are highly attenuated. Izuka et al. (1989)

demonstrated that a stable, attenuated phenotype could be attained when nucleotides 564 to 726 in the 5'-NTR of the Poliovirus Mahoney genome was deleted (50). Strains carrying long deletions are more stable than those carrying point mutations or short insertions (51).

From the analysis of nucleotide (nt) sequences present in the three poliovirus Sabin strains, nucleotide substitutions which were critical in attenuating mutations in the virulent strains isolated from cerebrospinal fluid were identified. There were 57 nucleotide substitutions distinguishing the Sabin 1 strain from its parent strain. The most critical molecular determinant of the attenuated phenotype of Sabin 1 was nucleotide (nt) 480 in the IRES region. This nt. affects the folding of the 5'-NTR structure responsible for neurovirulence (52). For Sabin 2 strain, the virulence determinant appeared to be at position 481 within the IRES region whereas for Sabin 3, the main determinant for the attenuated phenotype was established to be nt. 472 (53). Based on the high homology of the 5'-NTR between PV and EV-A71, a genetically stable LAV can be rationally designed through partial deletion of the 5'-NTR region in the EV-A71 genome (9).

Arita et al. (2005) incorporated 3 mutations in the 5'-NTR (nt. 485, 486, 474), 2 mutations in the RdRP (Tyr-73, Cys-363) and a single site mutation in the 3'-NTR (nt. 7409) of the EV-A71 BrCr strain (S1-3'). Although the strain had reduced pathogenicity, cynomolgous monkeys administered with the S1-3' strain still demonstrated mild neurological manifestations with flaccid paralysis. Hence, the use of this strain as a LAV was discontinued (54). The VP1-145 amino acid has been determined as a "hot spot" for evolutionary pressures on EV-A71 (55). In order to investigate the involvement of VP1-145 in the molecular basis of virulence in EV-A71, Nishimura and co-workers (2013) discovered that the amino acid at VP1-145 determined the binding of EV-A71 to the receptor P-selectin glycoprotein ligand (PSGL-1) present on leukocytes. If the amino acid was a Glycine (G) or Glutamine (Q) at VP1-145, the virus was able to bind to PSGL-1 whereas non-binding strains had a Glutamic Acid (E) at

VP1-145. They further discovered that VP1-145 acted as a molecular switch to regulate PSGL-1 binding through the modulation of VP1-244 that was located closely to VP1-145 (56). Interestingly, Caine et al. (2016) discovered that a mutation in VP1-244 (K244E) was crucial for mouse-adapted EV-A71 (mEV-A71) virulence, expanded tissue tropism and viral spread in adult interferon deficient AG129 mice. This new mouse model differed from young ICR and BALB/c mice as it permitted the systemic spread of mEV-A71 into multiple tissues, hence increasing brain viral loads in mice beyond 6 weeks old. They also found a new VP1 mutation (H37R) was important for K244E recovery in primate cell culture. The authors postulated that the H37R/K244E interaction is pertinent for replication in primate cells but the K244E mutant could replicate alone in a murine model (57).

Liu et al. (2014) also found that there were nine amino acid substitutions (H22Q, P27S, N31S/D, E98K, E145G/Q, D164E, T240A/S, V249I, A289T) present in the VP1 sequences when fatal and mild EV-A71 strains of sub-genotype C4 were compared (58). As such, these residues may be potential molecular determinants in EV-A71 that could change mild strains into fatal strains. Chang et al. (2012) also observed that the E145Q substitution at the VP1 was a common difference in the EV-A71 genomes of strains associated with mild and fatal cases of HFMD. Additionally, they also discovered that fatal strains had greater substitutions in the 5'-NTR and IRES regions (59). This was expected as these regions were responsible for receptor binding and translation of viral mRNA. The position of a certain specific amino acid in the genome may have a profound significance on virulence. For example, it was reported in Coxsackievirus B3 (CV-B3) that the presence of Asn at position 165 of the VP2 was responsible for the cardio-virulent phenotype (60). When Asn was replaced with Asp acid (VP2-162), there was a reduction of viral replication in cultured myocytes and fewer lesions per heart section *in vivo*. (61). Subsequently, it has been shown that the attenuated temperature

sensitive (ts) mutants demonstrated inhibited viral RNA synthesis, abnormal capsid polyprotein and defective viral particle assembly (62).

The molecular basis of pathogenesis in EV-A71 is still uncertain. It remains to be investigated if there is a universal molecular determinant present in every fatal EV-A71 sub-genotype strain or whether every fatal sub-genotype strain differs in virulence determinant(s). The analysis of the amino acid sequence of a fatal (EV-A71 strain 5865/Sin/000009) and a non-fatal (EV-A71 strain 5666/Sin/002209) strain isolated from the 2000 outbreak in Singapore showed that there is only a single nucleotide change at position 5262 in the 3A non-structural region. Comparative analysis revealed 99% amino acid similarity between the two strains except for the amino acid 1506. The fatal strain contains adenine (A) at position 5262 in the codon ACU that encoded for threonine, while the non-fatal strain contains guanine (G) in the codon GCU that encoded for alanine (63). This single amino acid could possibly contribute to the virulence of EV-A71 strain 41 (EV-A71 strain 5865/Sin/000009) sub-genotype B4. The mutation introduced at position 5262 did not completely eliminate the ability to replicate and form plaques in RD cells. There was some reduction of viral growth by site-directed mutations (SDM) at this site but the effect was less than the effects brought about by introducing SDM into nt. 475, 487 or a partial deletion created in the 5'-NTR region (PD). In addition, the A486G mutation which corresponds to A480G (Sabin 1), demonstrated *not* to be a significant virulence determinant in EV-A71. The mutation still produced more viral RNA copy number, plaques and VP1 than mutants 5262, 487, 475 and the partial deletant PD (64).

Li *et al.* (2011) compared the sequences of virulent and non-virulent strains and postulated that 4 residues at 2 positions in VP1 (VP1-145G/Q/R and VP1-164E), a residue on protease 2A (Lys in position 930) and four nucleotides at three positions in the 5'-NTR region (G in position 272, U in position 488 and A/U in position 700) are likely to contribute to the EV-A71 virulent phenotype (65). In another study, Yeh *et al.* (2011) reported that nucleotide

158 in the EV-A71 5'-NTR contributed to the virulence of the sub-genotype B1 virus. When cytosine at position 158 was substituted to uridine, this changed the conformation of the RNA secondary structure of stem loop II which led to reduced EV-A71 RNA translation and virulence in mice (66).

Subsequently, Wen et al. (2013) discovered that three positions (VP1-249V/I, Val^{P1148}/Ile^{P1148} in 3A and Ala^{P1728}/Cys^{P1728}/Val^{P1728} in 3C), were conserved among neuro-virulent strains but were different among mild strains of sub-genotype C4a. This implies that these 3 positions maybe potential virulence determinant(s). Interestingly, 2 of the 3 mild strains (SDLY11 and SDLY48) showed similar secondary structure predictions in the 5'-NTR region whereas all neuro-virulent strains (SDLY96, SDLY107 and SDLY153) had differing secondary structure predictions. Amongst all the 3 neuro-virulent strains, SDLY107 in particular varied in 4 positions (C^{P241}/T^{P241}, A^{P571}/T^{P571}, C^{P579}/T^{P579} in 5'-NTR and T^{P7335}/C^{P7335} in 3'-NTR), suggesting that these sites may also be molecular determinants of virulence (67).

Li et al. (2016) identified a critical mutation N1617D, in the 3C region when highly pathogenic strains were compared with lowly virulent strains. This could be due to conformational alterations in the active center of EV-A71, implicating that this aa. substitution could be a potential molecular determinant for the fatal sub-genotype C4a strains (68). Yuan et al. (2016) identified 23 out of 27 positively charged aa. within the VP1 region that could inhibit the production of infectious EV-A71 infectious virions. However, the inhibition could be overcome with second-site mutations, also within the VP1 protein. They discovered that conditional thermostable mutants could be constructed via changing the charge characteristics of VP1 as a K215A mutation in the GH loop decreased thermal stability. Their study showed that EV-A71 infection could be impaired by the inhibition of intra- or intermolecular interactions within the VP1 protein (69). In addition, the NIBSC scientists had constructed a genetically stable OPV strain by modifying the domain V in the 5'-NTR of the PV genome by

deleting any U-G base-pairs. They deduced that mutations in domain V would generate an attenuated PV. In addition, they transferred a crucial cis-acting replication element (cre) to a position close to the 5' end from the P2 region to decrease the loss of this part of the genome via recombination (70). Hence, the genetic stability of the attenuated PV strain would be increased and it would require two recombination events to replace domain V which was highly improbable. Such an approach could be adopted to further attenuate and stabilize the EV-A71 vaccine strain.

3-Dimensional Crystal structures

The 3D crystal structures of the virus-like particles (VLPs) for EV-A71 and chimeric EV-A71/CVA16 VLPs comprised linear and conformational epitopes of EV-A71 that are mostly found in both VLPs. Lyu et al. (2014) discovered that a minimum of eighteen amino acids from the VP1 BC loop (N-terminus) were temporally externalized, making it convenient for peptide insertion to generate recombinant EV-A71 virion. These foreign peptide insertions did not affect capsid structural changes, viral replication and un-coating process (71). Interestingly, a chimeric VLP containing substitution at four residues (K215L, E217A, K218N and E221D) within the GH loop of the SP70 epitope induced NtAb titers against *both* CV-A16 and EV-A71. The original VP1 GH loop in EV-A71 VLPs demonstrated a surface charge potential difference that was markedly different from the chimeric VLP, possibly attributed by the mutations in the GH loop which made it more exposed on the viral particle surface (72).

Wang and colleagues (2012) studied empty and mature EV-A71 viruses and found that empty viruses contained pockets that are collapsed in the EV-A71 capsid. This contributed to new discoveries about the EV-A71 un-coating process whereby the VP1 GH loop acts as an adaptor-sensor for attachment onto cellular receptors (73). Commercialization of such VLPs would not be too long in the future as there are a few licensed VLPs already commercially available such as those for human Papillomavirus (74) and Hepatitis B virus (HBV) (75). In

their subsequent study, Wang et al. (2013) constructed a virus containing nucleating peptides that could direct a calcium phosphate (CaP) bio-mineralization step. The engineered peptide was inserted between residues 100 and 101 on the surface of EV-A71, specifically on the VP1 BC loop. One of the nucleating peptides used was a phosphate chelating agent (N6p) that could raise the efficiency of EV-A71 to initiate CaP mineralization. The binding effect was attributed to phosphate-chelating domains (SVKRGTSVG and VGMKPSP). Interestingly, a mineral exterior that could promote thermo-stability and physicochemical characteristics could be biologically induced onto surfaces of vaccines under physiological conditions. Wang et al. (2013) demonstrated that their self-bio-mineralized vaccine could be kept at 37°C for about 1 week and at 26°C for more than 9 days (76). Genetic technology and bio-mineralization processes could provide improvisations for current vaccination programs, especially in low and middle income countries that are deficient in cold chain infrastructures. A Grand Challenge in Global Health by the Gates Foundation has highly recommended improving the thermo-stability of vaccines.

Bivalent and Trivalent Vaccines against Enteroviruses

The predominant enteroviruses circulating and causing frequent HFMD epidemics are EV-A71 and CV-A16. Hence, biopharmaceutical organizations such as the University of Queensland (Australia) and Hualan Biological Engineering Inc are developing the EV-A71/CV-A16 bivalent vaccine (Table 2). The efficacy and immunogenicity of the bivalent EV-A71/CV-A16 vaccine were evaluated and the results have been encouraging. Cai et al. (2014) found that mice immunized with inactivated bivalent vaccine produced sera that neutralized EV-A71 and CV-A16 *in vitro*. Immunization with the monovalent vaccine elicited protection against either EV-A71 or CV-A16, but passive immunization with the bivalent vaccine protected mice against severe EV-A71 and CV-A16 infections (77). Sun et al. (2014) also showed that the immune sera from animals vaccinated with the bivalent EV-A71/CV-A16 vaccine could induce passive

protection to newborn mice against lethal challenge with 14 LD₅₀ (Lethal dose, 50%) of EV-A71 and 50 LD₅₀ of CV-A16 (78). A trivalent inactivated EV-A71/CV-A16/CV-A6 vaccine was found to confer full protection from lethal challenges against CV-A6 and CV-A16 infected mice. The mice were evaluated in active and passive immunization studies to test the efficacy of the trivalent vaccine (79).

Besides inactivated bivalent vaccines, there are bivalent VLP vaccines being produced by different research groups. Such an example can be found with the secreted form of a bivalent EV-A71/CV-A16 VLP vaccine utilizing a baculovirus-insect cell expression system. Gong et al. (2014) reconstructed three-dimensional (3D) bivalent VLP structures and discovered that these structures resembled natural empty particles of EV-A71 and 135S-like expanded particles of CV-A16. The cryo-electron microscopy results also showed that the linear neutralizing epitopes and conformational epitopes were well preserved in the bivalent VLPs. In addition, immunogenicity tests were carried out in mice with the monovalent EV-A71 VLPs, monovalent CV-A16 VLPs and bivalent EV-A71/CV-A16 VLPs. They discovered that mice immunized with the VLP bivalent/composite-adjuvant (alum and CpG-oligodeoxynucleotides) vaccine was able to induce high NtAb titers ranging from 1:160 to 1:320 against four strains of EV-A71 (804232Y, 8052303F, 804251Y, 8061001Y) and two strains of CV-A16 viruses (705212F, 705213F) (80).

Zhao et al. (2015) also designed bivalent VLPs by replacing the SP70 epitope within the VP1 capsid protein of EV-A71 with that of CV-A16. The ChiEV-A71 VLPs produced in *Saccharomyces cerevisiae* demonstrated similarities in morphology and protein composition as the EV-A71 VLPs. BALB/c neonatal mice immunized with the ChiEV-A71 VLPs showed strong cellular immunity as indicated by the enhanced production of IFN- γ , IL-2, IL-4, and IL-6 in splenocytes. In addition, passive immunization with anti-ChiEV-A71 VLP sera conferred full protection against lethal challenges with both CV-A16 and EV-A71 in neonatal mice (81).

Their study could provide a reference to the design of future multivalent vaccines against EV-A71 and other Coxsackieviruses.

It is intriguing that ADE was observed when mice were challenged with EV-A71 prior to passive transfer of anti-CV-A16 VLP sera. However, no ADE was seen when mice received passive transfer of antisera against the bivalent EV-A71/CV-A16 VLP vaccine formulation. Indeed, the bivalent vaccine conferred full protection against lethal challenge by either EV-A71 or CV-A16 (82). Immunogenicity was compared between monovalent EV-A71 and CV-A16 IVs, monovalent EV-A71 and CV-A16 VLPs and together with bivalent EV-A71/CV-A16 VLPs. The study showed that the immune sera from the bivalent VLP vaccinated mice could protect neonatal mice from lethal challenges with either EV-A71 or CV-A16. In addition, the NtAbs were able to cross protect against other sub-genotypes of EV-A71 or CV-A16 (78). Recent large scale HFMD outbreaks in China have increasingly isolated CV-A10 as the main etiological agent. Liu et al. (2015) produced CV-A10 VLPs that could stimulate the production of NtAbs against CV-A10 infection *in vitro* (83). These studies showed that bivalent vaccination is indeed a promising approach for HFMD vaccine development as it has added advantages of being highly immunogenic, noninfectious, and accessible to quality control.

Nevertheless, there remains considerable interest as to which HFMD-causing serotype should be selected in a bivalent or trivalent vaccine. As EV-A71 and CV-A16 were found to co-circulate during HFMD outbreaks and most of the cases were due to EV-A71 and CV-A16, it would be desirable for a bivalent EV-A71/CV-A16 vaccine to be produced (84). In addition, the bivalent vaccine should be able to protect against the EV-A71 sub-genotype C4 or B4 as these sub-genotypes were the predominant ones causing fatal HFMD. Moreover, NtAbs elicited by C4 and B4 have been found to cross neutralize against other EV-A71 sub-genotypes (42, 85). A study by Chou et al. (2012) demonstrated that their IV based on the sub-genotype B4 could elicit cross-neutralizing antibodies against sub-genotypes B1, B5 and C4A with NtAb

titers ranging from 1:64 to 1:1280 (85). It greatly simplifies the choice of vaccine strain for EV-A71 as immunization with one sub-genotype could potentially cross-protect against all other sub-genotypes. This is in contrast to the polio vaccine whereby 3 serotypes were required to construct the oral polio vaccine. In addition, the chosen strain should be able to grow to high titers in a FDA approved cell line.

It is also important to address any manufacturing problems that may occur in the vaccine production process. The chosen bivalent vaccine comprising EV-A71/CV-A16 strains has to show strong genetic stability during passages based on the nucleotide sequences of the master virus seed, working seed banks and virus harvested from the production lots. These nt. sequences have to be identical with that of the original isolate (86). It is well known that serum protein is needed for optimal growth in cells as it provides a source of nutrients and growth factors. However, large amounts of serum proteins in the cell culture medium could make downstream purification significantly more difficult. Hence, virus production from cell culture grown in serum-free (SF) medium is preferable (87). Nevertheless, growth in SF medium may not represent optimal conditions for cell growth and high viral titer.

An outstanding manufacturing challenge would be the lack of financial support and technology transfer from foreign vaccine manufacturers. Currently, roller-bottles, cell factories and micro-carrier bioreactors are used for manufacturing bioprocesses. To produce more than 2 million EV-A71 vaccine doses annually, the micro-carrier bioreactor technology is the most ideal technology, but assembling skillful technical staff, and validating manufacturing infrastructure would require expensive investments of up to USD 35 million. In addition, new technologies such as “Wave” by Wave Biotechnology (GE Healthcare), “TideCell” by CESCO Bioengineering Co (Taiwan), single-use bioreactors by Hyclone (USA) and Sartorius Stedim Biotech (Germany) could provide easy start-up and disposable cell culture technology (88).

Other alternatives would be the usage of micro-carrier beads that increase surface area for cell growth (89).

It would be desirable to produce a cost effective bivalent EV-A71/CV-A16 or trivalent EV-A71/CV-A6/CV-A10 vaccine. As there remains insufficient data for the costing of a bivalent vaccine, forecasting of monovalent EV-A71 vaccines has been reported. Chong et al (2012) indicated that they could manufacture 50,000 doses of EV-A71 vaccine at 0.40 USD per dose (86). Interestingly, Lee et al (2010) projected that the EV-A71 monovalent vaccine could be sold at \$50 or \$75 US dollar per dose (90). However, the cost of immunization is more likely to be ~ \$30 per dose with 2 doses needed for primary immunization as reported by Mao et al. (2016) (91). Due to such a high profit margin, this may generate interest among global biopharmaceutical companies in the manufacturing of a bivalent EV-A71/CV-A16 vaccine (92).

Conclusion

The absence of FDA-approved vaccines for prevention of HFMD caused by EV-A71 has intensified research into EV-A71 vaccine development. Several novel vaccine candidates have been examined in this review and may have the potential to be developed further due to their high efficacy of protection or their potency against fatal HFMD infections. The IV is the lead vaccine candidate to enter the China market but it has only 80% efficacy against severe HFMD. There remains a greater need to understand how genomic changes brought about by spontaneous mutations of the viral genome could impact pathogenicity and antigenicity. The analysis of the 3D crystal structures of EV-A71 sub-genotypes should further enable the identification of critical nucleotides that may lead to amino acid changes which could alter the 3D crystal structures of EV-A71 and lead to changes in pathogenicity and antigenicity. Such genetic changes may contribute to distinct antigenic properties of different sub-genotypes. This

would inadvertently impact the rational design of vaccines based on a greater understanding of structure and function relationships.

Abbreviations

EV-A71, Enterovirus-A71; PV, Poliovirus; 5'-NTR, 5'- non coding region; nt, nucleotide; DNA deoxyribonucleic acid; cDNA, complementary DNA; kb, kilobase; kDa, kilodaltons; ORF, open reading frame; OPV, oral poliovirus vaccine; RNA, ribonucleic acid; IV, inactivated vaccine; LAV, live attenuated vaccine; VLP, virus-like particle; GMT; Geometric mean titers; wt, wild type; 3DPol, 3D Polymerase.

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Authors' contributions

PT Yee and CL Poh conceived and designed the manuscript. PT Yee prepared the manuscript and CL Poh provided critique and amendments to the manuscript. All authors have read and approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

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