



## Therapeutic and prevention strategies against human enterovirus 71 infection

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### Abstract

Human enterovirus 71 (HEV71) is the cause of hand, foot and mouth disease and associated neurological complications in children under five years of age. There has been an increase in HEV71 epidemic activity throughout the Asia-Pacific region in the past decade, and it is predicted to replace poliovirus as the extant neurotropic enterovirus of highest global public health significance. To date there is no effective antiviral treatment and no vaccine is available to prevent HEV71 infection. The increase in prevalence, virulence and

geographic spread of HEV71 infection over the past decade provides increasing incentive for the development of new therapeutic and prevention strategies against this emerging viral infection. The current review focuses on the potential, advantages and disadvantages of these strategies. Since the explosion of outbreaks leading to large epidemics in China, research in natural therapeutic products has identified several groups of compounds with anti-HEV71 activities. Concurrently, the search for effective synthetic antivirals has produced promising results. Other therapeutic strategies including immunotherapy and the use of oligonucleotides have also been explored. A sound prevention strategy is crucial in order to control the spread of HEV71. To this end the ultimate goal is the rapid development, regulatory approval and widespread implementation of a safe and effective vaccine. The various forms of HEV71 vaccine designs are highlighted in this review. Given the rapid progress of research in this area, eradication of the virus is likely to be achieved.

**Key words:** Human enterovirus 71; Infection; Therapy; Prevention; Drugs; Vaccine

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**Core tip:** This review focuses on therapeutic and prevention strategies for the control of human enterovirus 71 infection. Therapeutic strategies highlighted include natural products, synthetic antivirals, immunotherapy, and the use of oligonucleotides. Prevention strategies such as surveillance, physical prevention, and vaccine development form the second part of the review.

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## INTRODUCTION

Human enterovirus 71 (HEV71) is a member of the human enterovirus A species within the genus *Enterovirus* of the family *Picornaviridae*. It is a positive-stranded RNA virus of approximately 7500 nucleotides. The viral genome contains an open reading frame (ORF) encoding a polyprotein of 2194 amino acids. The ORF is divided into three regions: P1 encodes four structural proteins (VP1-VP4); P2 (2A-2C) and P3 (3A-3D) encode seven non-structural proteins. The ORF is flanked by 5' and 3' untranslated regions. A poly-A tail of variable length is covalently attached to the 3' terminus of the genome<sup>[1]</sup>.

Since its discovery in 1969, HEV71 has been identified as the cause of epidemics of hand-foot-and-mouth disease (HFMD) associated with severe neurological complications, including aseptic meningitis, brainstem encephalitis, acute flaccid paralysis and neurogenic pulmonary oedema, in children under five years of age<sup>[1]</sup>. There has been a large increase in HEV71 epidemic activity throughout the Asia-Pacific region since 1997. A large epidemic occurred in Taiwan in 1998, with  $1.3 \times 10^5$  cases of HFMD, 405 cases of severe neurological disease and 78 fatalities attributed to HEV71 infection<sup>[2-4]</sup>. In 1999, a large HFMD outbreak occurred in Perth, Western Australia, with approximately  $6 \times 10^3$  cases reported and 29 cases of severe neurological disease identified<sup>[5]</sup>. From 2008 to 2011, circulating HFMD outbreaks occurred throughout mainland China, increasing the annual number of HFMD cases from 488955 (126 deaths) to 1619706 (509 deaths)<sup>[6]</sup>. In 2010, the largest recorded outbreak of HEV71-associated HFMD occurred in the country, comprising more than 1.7 million cases, including 27000 patients who exhibited severe neurological complications, and 905 deaths<sup>[7]</sup>. Smaller epidemics have been detected in the United States and European countries, such as Austria, Germany, France, Norway, United Kingdom, Hungary and Greece<sup>[8-14]</sup>.

The reasons for the emergence of HEV71 as a cause of large epidemics of HFMD and acute neurological disease in the Asia-Pacific region remain elusive. Upon successful completion of the WHO-sponsored eradication of poliomyelitis, HEV71 will become the extant neurotropic enterovirus of highest global public health significance. However there are currently no effective clinical therapies or vaccine for HEV71 associated HFMD. Symptoms such as fever, encephalitis and meningitis are eased by supportive medication. In some cases viral infections are treated with broad-spectrum antiviral drugs, including Ribavirin, Ganciclovir, and Acyclovir<sup>[15]</sup>. These common remedies only partially alleviate the symptoms instead of controlling the infections, and usually come with high cytotoxicity. Although ribavirin has been reported to inhibit virus production *in vivo*, a very high dose is used for treatment, which may raise safety concerns. Other than symptomatic treatment, intravenous immunoglobulin (IVIG) is clinically used to neutralise the virus and to non-specifically suppress

inflammation. Considering the morbidity and mortality caused by the disease, it is important to develop new specialised drugs and ultimately a safe and effective vaccine for the control of HEV71 infection. This review focuses on the efforts and progress towards development of effective therapeutic and prevention strategies.

## THERAPEUTIC STRATEGIES

In recent years, significant amount of effort has been made to develop antiviral drugs for the treatment of HEV71 associated HFMD. Promising candidates have been identified through the screening of natural therapeutic products, repositioning of existing antiviral drugs, as well as the development of new synthetic compounds. Many of these drugs show anti-HEV71 activity *in vitro*, and some have been evaluated in animal models. However, clinical application of these drugs is not yet available.

### Natural therapeutic products

Natural therapeutic products have been used in many countries in Asia for centuries, and have gradually been adopted by Western medical treatment and health care<sup>[16,17]</sup>. The WHO estimates that approximately 80% of the global population still relies on traditional medicine for primary health care<sup>[18]</sup>. As such, the search for new bioactive molecules in plants is still an active part of pharmaceutical research in many key therapeutic areas, including immunosuppression and infectious disease<sup>[19]</sup>. Antiviral activities have been identified in several hundred natural compounds worldwide. Compared to synthetic pharmaceutical drugs, an advantage of natural molecules is the exclusion of extra chemical synthesis. This may reduce the cost of production, which is particularly attractive to affected patient population from low income countries.

Most natural therapeutic products work as a mixture, and thus it is difficult to characterise the detailed antiviral mechanisms and to further develop into effective clinical drugs. Up till recently, no single compound has been identified to potently inhibit HEV71. However, during the HFMD outbreaks in China, traditional Chinese medicines have demonstrated therapeutic efficacy by ameliorating the symptoms of the disease and/or shortening the course of the disease<sup>[20]</sup>. Most of the herbs with reported therapeutic effectiveness have been used traditionally or folklorically for inflammatory and/or infectious diseases. As disease outbreaks become more common in China, a significant increase of research in this area followed. Table 1 compares natural therapeutic products that have been well studied.

**Hydrolysable ellagitannins:** The most widely published natural molecules in association with HEV71 infection are ellagitannins, from the family of hydrolysable tannins. Ellagitannins are characterised by the presence of one or more hexahydroxydiphenoyl

**Table 1** Natural therapeutic products tested for anti-human enterovirus 71 activity

Natural product (Group)	Tested	Possible mechanism	Advantages	Disadvantages	Ref.
Hydrolysable Ellagitannins	<i>in vitro/in vivo</i>	Inhibit viral absorption/penetration	No obvious side effects	Weak oral activity	[21-30]
Flavonoids	<i>in vitro</i>	Inhibit viral RNA/protein synthesis	Low escape mutants	Mechanism not clear	[18,31-35]
Alkaloids	<i>in vitro/in vivo</i>	Inhibit protein synthesis	No obvious side effects	Mechanism not clear	[36-38]
Deferoxamine	<i>in vitro/in vivo</i>	Upregulation of B cells	Previous US FDA approval for treatment of iron overload	N/A	[39,40]

N/A: Not available; US FDA : United States Food and Drug Administration.

(HHDP) unit(s) on a glucopyranose core. The HHDP group is biosynthetically formed through intramolecular, oxidative C-C bond formation between neighboring galloyl groups in galloylglucoses<sup>[21]</sup>. They are easily hydrolysed, either enzymatically or with acid, to liberate a stable ellagic acid as the dilactone form of hexahydroxydiphenic acid. Hydrolysable ellagitannins have previously shown medicinal values and antiviral effects<sup>[22-25]</sup>.

Treatment with hydrolysable ellagitannins such as corilagin<sup>[26]</sup>, geraniin<sup>[27]</sup>, punicalagin<sup>[25]</sup> and chebulagic acid<sup>[28]</sup> enhanced the survival of HEV71-infected cells *in vitro* with low cytotoxicity. Further, geraniin, punicalagin and chebulagic acid was shown to greatly prolong the survival time and reduce mortality of HEV71-infected mice. Virus replication in the muscle of treated mice was shown to be significantly inhibited. In general, treatment did not cause any obvious side effects in the mice and full recovery was observed after two weeks. The antiviral mechanism of chebulagic acid against herpes simplex virus-1 (HSV-1) was previously published<sup>[22]</sup>. It was found to block interactions between cell surface glycosaminoglycans and HSV-1 glycoproteins, and could prevent binding, entry, and cell-to-cell spread, as well as secondary infection. Based on these observations, it is possible that chebulagic acid activity against HEV71 is related to the inhibition of viral absorption and/or entry. Further studies are required to elucidate the anti-HEV71 mechanism of hydrolysable ellagitannins, but results thus far suggest that they constitute a potential source for antiviral discovery, particularly in the field of HEV71 infection. Interestingly another hydrolysable tannin, punicalin, did not demonstrate obvious antiviral efficacy. This prompted the suggestion of key structural requirements for anti-HEV71 activity<sup>[28]</sup>. Although the *in vitro* antiviral activity of corilagin seemed promising, oral administration of corilagin was not shown to induce significant biological activity<sup>[29,30]</sup>. On the contrary, intraperitoneally administered geraniin, punicalagin and chebulagic acid demonstrated good inhibitory effects on HEV71<sup>[25,27,28]</sup>. This may have been due to the difficulty in the absorption and metabolism of corilagin by intestinal microflora. The incubation of tannins with anaerobic microflora in faeces of animal led to the hydrolysis of the compound into metabolites including gallic acid and ellagic acid<sup>[30]</sup>. To circumvent this problem, *in vivo* studies using intravenous or

intraperitoneal administration may be required.

**Flavonoids:** Another group of compounds commonly tested for anti-HEV71 activity are the flavonoids. Flavonoids are a broad class of low molecular weight secondary metabolites that are present in all vascular plants. The flavonoid structure is usually characterised by a C6-C3-C6 carbon skeleton<sup>[31]</sup>. These phenolic compounds are known to be responsible for the bioactivities of plant crude extracts to confer protection against UV radiation, pathogens, and herbivores<sup>[32]</sup>. Their relatively low toxicity and strong bioactive potential to increase human health prompted many studies in the field of pharmaceutical drug development.

Chrysosplenetin and penduletin<sup>[33]</sup>, 7-hydroxyisoflavone<sup>[34]</sup>, chrysin and its phosphate ester<sup>[18]</sup>, epigenin and its analog luteoline<sup>[35]</sup>, are flavonoids that have all been shown to exhibit *in vitro* anti-HEV71 activity. Experimental evidence indicated that these compounds could inhibit viral RNA and protein synthesis. To understand the mechanism of action, Zhu *et al*<sup>[33]</sup> attempted to select chrysosplenetin- and penduletin-resistant HEV71 through continuous passage in the presence of the compounds. However, after 13 passages, HEV71 remained sensitive to the compounds. Although the mechanism of action is still unclear, time-of-addition studies suggested that flavonoids function in post virus-attachment, during the early stages of virus infection<sup>[33-35]</sup>.

**Alkaloids:** Alkaloids have also been shown to possess anti-HEV71 activities. Liu *et al*<sup>[36]</sup> found that lycorine, one of the most abundant alkaloids of Amaryllidaceae, inhibited HEV71 replication in cultured cells, and lycorine treatment significantly enhanced the survival rate of HEV71-infected mice. Further investigation suggested that the drug inhibits the elongation of viral polyprotein during protein synthesis, and may lead to imbalanced synthesis of viral proteins and interrupted packaging of the virus. Matrine, a quinolizidine alkaloid, is one of the main active components of the root of Chinese *Sophora* herb plants<sup>[37]</sup>. It proved effective in reducing the mortality rate of HEV71-infected mice<sup>[38]</sup>. Treatment with matrine delayed the appearance of paralysis, reduced the clinical scores and prevented other symptoms of the infected mice compared with that of the placebo. Virus replication in mouse muscle tissues was significantly decreased and no obvious side effects

**Table 2 Synthetic antiviral compounds tested for anti- human enterovirus 71 activity**

Synthetic antivirals	Tested	Mechanism	Advantages	Disadvantages	Ref.
Pre-infection					
Pleconaril	<i>In vivo</i>	Prevents attachment by binding to viral capsid	High oral availability	Varied capacity of inhibition	[47-49]
BPROZ	<i>In vitro</i>	Prevents attachment by binding to viral capsid	High oral availability	Resistant mutants	[49-54]
Soluble and anti-SCARB2/PSGL-1	<i>In vitro</i>	Prevents attachment	N/A	N/A	[55-57]
Lactoferrin	<i>In vitro / in vivo</i>	Prevents entry by binding to VP1/ cellular receptor	No obvious side effects (animal)	Mechanism not clear	[58-62]
Suramin	<i>In vitro</i>	Prevents attachment	May inhibit other multiple stages of HEV71 life cycle	Mechanism not clear	[63]
Peptides (SP40)	<i>In vitro</i>	Prevents attachment by binding to glycosaminoglycans	Small size, high activity/specificity, low toxicity	Low bioavailability	[64-66]
Post-infection					
Rupintrivir	<i>In vitro / in vivo</i>	Inhibits viral 3C protein	Low quantity, low toxicity, high barrier for drug resistance	Lack efficacy in natural infection	[67,68]
DTrip-22	<i>In vitro</i>	Inhibits viral 3D polymerase activity	Broad spectrum activity	N/A	[69]
Aurintricarboxylic acid	<i>In vitro</i>	Inhibits viral 3D polymerase activity	N/A	N/A	[76-81]
NITD008	<i>In vitro / in vivo</i>	Inhibits viral 3D polymerase activity	More potent than ribavirin in vivo	May have toxicity issue, resistant mutants	[82,83]
Sorafenib	<i>In vitro</i>	Block virus induced activation of ERK/p38 signalling pathways	Licensed for cancer treatment	N/A	[84,85]

N/A: Not available.

were observed.

**Deferoxamine:** Besides plants, marine microorganisms are also a major source for natural products<sup>[39]</sup>. Deferoxamine (DFO), a marine natural product derived from *Streptomyces pilosus*, was found to compensate for the decreased levels of B cells caused by HEV71 infection in mice, and to improve the levels of the neutralising antibodies against the virus<sup>[40]</sup>. The clinical symptoms, muscle damage and mortality were ameliorated by DFO treatment. Interestingly DFO did not significantly inhibit viral replication in Rhabdomyosarcoma (RD) cells. In contrast, viral replication in the muscle tissues of DFO-treated mice was slightly inhibited. These results suggested that the possible mechanism of DFO activity against HEV71 in infected mice was through the upregulation of B cells, and not the direct inhibition of HEV71.

**Other natural products:** Other natural products shown to exhibit antiviral activity against HEV71 include *Glycyrrhiza* spp. and its active component glycyrrhizic acid<sup>[20,41]</sup>, *Fructus gardenia* and its primary component geniposide<sup>[42]</sup>, chlorogenic acid<sup>[43]</sup>, the *Ganoderma lucidum* triterpenoids, Lanosta-7,9(11),24-trien-3-one,15;26-dihydroxy and Ganoderic acid Y<sup>[15]</sup>, and hederasaponin B<sup>[44]</sup>. Whilst the *in vitro* results of these compounds looked promising, *in vivo* studies were not performed.

### Synthetic antiviral compounds

A growing body of literature on synthetic anti-HEV71

drug development has been published in recent years, but most of these drugs are still in the early phase of development and need further optimisation of their pharmacokinetics and absorption, distribution, metabolism, excretion, and toxicity profiles. Ribavirin, a wide spectrum synthetic antiviral, was reported to reduce mortality caused by HEV71 in Institute for Cancer Research (ICR) mice<sup>[45]</sup>. However, the dosage used was much higher than the clinical recommended dosage prescribed to adults with Hepatitis C Virus (HCV) infection. Given that most HEV71 infections affect children younger than 5 years old, high dose of ribavirin may raise serious safety concerns.

The life cycle of HEV71 generally involves virus attachment, uncoating and entry, polyprotein translation and cleavage, viral RNA replication, and virus assembly. These critical steps are currently considered targets for synthetic antiviral development. Lead compounds that inhibit virus attachment, uncoating and entry are being actively pursued and may be used as potential prophylactic against HEV71, whereas inhibitors of post-infection stages may be suitable for treatment. Both pre- and post-infection inhibitors of HEV71 are discussed in detail below and further summarised in Table 2.

**Pre-infection inhibitors:** The most widely studied chemical structures amongst capsid binding molecules as antiviral agents for HEV71 are the series of "WIN" compounds<sup>[46]</sup>. Pleconaril (WIN 61893) was the first of a new generation of metabolically stable capsid



function inhibitors. In a mouse model of infection following intracranial inoculation of enteroviruses, pleconaril reduced viral titres in all affected organs and prevented death in animals. Furthermore, there was high oral bioavailability in humans and other animals<sup>[47,48]</sup>. However, the HEV71 inhibition capacity of pleconaril could vary for different isolates of the virus. It was nearly ineffective in neutralising HEV71 isolates from the outbreak in Taiwan<sup>[49]</sup>. Using pleconaril as a template for computational drug design, a Taiwanese group succeeded in discovering a new class of pyridyl imidazolidinones with anti-HEV71 activity. A series of imidazolidinone derivatives, designated BPROZ (e.g., BPROZ-194, BPROZ-103 and BPROZ-074), demonstrated effectiveness against HEV71 infection<sup>[49-54]</sup>. Their therapeutic potential is still under active investigation.

The soluble form of HEV71 receptors, SCARB2 and PSGL-1, has been shown to block virus-host interaction<sup>[55,56]</sup>. It was proposed that these soluble receptors could act as molecular decoys of cell-associated receptors<sup>[57]</sup>. Antibodies against these receptors have also been shown to inhibit *in vitro* virus infection<sup>[55,56]</sup>. However, further studies are required to determine the potential of these molecules as therapeutic antivirals. In their study, Weng *et al*<sup>[58]</sup> demonstrated that lactoferrin (LF) inhibited HEV71 infection *in vitro* and *in vivo* by binding to the VP1 protein of HEV71, as well as to host cells. The anti-HEV71 mechanism of LF is unclear, but may relate to the prevention of viral entry by blocking cellular receptors and/or by direct binding to the virus particles, as suggested by the above finding. Binding of LF to several different cell ligands such as heparan sulfate, chondroitin sulphate and nucleolin has been reported<sup>[59-61]</sup>. However, antiviral activity of LF analogues is only partly related to their affinity for heparin sulfate<sup>[62]</sup>. Although lactoferrin has not been approved for therapeutic purposes, it could be considered an agent for preventing virus entry. Another group of researchers screened a library of compounds and identified suramin as having the ability to inhibit HEV71 proliferation by blocking the attachment of HEV71 to host cells, as well as affect other steps of the HEV71 life cycle<sup>[63]</sup>.

Peptides have also been used as therapeutic agents to block viral attachment or entry into host cells. A major advantage is their small size and their high activity and specificity when compared to antibodies and other larger molecules. Peptides accumulate in lesser quantity in tissues, and have very low cell toxicity when compared to synthetic molecules<sup>[64]</sup>. A 15-mer peptide spanning from position 118 to 132 in the VP1 capsid region, SP40, exhibited antiviral activity in all three genotypes of HEV71 (genotypes A, B and C), coxsackievirus A16 (CVA16) and poliovirus Mahoney (PV1)<sup>[65]</sup>. It also reduced viral induced CPE and viral RNA synthesis in Vero, HeLa and HT-29 cell lines in a dose-dependent manner. Data from further research suggested that the SP40 peptide could have interacted with cell surface glycosaminoglycans and prevented

HEV71 attachment. A major disadvantage of peptides is their low bioavailability due to their rapid degradation in the gastrointestinal system. To circumvent this issue, new formulations such as the D-isomer peptide<sup>[64]</sup>, addition of N-terminal pyroglutamate and C-terminal homoserine lactone to the peptide, are being developed to improve the resistance to peptidase<sup>[66]</sup>.

Attachment and entry inhibitors stop the virus from entering cells, and therefore may be useful as prophylactic agents. However, a major obstacle of this approach is for it to be cost-effective for resource-limited countries where large outbreaks frequently occur. Furthermore, the effectiveness of the drug itself would be highly dependent on the timing of the treatment provided. It is a challenge to deliver a sufficient amount of the inhibitor to the targeted site early enough to prevent disease progression, or to prevent the spread of infection to others.

**Post-infection inhibitors:** Various synthetic antiviral compounds were designed to target post-entry stages of the HEV71 life-cycle. The anti-HEV71 activity of rupintrivir, an irreversible peptidomimetic inhibitor of viral 3C protein, has been evaluated in a mouse model<sup>[67]</sup>. Complete protection against HEV71-induced cell death was observed at low nanomolar concentrations, with very little cell toxicity. Consistent with the symptoms, a significant decline in viral RNA was witnessed in intestine, lung, muscle, brain stem, and cardiac muscle when rupintrivir was administered *in vivo*. Rupintrivir also significantly improved the integrity of limb muscle structure and suppressed the expression of VP1 in infected mouse muscle. Another potential clinical advantage is the high barrier for emergence of drug resistance, as tested by the researchers<sup>[67]</sup>. However, it is worth noting that a previous clinical trial for rupintrivir for the treatment of human rhinovirus infection was halted due to a lack of efficacy in natural infection studies<sup>[68]</sup>.

Several compounds were found to inhibit the 3D polymerase. DTriP-22, a piperazine-containing pyrazolo [3,4-d] pyrimidine derivative, was shown to inhibit HEV71 RNA accumulation during virus infection, but not IRES-driven translation<sup>[69]</sup>. It may interfere with 3D activity by obstructing the nucleoside triphosphate entry cavity of 3D polymerase but not by incorporation into the growing RNA chains. This compound is considered novel because most other polymerase inhibitors that exhibit anti-enterovirus activity are nucleoside analogues<sup>[70-75]</sup>. DTriP-22 has a broad spectrum activity against RNA viruses, including different genotypes of HEV71, coxsackieviruses A and B, and echovirus 9<sup>[69]</sup>. Aurintricarboxylic acid (ATA), a polyanionic compound originally reported to be an inhibitor for the replication of HIV, HCV and SARS-CoV<sup>[76-80]</sup>, also exhibited the ability to inhibit HEV71 3D polymerase<sup>[81]</sup>. Results showed that ATA slows down viral RNA synthesis at early stages after a single round of viral replication in HEV71-infected cells. However, ATA did not inhibit the activity of HEV71 viral

2A/3C protease activity. A nucleoside analog, NITD008, has been reported to selectively inhibit viruses within the family *Flaviviridae*<sup>[82]</sup>. Although NITD008 showed efficacy in a dengue mouse model, it was not further developed due to the adverse findings observed in a preclinical toxicity study<sup>[83]</sup>. Deng *et al*<sup>[83]</sup> reported that NITD008 potently inhibits HEV71 in cell culture and in a mouse model, and demonstrated the feasibility that this compound could potentially be developed for HEV71 therapy, if the toxicity issue is resolved. Their data further showed that mutations in viral 3A and 3D polymerase regions could confer resistance against NITD008, suggesting an intimate crosstalk between 3A and 3D during viral replication.

Sorafenib, previously known as BAY 43-9006 and marketed commercially as Nexavar, is a multi-target tyrosine and serine-threonine kinase inhibitor currently used in cancer therapy<sup>[84]</sup>. A significant reduction of infectious HEV71 titres and viral RNA was observed in infected cells when sorafenib was added 1 and 3 h post-infection. However, no difference was seen compared to non-treated cells when sorafenib was added 2 h pre-infection and during virus adsorption. Experimental data indicated that sorafenib treatment was able to block the HEV71 mediated CPE through blocking of virus induced activation of the ERK and p38 signaling pathways. A previous study has shown that HEV71 infection induced cyclooxygenase-2 (COX-2)/prostaglandins (PG) E<sub>2</sub> expression *via* mitogen-activated protein kinases (MAPKs) including ERK and p38, and further that inhibition of HEV71-induced COX-2/PGE<sub>2</sub> expression may reduce CNS inflammation<sup>[85]</sup>. Thus it was proposed that sorafenib treatment may alleviate HEV71-induced inflammatory responses<sup>[84]</sup>. Further *in vivo* studies are required to validate the effectiveness of the drug.

### Other therapeutic strategies

**Immunoglobulin:** A number of animal studies have shown that neutralising antibodies stimulated by immunisation with inactivated virus, virus-like proteins, or VP1 subunit vaccines, are cross-protective against heterologous strains of HEV71 and can passively protect mice and monkeys (see section on vaccine development). Further, studies on patients have indicated that HEV71 infection is cleared by humoral immunity, and clinical trials have shown the presence of neutralising antibodies in the serum of immunised healthy adults and children<sup>[86-88]</sup>. The significant involvement of neutralising antibody responses in the control of HEV71 infection in humans would render IVIG treatment an ideal complimentary therapeutic agent. In fact since the year 2000, IVIG has been used in China as the last resort for treatment of severe cases of HEV71 infection, with some measure of success<sup>[89]</sup>.

However, treatment of patients with IVIG has its disadvantages. Besides the risk of transmitting human pathogens using pooled human sera, necessitating screening and treatment, it also requires donor availability. Other disadvantages include batch to batch

variability, and the presence in the serum of virus specific but non-neutralising antibodies<sup>[90]</sup>. A phenomenon termed antibody-dependent enhancement (ADE) was recently confirmed in experimental and clinical settings<sup>[91,92]</sup>, in which sub-neutralising concentration of antibodies was evidenced to enhance HEV71 infection in Fc receptor-bearing human monocytes and contributed to exacerbation of HEV71 infection in mice. The wide existence of cross reactivity between enterovirus antibodies may also become the underlying risk for HEV71 ADE infections.

A solution would be to exploit future passive immunotherapy based on monoclonal antibodies (mAb) produced in cell culture. They offer a selective advantage over pooled human sera that are more commonly used in IVIG treatment by reducing the risks mentioned above. Based on the success of a United States Food and Drug Administration (FDA) approved humanised mAb for respiratory syncytial virus infection of the lower respiratory tract<sup>[93]</sup>, a similar approach was taken to develop neutralising anti-HEV71 mAb for the treatment of severe HFMD caused by HEV71<sup>[89]</sup>. Using previously identified peptides containing amino acids of the VP1 region known to be potent in eliciting neutralising antibody<sup>[94,95]</sup>, a mAb (clone 22A12) with strong neutralising activity against HEV71 in an *in vitro* neutralisation assay was successfully generated. Because clone 22A12 is a murine antibody, further work for the chimerisation and/or humanisation of the antibody is currently underway to reduce human anti-mouse antibody response for therapeutic application. Another group of researchers generated and characterised several mAbs by immunising mice with purified HEV71 virus, strain Henan2<sup>[96]</sup>. They identified a mAb, clone 4E8, with strong neutralising activity against HEV71 and that specifically reacted with synthetic peptides containing amino acids 240-250 and 250-260 of VP1 by Enzyme-Linked Immunosorbent Assay (ELISA) assay. Clone 4E8 partially protected mice against the lethal challenge of HEV71 strain Henan2. Kiener *et al*<sup>[90]</sup> succeeded in isolating a novel mAb against HEV71 that targets a conformational neutralisation epitope outside of VP1. The mAb 10D3 targets the highly conserved "knob" region of VP3. The protective efficacy of mAb 10D3 was evaluated and verified by an animal challenge experiment using a lethal dose of HEV71. All mice prophylactically treated with mAb 10D3 survived the lethal challenge without showing any disease symptoms.

Several factors have to be considered when using mAbs instead of polyclonal serum. First, due to the antigenic variability of circulating strains, the mAb must cross-neutralise all existing subtypes to be useful. Second, there is a risk of escape mutations, which may be circumvented by administering two or more antiviral mAbs against non-overlapping epitopes. A combination of synergistic mAbs may also reduce the required dosage<sup>[97,98]</sup>.

The use of non-human immunoglobulins in the

treatment of HEV71 infection has also been investigated. Immunoglobulin Y (IgY) antibodies are the predominant serum immunoglobulin in birds, reptiles, and amphibians, and are transferred from serum to egg yolk in the females to confer passive immunity to their embryos and neonates<sup>[99]</sup>. The potential of orally administered IgY for the prevention and treatment of many pathogens has been widely reported<sup>[100-103]</sup>. It was found that chicken as bio-factory can produce a higher yield of IgY antibodies compared to the production of IgG in mammals. In HEV71-infected ICR mice, a survival rate of 98.3% was achieved when the challenged mice were given intraperitoneal injection 1 to 3 d post-infection for 3 consecutive days with a purified IgY antibody at neutralisation titre of 128 or more<sup>[104]</sup>. Oral administration at a higher dose also conferred protection to infected mice. The study suggested that IgY in the form of an egg-yolk-added drink, yolk powder tablet, or capsule, can potentially be used to prevent the early infection of HEV71.

**Adoptive transfer of macrophage:** The adoptive transfer or activation of macrophages has been used in the immunotherapy of cancer, liver ischemia, reperfusion injury and pneumonia<sup>[105-108]</sup>. Liu *et al*<sup>[109]</sup> showed that the adoptive transfer of macrophage cells from adult mice can partly protect young mice from lethal HEV71 infection. The macrophages displayed anti-HEV71 activity *in vitro* and could alleviate the pathology of infected mice, possibly by engulfing the virus directly through phagocytosis. The application of macrophages in antiviral therapy *via* adoptive transfer is a novel proposal. Unlike human macrophage, murine macrophage can be easily obtained either from the peritoneal cavity or grown from bone marrow precursor cells. Technology for the isolation or growth of large scale human macrophage is still unavailable. Future studies using activated macrophage derived from peripheral blood monocytes of adults were proposed.

**Interferons:** The effectiveness of interferons (IFNs) in the treatment of HEV71 infection has been studied with contradictory findings. Liu *et al*<sup>[110]</sup> demonstrated that early treatment of HEV71-infected newborn mice with a recombinant murine IFN- $\alpha$  resulted in an increased survival rate. However another study demonstrated that HEV71 2A<sup>pro</sup> could be an IFN antagonist, because it reduces the expression level of the type I IFN receptor<sup>[111]</sup>, making it questionable whether type I IFN will be active against HEV71 infection. There are about 20 different human type I IFNs identified to date<sup>[112]</sup>. Although they are highly homologous in amino acid sequence and share the same receptors, the biological effect of each IFN is apparently different. It has been shown that the anti-HEV71 activities of various IFN subtypes differ from each other<sup>[113]</sup>. Based on their antiviral activities, they can be divided into three subgroups: IFNs with high anti-HEV71 activities at low

concentrations, IFNs with moderate anti-HEV71 activity at high concentrations, and IFNs with nearly no antiviral activities. Hung *et al*<sup>[114]</sup> showed that the 3C<sup>pro</sup> of HEV71 was able to cleave IRF9, a host protein involved in the signaling cascade triggered by type I IFN. They found that HEV71 could be effectively inhibited by a combination of IFN- $\alpha$  and a 3C<sup>pro</sup> inhibitor such as rupintrivir.

**All-trans-retinoic-acid:** Most HEV71-infected children present with vitamin A (VA) deficiency, which is associated with decreased immunity and more severe pathogenic conditions<sup>[115]</sup>. It was shown that serum IFN- $\alpha$  levels were markedly reduced and positively related to the lack of VA in HEV71-infected children. The active VA metabolite, all-trans-retinoic acid (ATRA), is the natural ligand for the retinoic acid receptors (RAR). In various *in vitro* systems, ATRA has been shown to regulate the expression of a number of IFN-stimulated genes, including retinoid-induced gene I (RIG-I), a pattern recognition receptor involved in the innate immune response of the host<sup>[116-118]</sup>. It was proposed that the inhibition of RIG-I-mediated type I IFN responses may contribute to the pathogenesis of HEV71 infection<sup>[119]</sup>. Chen *et al*<sup>[120]</sup> demonstrated that ATRA is a potent IFN inducer that effectively inhibits HEV71 and significantly regulates the RIG-I signalling pathway in the human monocytic cell line. They proposed that the antiviral effect of ATRA occurred through a RAR- $\alpha$  pathway, and further suggested that ATRA may directly contribute to anti-HEV71 infection by reinforcing innate immunity.

**Oligonucleotides:** Previous reports have described the antiviral effects of RNA-based therapeutics, such as siRNA, shRNA and miRNA, targeting the VP1, 3D, 2C genes, or the 3' UTR of the HEV71 genome, resulting in antiviral activity<sup>[121-128]</sup>. However, whilst plasmid-derived shRNAs are widely used for laboratory studies, they are not suitable for antiviral therapy. Further, the limitations of RNAs are short half-life and the requirement of a delivery agent that may be toxic to the host. There is currently no approved marketed siRNA drug. On the contrary, the use of antisense oligodeoxynucleotide (ASODN) technology to inhibit pathogen replication has shown promising results. Since the United States FDA approved the first antisense drug, Fomivirsen, for the treatment of cytomegalovirus (CMV) retinitis in 1998, more than 30 types of ASODNs have been evaluated in clinical trials<sup>[129]</sup>.

Unmodified oligonucleotides are highly unstable *in vivo* due to rapid nuclease digestion. In order to circumvent this problem, a number of chemically modified oligonucleotides such as classic phosphorothioate oligonucleotides, phosphorodiamidate morpholino oligomers, locked nucleic acids, and gene-silencing oligonucleotides have been developed<sup>[130]</sup>.

Liu *et al*<sup>[131]</sup> designed and tested 5 antisense

phosphorothioate oligonucleotides targeting the 5'-terminal conserved sequence found in HEV71 RNA. One of the oligonucleotides, EV5, effectively inhibited HEV71 amplification both *in vitro* and *in vivo* in a sequence-specific and dose-dependent manner. It was also capable of providing effective protection to HEV71-infected mice and inhibited virus replication in the lungs, intestines, muscle, but not brain, of infected mice. Tan *et al.*<sup>[132]</sup> tested 3 octoguanidium dendrimer conjugated-morpholino oligomers (vivo-MOs) that are complementary to the HEV71 IRES (vivo-MO-1 and -2) and 3D polymerase (vivo-MO-3). Vivo-MO-1 and -2 showed significantly reduced plaque numbers, viral RNA copies, and viral capsid expression in RD cells in a dose-dependent manner. In contrast, vivo-MO-3 exhibited less antiviral activity. Both vivo-MO-1 and 2 remained active when administered within 4 h before or 6 h after HEV71 infection. Resistant mutants arose after serial passages in the presence of vivo-MO-1, but not vivo-MO-2. Thus vivo-MO-2 was proposed to be a favourable candidate for further development as an antiviral agent.

## PREVENTION STRATEGIES

HEV71 is highly contagious and can be isolated from throat swabs, rectal swabs, and stool specimens of sick children. Virus shedding can persist for nearly 4-5 wk in the respiratory tract and through faeces<sup>[133,134]</sup>. As a result, HEV71 transmission may occur not only through direct contact with infected people, but also contact with respiratory secretions or faeces of an infected person. The virus can subsequently spread from one person to another through the faecal-oral route by contaminated hands or objects<sup>[135]</sup>, rapidly causing outbreaks. Due to the long periods of viral shedding in children, HEV71 is frequently transmitted in families, kindergartens, and schools<sup>[136]</sup>. Therefore to successfully control the devastating outcome of HEV71 epidemics, prevention of infection remains the top priority.

### Surveillance

Until a vaccine becomes available, the best way to prevent HEV71 infection is through infection control practices such as hand-washing, disinfection and social distancing during epidemics<sup>[137]</sup>. Early intervention can lessen the spread of the virus. For these actions to be effective, adequate clinical and laboratory surveillance of HEV71 activity and identity in the community is essential to provide early warning of impending epidemics. As such many countries in the Asia-Pacific region, including Japan, Malaysia, Singapore, Taiwan, Vietnam and China, have implemented heightened surveillance for HEV71<sup>[138-142]</sup>. HFMD has now become a notifiable disease in many countries in the region. However, since other enteroviruses such as CVA8, CVA10, and CVA16 can also cause HFMD, concurrent virological surveillance may provide invaluable molecular epidemiological data to help track the spread of the virus across the

region<sup>[143]</sup>. In some instances surveillance programs have provided information that resulted in early control of HEV71 epidemics and reduced the total number of cases of acute neurological disease<sup>[144]</sup>.

### Physical prevention

Transmission of the viruses responsible for HFMD, including HEV71 and CVA16, is mainly through the faecal-oral route. Therefore the first line of defense is to contain the disease causing agent. Infected children are quarantined and non-infected children are also kept from crowds. During the 2000 outbreak in Singapore, spread of viruses was prevalent in child-care centres. One of the measures taken to break the chain of transmission was a 2-wk nationwide closure of preschool centres<sup>[145]</sup>. However, it was suggested that even though such controls may decrease the peak incidence of disease, the outbreak may be prolonged, and therefore the overall number of cases may not be lowered<sup>[143]</sup>.

Health education plays an important role to inform and educate parents about the virus infection and prevention strategies. It should focus on observance of good personal hygiene, and cleaning and disinfection of premises and articles. Alcohols are widely used as active ingredients in many hand disinfectants. However, their effectiveness is largely dependent on the type and concentration used. A recent study showed that 95% ethanol instead of 70%-95% isopropanol has the most virucidal activity against HEV71, but did not result in complete inactivation of HEV71<sup>[146]</sup>. Further, high concentration of ethanol may cause skin irritation and a decrease in antibacterial activity. New formulations are needed for routine use to prevent the spread of enteroviruses.

### Vaccine development

Similarities between HEV71 and poliovirus in many virological and clinical aspects have strongly suggested that a vaccine strategy, similar to that against poliovirus infection, could be effectively adopted to control HEV71 infection. Because it mainly threatens the children in developing countries, an ideal HEV71 vaccine would have to be inexpensive, safe, convenient to administer, and acceptable to parents. In addition, a successful vaccine strain would also provide cross-protection to different HEV71 genotypes.

**Live-attenuated vaccine:** Based on the similarities between PV and HEV71, Arita *et al.*<sup>[147]</sup> developed a HEV71 attenuated strain carrying mutations in the 5'- and 3'-untranslated regions and 3D polymerase, based on the temperature-sensitive determinants of poliovirus Sabin 1 vaccine strain. The EV71 (S1-3') strain, which belongs to HEV71 genotype A, was characterised by attenuated neurovirulence and limited spread of virus. In a subsequent study, cynomolgus monkeys inoculated with EV71 (S1-3') *via* the intravenous



route had a mild neurological symptom in the form of tremor, but survived lethal challenge by virulent HEV71 (BrCr-TR) without exacerbation of the symptom<sup>[148]</sup>. The immunised monkey sera demonstrated a broad spectrum of cross-genotype neutralising activity, including genotypes A, B1, B4, C2, and C4. Although EV71 (S1-3') demonstrated promise as a live attenuated vaccine against HEV71, the vaccine itself was not completely attenuated, as evidenced by mild neurological symptoms and isolation of virus from the spinal cord.

Due to the lack of proof-reading activity by enteroviral 3D polymerase, a high incidence of error leading to random mutations occur during replication. This phenomenon makes it easier for the reversion of mutants to wild-type virus. To overcome this issue, researchers have explored the possibility of replacement or deletion of bigger fragments. Replacement of the PV internal ribosome entry site (IRES), with that of a non-neurotropic human rhinovirus (HRV), was found to stably attenuate PV in animal models<sup>[149,150]</sup>. In HEV71, it was shown that deletion of stem-loop domain Z within the 3'-untranslated region attenuates the growth of a HEV71-HRV2-IRES chimera in neuroblastoma cells<sup>[151]</sup>. Another strategy employed to generate stably attenuated vaccine strains is to increase the replication fidelity of the 3D polymerase. Mutations at amino acid positions G64R and S264L in the HEV71 3D polymerase have recently been shown to increase replication fidelity and the genetic stability of the HEV71 genome by greater than ten-fold during growth in cell culture<sup>[152]</sup>. Further, the HEV71 3D-G64R and 3D-S264L mutant virus populations were attenuated in a mouse model of HEV71 infection<sup>[153]</sup>.

**Inactivated vaccine:** In response to the Bulgarian outbreak in 1975, a formalin-inactivated HEV71 vaccine was developed, but was not used to control the epidemic<sup>[154]</sup>. However since then, the value of inactivated vaccine for the effective control of HEV71 has been shown by various researchers. Suckling mice immunised with the adjuvant-carrying formaldehyde-inactivated mouse-adapted HEV71 vaccine were effectively protected from lethal virus challenge and disease<sup>[155]</sup>. Another experimentally inactivated vaccine produced using the FY-23K-B strain of HEV71 was capable of inducing an immune response and offered protection to rhesus monkeys against future virus attacks<sup>[156]</sup>. Additionally, passive transfer of serum from formalin-inactivated and heat-inactivated virus vaccine immunised adult mice, could provide protection against HEV71 challenge in neonatal mice<sup>[157,158]</sup>. The efficacy of this model of maternal vaccination-neonatal challenge is consistent with the results of other similar studies using maternal vaccination to protect offspring from infectious disease<sup>[159-162]</sup>. Bek *et al*<sup>[159]</sup> provided the first demonstration of cross-genotype protective efficacy of a candidate HEV71 vaccine which

suggested that inactivated vaccines may confer broad protection against HEV71 infection. On the other hand, another study showed that HEV71 type specificity of neutralisation was unidirectional<sup>[163]</sup>. The antisera used against newly emerging subgenogroups could cross-neutralise their ancestor subgenogroups, but not vice versa. Chen *et al*<sup>[164]</sup> demonstrated that co-immunisation of a formaldehyde-inactivated HEV71 vaccine with a commercial pentavalent vaccine that contained inactivated polio vaccine, did not interfere in antibody production nor protective efficacy of the HEV71 vaccine. This indicates that the two vaccines are compatible after co-immunisation, and that formaldehyde-inactivated HEV71 vaccine may be used in designing multivalent vaccines.

Due to their inability to replicate, inactivated HEV71 vaccines are favoured over the live attenuated vaccines for safety reasons. However, the manufacturing costs of inactivated vaccines and potential supply problems cause substantial difficulties in practical implementation, particularly in developing countries. Further, viruses are sensitive to chemical treatment and neutralising epitopes could be destroyed during inactivation, as it is reported in formalin inactivated C4D HEV71 vaccine strain<sup>[165]</sup>. Nevertheless, research and development of HEV71 inactivated vaccines have progressed further than the other types of HEV71 vaccines, with some currently in phase III clinical trial<sup>[166]</sup>.

**Subunit vaccine:** Like all enterovirus the antigenic diversity of HEV71 is caused by variations within capsid proteins VP1, VP2 and VP3, but the VP1 protein displays a number of important neutralising epitopes<sup>[157,167,168]</sup>. Key neutralising antibody determinants have been found in the N-terminal half of VP1 when tested with high titre human neutralising antibodies<sup>[169,170]</sup>. The potential safety advantage of subunit vaccines over conventional whole virus vaccines has prompted researchers to query whether the VP1 subunit of HEV71 is sufficient to provoke adequate protective immunity against viral infection. Different delivery systems have been tested for their suitability in expressing the VP1 and to stimulate immune response. They include recombinant VP1 protein expressed in *Escherichia coli* BL21<sup>[157]</sup>, recombinant Newcastle disease virus capsid displaying VP1<sup>[171]</sup>, and VP1 expressed in yeast *Pichia pastoris*<sup>[172]</sup>. All induced high levels of neutralising antibodies.

The mucosal immune system serves as the first line of defense against HEV71 as it initiates disease following implantation in the gut mucosa<sup>[173]</sup>. Thus an oral vaccine for immunisation against HEV71 has its advantages over injected vaccines. Oral subunit vaccines stimulate production of mucosal antibodies more effectively than is the usual case with injected vaccines<sup>[174]</sup>. Oral administration is also widely accepted in children who need a HEV71 vaccine. The use of attenuated *Salmonella* as a vector for the VP1 subunit demonstrated the advantages of oral vaccine vectors<sup>[175]</sup>.

Yu *et al*<sup>[176]</sup> showed that VP1-expressing *Bifidobacterium longum*, a gastrointestinal probiotic, can confer protection from the mother to neonatal mice, suggesting the potential of this recombinant *B. longum* as an oral vaccine against HEV71 infection. Transgenic plants and animals are possible alternatives to prokaryotic and eukaryotic vectors. They offer a palatable oral delivery system that can elicit a good mucosal immune response as well as systemic humoral and cellular immune responses, making it particularly suitable for protecting against infectious agents intruding *via* the mucosal surface<sup>[177,178]</sup>. In one study, transgenic tomato fruit expressing the VP1 subunit was developed as a free-feeding oral vaccine<sup>[179]</sup>. Serum from immunised mice was able to neutralise the infection of HEV71 in RD cells. In another study, the bovine  $\alpha$ -lactalbumin promoter and  $\alpha$ S1-casein signal peptide sequence were fused with the VP1 cDNA to generate transgenic mice with mammary gland-specific VP1 expression<sup>[180]</sup>. Expression of the HEV71 VP1 capsid protein was shown to be highly specific to the mammary gland and was secreted in the milk of transgenic mice, reaching satisfactory expression level for oral vaccine development and is much higher than that achieved in bacterial or transgenic plant system<sup>[157,158,179,181,182]</sup>.

Gastric acid and enzymatic digestion are major concerns for oral vaccines because they may interfere with vaccine conformation and absorption. Moreover, it is difficult to determine the precise dose of antigens for immunisation, since competition with food and microbial antigens interferes with the absorption rate of vaccine components. Many strategies have been employed to improve oral vaccine delivery, including the use of tissue-specific promoters, mucosal immune adjuvant, liposomes, and N-trimethyl chitosan nanoparticles<sup>[173,174]</sup>. New strategies are necessary to achieve a high level of expression of VP1 protein in the correct antigen conformation. If these prototypes can be refined to yield similar immunogenicity levels as inactivated vaccines, they could become strong preventive options.

**Synthetic peptide:** Epitope-based vaccination using synthetic peptides is another area under intense investigation for the delivery of precise vaccine components to the immune system. A series of overlapping synthetic peptides spanning the VP1 capsid protein of HEV71 was used to immunise BALB/c mice in order to identify neutralising linear epitopes<sup>[94]</sup>. Peptides containing amino acids 163–177 and 208–222 of the VP1 were capable of eliciting neutralising antibodies against HEV71. Additionally, mouse antisera raised against the peptide 208–222, designated SP70, demonstrated *in vivo* passive protective efficacy in BALB/c mice<sup>[183]</sup>. Hydrophobic profile assays showed that this highly conserved sequence is located within the major hydrophilic regions and is expected to be exposed at the surface of the protein, hence making it a promising and attractive candidate for

synthetic peptide-based HEV71 vaccine<sup>[94]</sup>. Further, the amino acid sequence represented by SP70 was totally conserved amongst 25 HEV71 strains from subgenogroups A, B1–B5 and C1–C4, which suggested possible cross-protection against infectivity of all HEV71 strains. A different delivery approach for the synthetic peptide was explored using adenovirus (Ad) vectors<sup>[184]</sup>. Compared to the recombinant GST-fused SP70 protein, immunisation with the Ads containing SP70 elicited higher SP70-specific IgG titres, higher neutralisation titres, and conferred more effective protection to neonatal mice.

Nevertheless, mouse antisera raised against HEV71 whole virions provide higher *in vivo* passive protection to suckling mice against lethal HEV71 challenge when compared with the anti-SP70 antisera, possibly due to higher titres of neutralising antibodies elicited by several neutralising epitopes located on the virus other than that represented by the synthetic peptide SP70 alone. Further, the short epitopes can easily change to avoid antibody mediated neutralisation. To circumvent this issue, 6 peptides without cross-reactivity were selected and combined into three vaccine candidates and applied in further evaluation in neonatal mice<sup>[185]</sup>. The Vac6 comprising the peptides of P70–159, P140–249, P324–443 and P746–876 of the structural proteins could provide effective protection on pups against virus infection.

**Virus-like particles:** Another method of vaccine development is the construction of virus-like particles (VLPs). The baculovirus expression system is the most widely used platform for generating VLPs. To assemble the HEV71 VLPs, the P1 polyprotein needs to be cleaved by viral protease 3CD into individual structural proteins. Hu *et al*<sup>[186]</sup> developed VLPs by co-expressing the P1 and 3CD regions of HEV71 in the pFastBac™ Dual vector, which contains two strong baculovirus promoters, polyhedron (PPH) and p10 (Pp10). The P1 region was controlled by a strong baculovirus promoter, PPH, whilst the protease 3CD was controlled by weak promoters such as CMV promoter or baculovirus IE1 promoter. The expressed 3CD successfully cleaved P1 *in vitro* and *in vivo*. Also, the co-infection in insect cells resulted in crystalline virus-like particle structures morphologically resembling the authentic HEV71 aggregates. A patent for these recombinant baculoviruses has been applied for in Taiwan, the United States and mainland China<sup>[166]</sup>.

In a study using monkeys, Lin *et al*<sup>[187]</sup> found that VLPs and formalin-inactivated vaccines generated comparable amount of HEV71-binding antibodies measured by ELISA, and induced memory T and B cell responses. However, monkeys immunised with inactivated HEV71 virus showed relatively greater neutralisation titre, proliferation, and cytokine production than those immunised with VLPs. This may be partially due to the conformation difference between VLPs and viral particles, which was not detected under the

**Table 3 Comparison of human enterovirus 71 vaccine strategies**

Vaccines	Tested	Advantages	Disadvantages	Ref.
Live-attenuated vaccine	<i>In vitro / in vivo</i>	Broad spectrum, low cost	Incomplete attenuation	[143-149]
Inactivated vaccine	<i>In vitro / in vivo / clinical trial</i>	Inability to replicate	High cost	[150-162]
Subunit vaccine	<i>In vitro / in vivo</i>	Safe to use	Low immunogenicity	[153,154,163-178]
Synthetic peptides	<i>In vitro / in vivo</i>	Small and safe to use	Low immunogenicity, escape mutants	[94,179-181]
Virus-like particles	<i>In vitro / in vivo</i>	Safe to use	Unstable, need purification, high cost	[162,182-185]
DNA vaccine	<i>In vitro / in vivo</i>	Most resemble native virus, fast production, low cost, can be manipulated	Low neutralising effect	[153,186-190]

assays performed. Even though immunisation with VLPs has less of a response than inactivated vaccine, nevertheless they provide a safer method for preventing viral infection with regards to clinical treatment.

The main problem associated with VLPs is their stability, purification and cost. At present, the VLPs are mostly developed using insect cells and the strict culture conditions limit the required large scale of vaccine production. Thus, transgenic plants or yeast that can produce VLPs to be delivered by either oral administration or injection may prove to be promising platforms. Recently Li *et al*<sup>[188]</sup> coexpressed the P1 and 3CD regions in *Saccharomyces cerevisiae* to yield VLPs. The *S. cerevisiae* system is a low cost platform and it is easy to scale-up production. As a eukaryotic expression system, it benefits from the processes of protein expression, folding, and modification, which are lacking in prokaryotic expression systems. Compared to the insect cell expression systems, the use of stable yeast transformants avoid the generation of initial large quantities of recombinant baculoviruses. In laboratory conditions, however, the yield of *S. cerevisiae*-derived VLPs was not sufficient for clinical use. However the use of fermentation engineering and automation control, which have been used for the production of other types of VLPs<sup>[189]</sup>, may overcome this issue. The patent for *S. cerevisiae* production of VLPs has been applied for in China<sup>[166]</sup>.

**DNA vaccine:** DNA immunisation offers many advantages over the traditional forms of vaccination. It is able to induce the expression of antigens that resemble native viral epitopes more closely than standard vaccines do, since live attenuated and inactivated vaccines are often altered in their protein structure and antigenicity. Plasmid vectors can be constructed and produced quickly, at relatively lower cost, and the coding sequence can be manipulated in many ways. Further, DNA vaccines encoding several antigens or proteins can be delivered to the host in a single dose at low quantity to induce immune responses. They are also very temperature stable making storage and transport much easier.

Tung *et al*<sup>[190]</sup> developed a HEV71 DNA vaccine by inserting the VP1 gene into a eukaryotic expression vector and evaluated the immune response in mice.

They showed that whilst anti-VP1 IgG level was increased in immunised mice, the level declined after boosting immunisation. Further, although the anti-VP1 IgG exhibited neutralising activity against HEV71, the neutralising effect of the sera of mice immunised with the VP1 DNA vaccine was much lower than that of HEV71-infected human serum. Another DNA vaccine was developed by inserting the entire VP1 gene into plasmid pcDNA3<sup>[157]</sup>. Intramuscular administration elicited a high and stable level of neutralisation titre in both ICR and BALB/c mice, which could be detected post-immunisation. However, it induced a weaker immune stimulation compared to whole virus particles.

Various strategies to increase the immune stimulation ability of DNA vaccines have been explored. Amongst these are the incorporation of immunostimulatory sequences in the backbone of the plasmid, co-expression of stimulatory molecules, use of localisation/secretory signals, and an appropriate delivery system, as well as adjuvants and optimisation of transgene expression<sup>[191-194]</sup>. While therapeutic and prophylactic DNA vaccine clinical trials are underway for a variety of infectious diseases and cancers, the scientific basis of DNA vaccines has yet to be clearly defined. If DNA vaccines pass all scientific and regulatory scrutiny, they promise to be products of the next generation. A comparison of DNA vaccine with other vaccine strategies is shown in Table 3.

## CONCLUSION

During the past decade, HFMD and associated neurological complications caused by HEV71 infection have resulted in the loss of many paediatric lives in the Asia-Pacific region. Whilst a significant amount of research have been published in the field of HEV71 antivirals and vaccine development lately, an effective therapeutic and/or prevention strategy is still elusive. Various groups of natural compounds have demonstrated anti-HEV71 activities. However more work is needed to characterise the detailed antiviral mechanisms and to further develop into effective clinical drugs. The use of synthetic antiviral compounds in clinical setting has been hampered by potential adverse effects to the host and emergence of drug resistance mutants. New strategies such as computer-aided drug

design, screening of licensed drugs against HEV71 infection, and combination therapy targeting different replication steps of HEV71, may play an important role in antiviral drug development.

The recent identification of HEV71 receptors SCARB2 and PSGL-1 will enable the development of humanised transgenic mice for testing of antivirals and vaccines. Vaccine candidates in the form of inactivated HEV71 have progressed into clinical trials and look most promising. However, the unit cost of inactivated HEV71 vaccines is likely to be high, restricting their usefulness in resource-limited countries in Southeast Asia. By contrast, self-propagating live attenuated vaccines can be produced at much lower unit cost and are thus likely to be more cost-effective for use in vaccine prevention programs in developing countries and in regional and global control strategies. However, in order for this potential to be realised, it will be necessary to design a HEV71 vaccine in which attenuation is fully defined and which possesses a demonstrably higher stability and safety profile than the oral polio vaccine. Together with a good surveillance program, these strategies will hopefully lead to the containment and eradication of HEV71.

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