Attenuation of Virulence as Antimicrobial Strategy

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Vaccination for viral diseases has markedly reduced mortality worldwide for more than 200 years. Nevertheless, the future is abounding with challenges as there remain many diseases that do not yet have effective vaccines. Although there are more vaccines undergoing clinical trials, humans remain vulnerable to the existing 180 or so viruses that have no effective vaccines. Therefore, increased research is required in the development of new and better vaccines. Of increasing interest in recent years would be the design of live attenuated vaccines (LAV). This type of vaccine is cheaper to produce, induces excellent immunogenicity, does not need boosters and confers live-long immunity. With a growing understanding of the molecular basis of virulence in diseases, safe and effective LAVs can be developed through attenuation, optimization of immunogenicity and genetic stability. In this paper, attenuation of virulence for viruses such as Poliovirus, Enterovirus 71, Coxsackievirus B3 and B4 are examined.

\textbf{Keywords}: Poliovirus; Enterovirus 71; Coxsackievirus B3; Coxsackievirus B4; attenuation

1. Poliovirus

1.1 Poliovirus vaccine

Since the 1950s, there have been two safe and effective vaccines against poliomyelitis. The first vaccine developed in 1952 by Dr Jonas Salk was the inactivated polio vaccine (IPV). The IPV was made from formalin-inactivation of three wild-type virulent polio strains which are the Mahoney (type 1), MEF-1 (type 2) and Saukett (type 3) \cite{1}. The second effective vaccine would be the live attenuated oral polio vaccine (OPV). The Oral Polio Vaccine (OPV) is an attenuated vaccine which has reduced worldwide poliomyelitis caused by poliovirus (PV) infection. The IPV was the only poliovirus vaccine available until licensure of the Oral Poliovirus Vaccine (OPV) in 1962 \cite{2}. There was a need for an OPV in developing countries as it is a live attenuated vaccine which has long-lasting immune response and regular boosters are not needed. One time oral administration of the OPV was found to provide lifelong protection against poliomyelitis. Elimination of poliomyelitis in the developing world was achieved mainly through mass vaccination with the OPV despite its ability to revert to the wild type in 1 out of 750, 000 vaccines \cite{3}.

Due to the success of global OPV vaccination programs, the World Health Assembly declared in 1988 that polio should be eradicated by the year 2000, aligned with the success of the small pox eradication program. However, the eradication deadline was repeatedly postponed and has not been met till today. This is due to the fact that there are several countries where polio persistently remained endemic such as in Nigeria whereby the population remained resistant to polio immunization or India where OPV had low efficacies due to high population and poor sanitation \cite{4}. Therefore, there is a need to have a greater understanding of the molecular determinants of neurovirulence in PV to develop new and better vaccines based on genetic manipulations to render the virus non-pathogenic, yet containing similar antigenic structures to the wild type PV.

1.2 Molecular Determinants of Neurovirulence

The molecular determinants of neurovireulence in PV have been determined. The polio virus is an enterovirus from the family \textit{Picornaviridae}. The PV has a 5' non-translated (NTR) cloverleaf structure and a 3'-poly (A) tail. Domain I is important for virus replication and domains II-VI encompass the internal ribosome entry site (IRES) that directs translation of mRNA by internal ribosome binding (Figure 1). If there are mutations in the 5'-NTR, this decreases multiplication efficiency, alters cell tropism and attenuates virulence \cite{2}.

There are three attenuated strains being used as OPV: Sabin 1 was derived from the Mahoney strain, Sabin 2 was derived from the P172 strain and Sabin 3 was derived from the Leon strain. Identification of the genetic determinants of attenuation of the Sabin OPV strains has been comprehensively reviewed \cite{2}. The complete sequences of the three PV genomes and the development of infectious poliovirus complementary Deoxyribonucleic Acid (cDNA) clones have led to the systematic investigations of the critical mutations responsible for the attenuated phenotypes of the Sabin OPV strains.

From the analysis of nucleotide (nt) sequences present in the three PV Sabin strains, nucleotide substitutions which were critical in attenuating mutations in the virulent strains isolated from cerebrospinal fluid were identified. There are 57 nucleotide substitutions distinguishing the Sabin 1 strain from its parent strain \cite{5}.
Fig. 1 Structure and genome of Poliovirus. All structural proteins: VP1, VP2 and VP3 are encoded by the P1 region of the genome. The P2 and P3 genes encode for seven non-structural proteins: 2A-2C and 3A-3D [2].

Among these nucleotide substitutions, the A480G in the IRES is the most important determinant of the attenuated phenotype of Sabin 1. Their studies strongly suggested that nt. 480 influences the formation of a highly ordered structure in the 5'-NTR that is responsible for neurovirulence [6]. Four other nucleotide substitutions contributing to the attenuated phenotype were mapped to the capsid region. There was one in VP4, one in VP3 and two in VP1. In addition, there was also one substitution that contributed to the temperature-sensitive phenotype mapped to the 3DPol region [7, 8].

However, there were only 2 nt. substitutions found in the Sabin 2 strain that appeared at position 481 within the IRES region and position 2909 within VP1 (Figure 2). For Sabin 3, a total of 10 nt. substitutions were found to differ from its parent strain, but only 3 substitutions appeared to be the main determinants for the attenuated phenotype (C274U in IRES, C2034U in VP3, and U2493C in VP1) [9]. Sabin 3 strain was also found to be the most genetically unstable of the three Sabin strains. As a result of the analysis of the molecular determinants of attenuation, in vitro construction of piconaviruses with reduced-virulence could be performed via the introduction of mutations in the 5'-NTR to reduce the efficiency of viral replication.

Fig. 2 Three genotypes of Poliovirus Sabin vaccine strains. All contain a determinant of attenuation in the 5' NTR of ssRNA mutation that reduces viral replication. They are nt. 480 in Sabin 1, nt. 481 in Sabin 2 and nt. 472 in Sabin 3 [1, 6]. The 5'NTR of EV71_41 is highly homologous to the Poliovirus genome at the three indicated positions of attenuation.

1.3 Recent advances in developing new polio vaccines

One of the most anticipated next generation vaccines is an IPV based on the attenuated Sabin poliovirus strains, producing a Sabin IPV (sIPV). The sIPV is produced from poliovirus strains that have an antigenic structure identical to the currently used wild-type strains but were rendered non-pathogenic by genetic manipulations. Of interest would be the manipulations of the IRES within the 5'-NTR to produce genetically-stable OPV strains such as the removal of U-G base-pairs in domain V of the 5'-NTR in the PV genome and insertion of the cis-acting replication element (cre) from the P2 region to a position near the 5' end to reduce the risk of loss of this part of the genome through recombination. Any single mutation in base-paired stems would weaken the domain V and generate a more attenuated virus. Figure 3 illustrates the production of genetically-stable OPV strains. In this way, two recombination events would be required to replace domain V which is highly unlikely [10].

In addition to that, thermostable PV virus-like particles (VLPs) that have similar antigenicity as the wild type PV but are stable enough to allow vaccine production are currently being constructed. VLPs are devoid of viral RNA but are produced naturally during PV infections. The external surface of a VLP is indistinguishable from that of a PV particle but several internal amino acid chains are disordered (Figure 4). The National Institute for Biological Standards and Control (NIBSC) are developing the production of thermostable PV virus-like particles (VLP) using recombinant mammalian, yeast, bacterial, baculovirus and plant expression systems in collaboration with several UK laboratories. They have identified several amino acid substitutions that stabilise VLPs without altering antigenicity and these VLPs appear as thermostable as the current IPV [10].
2. Enterovirus 71

There are approximately 100 serotypes of enteroviruses that are segregated into four classifications, based on their biological and molecular properties. The four human enterovirus species (A-D) are the polioviruses which belong to species C (3 serotypes), the non-polio species A enterovirus includes some coxsackie A viruses, EV71 and others (25 serotypes), species B includes coxsackie B viruses, Echoviruses and other enteroviruses and is represented by 63 serotypes, and species C is represented by more Coxsackie A virus and Enteroviruses (23 serotypes) and species D is represented by 5 serotypes. Enteroviruses are resistant to alcohol, organic solvents, freezing but can be inactivated by temperatures higher than 56°C. The enteroviruses that can cause the hand, foot and mouth disease (HFMD) are the Enterovirus 71 (EV71), Coxsackie type A16 (CVA16), CVA5, CVA8 and CVA10 [11]. Table 1 summarizes the various clinical manifestations of enteroviral infections.

![Fig. 3](image1.png)

**Fig. 3** Production of genetically-stable OPV strains. All the U-G base-pairs in domain V was removed to make it more genetically stable and the cis-acting replication element (cre) was moved from the P2 region to a position near the 5′ end to reduce the risk of loss of this part of the genome through recombination [10].

![Fig. 4](image2.png)

**Fig. 4** The difference between a VLP and virus particle. The external surface of a VLP is indistinguishable from that of a PV particle but several internal amino acid chains are disordered [10].

Coxsackie viruses are common etiological agents of HMFD but they do not generally cause neurological or cardiopulmonary disease. In contrast, EV71 is the main causative agent of serious neurological infections with fatal outcomes in several large scale outbreaks of HFMD [12]. The EV71 virus is classified as Human enterovirus A (HEV-A) species, belonging to the genus Enterovirus in the family *Picornaviridae*, together with some Coxsackie A viruses [13]. The EV71 virus is a non-enveloped icosahedral viral particle that contains a single-stranded, positive sense, polyadenylated viral Ribonucleic Acid (RNA) of approximately 7.4kb (Figure 5). The capsid is made up of 60 protomers, each consisting of 4 polypeptides that comprise the structural proteins, VP1, VP2, VP3 and VP4. Of all the
polypeptides, VP4 is located on the internal side of the capsid while VP1, VP2 and VP3 are located on the external surface of the EV71 virus [14].

The EV71 virus commonly causes the hand, foot and mouth disease (HFMD) in young children less than 6 years of age. Although EV71 started circulating as early as 1963 in the Netherlands, EV71 was first reported to be isolated in 1969 from the stool specimen of an infant with serious nervous system disease in California [16]. EV71 has caused more than 3 million infections worldwide and approximately 0.15% of the infections led to death [17]. Mild symptoms of EV71 infection in children range from fever (≥ 39°C), sore throat, loss of appetite and rash with vesicles on hands, foot and diaper area. In addition, rupture of the vesicles would lead to ulcers in the throat, mouth and tongue. EV71 can produce more severe symptoms such as aseptic meningitis, brain stem encephalitis, acute flaccid paralysis, neurogenic pulmonary oedema, delayed neurodevelopment and reduced cognitive function [12].

Table 1  Clinical manifestations of enteroviruses.

<table>
<thead>
<tr>
<th>Enterovirus Serotypes</th>
<th>Clinical Manifestations</th>
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<tbody>
<tr>
<td>Poliovirus 1 to 3</td>
<td>Paralysis</td>
</tr>
<tr>
<td>Echovirus 4, 6, 9, 11, 30; Enterovirus 70, 71</td>
<td></td>
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<tr>
<td>Poliovirus 1 to 3; Coxsackievirus A2, A4, A7, A9, A10, B1 to B6; Echovirus 1 to 11, 13 to 23, 25, 27, 28, 30, 31; Enterovirus 70, 71</td>
<td>Aseptic Meningitis</td>
</tr>
<tr>
<td>Coxsackievirus A5, A8, A10, A16, Enterovirus 71</td>
<td></td>
</tr>
<tr>
<td>Coxsackievirus A2 to A6, A8, A10</td>
<td>Hand, foot and mouth disease (HFMD)</td>
</tr>
<tr>
<td>Coxsackievirus A24, Enterovirus 70</td>
<td>Herpangina</td>
</tr>
<tr>
<td>Echovirus 2, 6, 9, 19</td>
<td>Acute hemorrhagic conjunctivitis</td>
</tr>
<tr>
<td>Coxsackievirus B1 to B5, Enterovirus 71</td>
<td>Encephalitis</td>
</tr>
<tr>
<td>Coxsackievirus B3</td>
<td>Meningoencephalitis</td>
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<tr>
<td></td>
<td>Pericarditis, myocarditis</td>
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Each symptom may potentially be caused by more than one enterovirus [15].

The lack of vaccines and antiviral drugs against EV71 highlights the urgency and significance of developing preventive and treatment agents against EV71 to prevent further fatalities. Research groups have developed experimental inactivated vaccines [18], recombinant VP1 vaccine [19], live attenuated vaccines [20, 21] virus-like particles [22], synthetic peptide vaccine [23, 24] and DNA vaccine [25]. The live attenuated vaccine is cheaper to produce, induces excellent immunogenicity, does not need boosters and confers live-long immunity. This is because the LAV elicits both the humoral and cellular immunity, alongside the innate and adaptive immunity.

Li et al. (2011) compared the sequences of virulent and non-virulent strains and concluded that four amino acids at two positions (Gly/Gln/Arg in position 710 and Glu in position 729) in the VP1, one (Lys in position 930) and four
nucleotides at three positions (G in position 272, U in position 488 and A/U in position 700) in the 5'-NTR region are likely to contribute to the EV71 virulent phenotype [26]. In another study, Yeh et al. (2011) reported that nucleotide 158 in the EV71 5'-NTR contributed to the virulence of the virus belonging to sub-genotype B1. A single nucleotide change from cytosine to uridine at position 158 caused an alteration in the RNA secondary structure of stem loop II which led to reduced viral translation and virulence in mice [27].

An earlier study published by Izuke et al. (1989) demonstrated that deletion from nucleotides 564 to 726 in the 5'-NTR of the poliovirus Mahoney genome was successfully expressed as a stable, less neurovirulent phenotype [28]. Mutant strains carrying long deletions maybe more stable and safer than those carrying point mutations, short deletions or short insertions [29]. Therefore, it is of interest that by changing the nucleotides in the virulence-associated positions of the viral genome, neurovirulence can be reduced and genetic stability increased to reduce the possibility of reversion to virulence. However, complete stability may be difficult to attain as there may be other virulent determinants of attenuation that have yet to be discovered. In addition, recombination between different group C enteroviruses occur so frequently in humans that exchange of the attenuation region maybe inevitable, especially if there is high circulation of viruses [30].

3. Coxsackievirus B3

Coxsackievirus B3 (CVB3), one of six CVB serotypes, is a member of the genus Enterovirus within the family Picornaviridae. The CVB3 virus is a non-enveloped icosahedral viral particle that contains a single-stranded, positive sense, polyadenylated viral Ribonucleic Acid (RNA) of approximately 7.4k b in length. The open reading frame is subdivided into three regions, P1, P2 and P3. The four viral capsid proteins: VP1 (281 aa), VP2 (263 aa), VP3 (238 aa), and VP4 (69 aa) are encoded within the P1 region. The P2 and P3 regions encode non-structural proteins that are involved in viral replication (Dunn et al., 2000). The near atomic structure of the CVB3 shows high similarity to that of poliovirus type 1 [31] and rhinovirus [32].

Coxsackie B viruses have been identified as a major cause of myocarditis in humans and are implicated in the pathogenesis of dilated cardiomyopathy [33]. Similar to EV71, the primary age group that suffer more severe clinical syndromes are children and neonates. Over a 4-year period, 45% of infant cases reported to the CDC Enteroviral Surveillance Program were attributed to CVB infection [34]. Hence, researchers are trying to elucidate the mechanism by which CVB cause lethal infections. The enteroviral capsid (VP1-VP4) has been shown to contain determinants contributing to the pathogenic phenotype of CVB4 [35], CVB3 [36], and the polioviruses (PVs) [37].

However, as exemplified by human immunodeficiency virus, laboratory strains are sometimes so phenotypically and genotypically distinct from those found in infected individuals that therapeutic or preventative measures effective against these laboratory strains do not always correlate with naturally circulating strains [38]. For example, it was reported that the presence of Asn at position 165 of VP2 was responsible for a cardiocirculatory phenotype [36]. However, upon analysis of multiple CVB3 clinical isolates, Dunn and co-workers (2000) discovered that all strains of CVB3 (cardiovirulent and non-cardiovirulent) encode Asn at position 165 [39]. It could be possible that the mapping of attenuation by Knowlton et al. (1996) that Asn165 was responsible for reduced virulence was attributed to laboratory strains and not naturally circulating ones.

In another example, Tu and colleagues (1995) observed that a U→C mutation at nt. 234 within the CVB3 5'-NTR resulted in attenuation of the cardiovirulent phenotype in mice [40]. However, Chapman et al. (1997) and Romero et al. (1997) have discovered that multiple CVB3 strains always had a U regardless of cardiocirculatory phenotype [41, 42]. It could be possible that the findings of Tu and colleagues were dependent on strains engineered by biological means in the laboratory and thereby, not found in naturally circulating CVB3 strains. Recent studies using artificially attenuated strains engineered in the lab have demonstrated that specific nucleotide(s) within the 5'-NTR are known to alter the virulent phenotype of the PVs [43], CVB1 [44], and CVB3 [40]. A more powerful tool in the study of the genetic basis of picornaviruses would be to engineer recombinant chimeric viruses derived from the cDNA clones of virulent and non-virulent viruses.

Dunn and colleagues (2000) carried out the first study to map the genetics of enterovirus virulence using wild-type strains [39]. They constructed six intratypic chimeric viruses in which 5'-NTR and capsid sequences of the infectious cDNA copy of the cardiocirculatory CVB3/20 genome was replaced by homologous sequences from non-cardiovirulent CVB3/CO or cardiocirculatory CVB3/AS strains (Figure 6). Chimeric strains were then tested for cardiovirulence by inoculation of C3H/HeJ mice. They discovered that replacement of the homologous region of non-cardiovirulent CVB3/CO with the cardiocirculatory CVB3/20 capsid coding region maintained the cardiovirulent CVB3/20 phenotype. However, recombinant virus containing the non-cardiovirulent CVB3/CO 5'-NTR alone or the 5'-NTR and capsid sequences together were not myocarditis, and infectious virus was not recovered from the murine heart. Chimeric viruses containing the cardiovirulent CVB3/AS 5'-NTR alone, capsid sequence alone, or both together preserved the myocarditic phenotype. Taken altogether, their results are consistent with previous studies that sequences within the CVB3 5'-NTR are the primary determinants of the myocarditic phenotype of CVB3 strains.
Coxsackievirus B4 (CVB4) is an enterovirus from the family Picornaviridae. The virus genome consists of a single-stranded RNA of positive polarity. The open reading frame which is flanked by the 5' and 3'-NTRs is divided into three regions, P1, P2, and P3. The four capsid proteins, VP1 through VP4, are encoded within the P1 region, while the non-structural proteins that are involved in viral replication, translation and processing of poly-protein are encoded within the P2 and P3 regions [35]. CVB4 is the etiological agent of type I insulin-dependent diabetes and pancreatitis [45]. The existence of variants within a single serotype further complicates the pathogenesis of coxsackievirus infections.

Previous studies have shown that a major determinant of CVB4 virulence is the 5' end of the genome, which encompasses both the 5'-NTR and the P1 region [46]. Ramsingh and co-workers (1995) demonstrated that Arg-16 of VP4 is a determinant of virulence. They constructed chimeric viruses (vCB424) from cDNA clones of virulent (CB4-V) and avirulent (CB4-P) strains (Figure 7). They then injected congenic B10.T6(R) mice with 10^4 PFU of virus and the mice were sacrificed at various times post-infection. Their pancreatic tissues were stained and fixed for further analysis. They discovered that pancreatic tissues from mice infected with vCB424 showed loss of groups of acini with the appearance of numerous small ductular structures of unknown origin, while that from CB4-P-infected mice contained well-preserved acini and islet cells. In addition, the chimeric virus exhibited a large-plaque phenotype, suggesting that the Arg substitution at position 16 of VP4 affected virus replication in vitro [46].
The top line depicts the structural organization of the coxsackievirus B4 (JVB strain) genome (Jenkins et al., 1987). A partial restriction map of the subclones used for the construction of chimeric cDNAs is shown. One pair of subclones contained the 5' UTR and the P1 regions of CB4-P and CB4-V as XbaI-HindIII fragments. Another pair contained the P2 and P3 regions and the 3' UTR of CB4-P and CB4-V as HindIII-SacI inserts [46].

Taken altogether, their results indicated that the recombinant virus (vCB424) containing Arg-16 of VP4 in an avirulent genetic background resulted in a phenotype that was intermediate between those of the two parental virulent (CB4-V) and avirulent (CB4-P) strains. This could be due to a gain of a positive charge at VP4-16, which may have affected the interaction of VP4 with the negatively charged viral genomic RNA thereby, affecting viral replication [46]. With this understanding of the molecular basis of virulence in CVB4, safe and effective LAVs against pancreatitis can be developed through optimization of immunogenicity and genetic stability.

Besides pancreatitis, CVB4 is also the etiological agent of type I insulin-dependent diabetes (T1D). This disease occurs when an immune cellular infiltration kills insulin-producing β-cells in the pancreatic islets involving host genetics [47, 48] and environmental influences [49, 50]. Symptoms of T1D were first described in ancient Greece by Aretaeus [51]. Unlike poliomyelitis, T1D incidences remained low and relatively constant till the 1940s, climbing rapidly only after the early 1950s [52].

It could be possible that before World War II, hygiene and sanitation was lacking and hence, this suppressed CVB4 replication within the pancreatic cells. Drescher et al. (2015) also hypothesised that this could be due to an increase in protective regulatory T cell (Treg) levels in the pancreas early in life that was maintained throughout one's life. This would have also suppressed host autoimmune effector T cells (Teff), thereby reducing T1D infections [53]. This was demonstrated in young NOD mouse which were protected from autoimmune-driven T1D when inoculated with CVB4. The protected mice had higher levels of Tregs (CD4+, CD25+, FoxP3+ T cells) and TGF-β2 in pancreatic lymph nodes and spleen than control mice [54]. Therefore, a suitable vaccine against T1D should ideally be one that increases Tregs and TGF-β2 levels relative to the Teff cell pool. There is no evidence that a single enterovirus is responsible for inducing T1D throughout the world. Hence, it would be good to proceed initially with an injectable inactivated vaccine containing several different enteroviral serotypes that could be followed with an attenuated version. This would fully stimulate the immune response as would natural infections but in a safe manner (Figure 8). This approach represents an initial step toward recognizing the importance of enteroviral infections associated with human T1D etiology and may provide information regarding the impact of a traditionally delivered vaccine approach upon the modulation of T1D-protective Treg and Teff activity.

**Fig. 8** Approaching vaccination for the suppression of T1D. (A) A pancreas with developing autoimmune insulitis is infected by an enterovirus. The damage to the islets from the host's lymphocytic infiltration permits enterovirus replication which leads to T1D onset. (B) An individual is exposed to enteroviruses in a vaccine induces a protective Treg population which suppresses the development of the autoimmune insulitis [53].
5. Conclusion

Currently, there are only an estimated 15 vaccines to combat the 200 viruses known to infect man. With the discovery of virulence determinants for Enterovirus 71, Coxsackieviruses B3 and B4, rational design of a live attenuated vaccine based on site-directed mutagenesis of specific nucleotides allows control of the primary structure of proteins. Therefore, it is of interest that by changing the nucleotides in the virulence-associated positions of the viral genome, neurovirulence can be attenuated and genetic stability increased to reduce the possibility of reversion to virulence. Increased studies are required in the development of new and better vaccines, whereby high efficacy, long-lasting immunity, minimal risk of vaccines, safe and easy production, low cost, dispensing the need for refrigeration and convenient delivery are the major goals in their design.

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