Review article

CLINICAL AND EXPERIMENTAL VACCINE RESEARCH

Experimental animal models for development of human enterovirus vaccine

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Enterovirus infections induce infectious diseases in young children, such as hand, foot, and mouth disease which is characterized by highly contagious rashes or blisters around the hands, feet, buttocks, and mouth. This predominantly arises from enterovirus A71 or coxsackievirus A16 infections and in severe cases, they can lead to encephalitis, paralysis, pulmonary edema, or even fatality, representing a global health threat. Due to the absence of effective therapeutic strategies for these infections, various experimental animal models are being investigated for the development of vaccines. During the early stages of research on enterovirus infections, non-human primate infections exhibited symptoms like those in humans, leading to their utilization as model animals. However, due to economic and ethical considerations, their current usage is limited. While enterovirus infections do not readily occur in mice, an infection model with mouse-adapted strain in neonatal mice has been employed. Cellular receptors have been identified in human cells, and genetically modified mice expressing these receptors have been used. Most recently, the utilization of Mongolian gerbil model is actively being considered and should be pursued for further animal model development. So, herein, we provide a summarized overview of the current portfolio of available enterovirus infection models, emphasizing their respective advantages and limitations.

Keywords: Enterovirus, Vaccines, Disease models

Introduction

Enteroviruses (EVs) are classified within the genus *Enterovirus of the Picornaviridae* family, exhibiting associations with various human and mammalian diseases. The *Enterovirus* genus comprises 15 species, including EV-A-L and rhinovirus A, B, and C. These entities are non-enveloped viruses with diameters of approximately 30–50 nm, displaying icosahedral capsids composed of 60 identical subunits. The genome of EV consists of an approximately 7,500-nucleotide-long single-stranded positive-sense RNA, encompassing the 5' untranslated region, the 3' untranslated region, and the open reading frame (ORF). The ORF of EV is composed of three regions: P1, P2, and P3. This ORF is translated into a polyprotein that is subsequently cleaved by proteolysis into functional viral proteins. The P1 region encompasses four structural proteins (VP4, VP2, VP3, and VP1) which constitute the viral capsid [1]. Additionally, the P2 and P3 regions give rise to non-structural involved in viral replication. The VP1 protein contains crucial neutralization epitopes that are utilized for virus serotype identification and evolutionary investigations [2]. EV enters host cells through specific recep-

tors, namely human P-selectin glycoprotein ligand-1 (PSGL-1) or scavenger receptor class B member 2 (SCARB2) and others [3-6].

Hand, foot, and mouth disease (HFMD), as well as respiratory infections, diarrhea, viral myocarditis, encephalitis, and aseptic meningitis, are induced by EV. Among these, enterovirus A71 (EV-A71) and coxsackievirus A16 (CV-A16), both belonging to the EV-A species, serve as primary pathogens responsible for HFMD cases globally [7-10]. The reporting of the EV-A71 epidemic began in the Netherlands in 1963 [11] and presently, this disease has become a severe and life-threatening ailment for children worldwide. EV-A71 carries a single serotype and has been classified phylogenetically into three genotypes (genotype A, genotype B, genotype C). The latter two genetic groups further differentiate into B1 to B5 and C1 to C5 sub-genotypes. Due to the lack of effective treatments for diseases caused by EV, vaccines have emerged as the most effective solution for preventing EV-related illnesses. In 2015, two inactivated EV-A71 vaccines were approved in China for the prevention of HFMD [12]. Additionally, both monovalent CV-A16 vaccines and bivalent EV-A71 and CV-A16 vaccines have demonstrated promising efficacy in preclinical studies for preventing severe HFMD [13]. While research on CV-B3 vaccines has been conducted over several years, there currently exists no vaccine capable of safeguarding children from CV-B3 infections leading to viral myocarditis [14]. Furthermore, epidemiological investigations reveal evolving trends in the prevalence characteristics of EVs. CV-A6 and CV-A10 are gradually replacing EV-A71 and CV-A16 as major pathogens HFMD, while the incidence of CV-B3 and CV-B5 is on the rise [15-17]. Although EV-A71 infections generally manifest as mild and self-limiting, at times, such infections escalate into central nervous system (CNS) infections involving aseptic meningitis, encephalitis, and acute flaccid paralysis.

Consequently, collaborative endeavors are currently underway for the research and development of EV vaccines aimed at preventing severe infections in children, necessitating the establishment of suitable animal models. Thus, this review investigates the establishment and types of various animal models utilized in vaccines.

Neonatal Suckling Mouse Model with Mouse Adapted Enterovirus Strains

While viral infections and host defense mechanisms have been proposed, the detailed mechanism of EV-A71's transmission and pathology remain incomplete [18]. In contrast to humans, viral replication in mice predominantly occurs in muscle and adipose cells and mice older than 3 weeks do not manifest sensitivity to EV-A71. However, neonatal mice have been routinely employed as models for virus-induced encephalitis caused by various neurotropic viruses such as flaviviruses [19,20], alphaviruses [21,22], and other enteric viruses [23-25]. In these models, viruses are either congenitally transmitted or administered within the first week of birth to generate desired infection and disease phenotypes in neonates. Therefore, similarly, a neonatal mouse model could be considered for exploring the applicability of an approach to EVs.

According to the research by Sickles et al. [26], coxsackievirus A is normally potent pathogenicity in neonatal mice, and they established the CV-A16 neonatal mouse model for evaluation of vaccine protective efficacy using a clinically isolated BJCA08/CA16 strain. The BJCA08 strain can induce a 100% fatality rate in neonatal mice under 5 days old and inciting clinical symptoms in murine subjects. The neonatal mouse model of EV-A71 infection developed by Chua et al. [27] utilizes a mouse-adapted strain (MP-26M) derived from the clinical isolate EV-A71-26M (sub-genogroup B3). This strain was generated by subjecting the EV-A71-26M virus to six serial passages in BALB/c mice [28,29]. Two mutations in the capsid proteins VP1 (G145E) and VP2 (K149I) were found to generate the mouse-adapted phenotype. The resulting MP-26M strain induces acute flaccid paralysis in neonatal BALB/c mice, accompanied by the development of severe skeletal muscle myositis [27]. A similar mouse model was developed using 1-day-old ICR mice, wherein the Taiwan EV-A71 strain 4643 (subgenogroup C2) was subjected to four serial passages to generate the mouse-adapted strain MP4 [30,31]. Histopathological examination of tissues from mice infected with MP4 revealed the presence of skeletal muscle myositis, along with evidence of neural loss and cell apoptosis in the spinal cord and brainstem. In this virus, two mutations responsible for the mouse adaptation of MP4 have been identified, located in the structural protein genes VP1 (G145E) and VP2 (K149M) [32].

While the neonatal mouse model has greatly enhanced the investigation of EV pathogenicity, validating vaccine efficacy using this model still presents challenges. The neonatal mouse model cannot be directly employed for vaccine candidate research since mice that are less than 1 week old have an imma-

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ture immune system, and the mice take about 2–3 weeks to mature into adulthood, during the vaccine administration. An alternative method for utilizing the neonatal mouse model in vaccine efficacy testing involves assessing the protective antibodies transferred from vaccinated parents to their offspring. Through this approach, the offspring of parent mice that have been vaccinated exhibit elevated titers of EV-A71-specific immunoglobulin G, reaching levels comparable to those observed in immunized parents. When exposed to EV-A71 virus, these neonatal mice do not experience CNS complications and even survive at lethal doses [33]. Nonetheless, there remains a necessity for animal models that offer a more precise assessment of vaccine efficacy beyond these indirect methods.

Mongolian Gerbil: A Rodent Model Close to the Age of An Adult Individual

As previously mentioned, a fully validated adult mouse model with complete relevance for confirming vaccine efficacy has not yet been established. Despite efforts to enhance mouse virulence through adaptation with EV-A71 clinical isolates, the adapted virus itself is unable to infect mice with a mature immune system after a certain age. On the other hand, Mongolian gerbil is a more desirable animal model for validating vaccine efficacy. Mongolian gerbils, belonging to the Gerbillinae subfamily, are rodents native to the Mongolian steppe. According to previous research, gerbils have been utilized as models for infections caused by a variety of viruses, including hantaviruses, La Crosse encephalitis virus, encephalomyocarditis virus, and hepatitis E virus [34,35]. In addition, infections with EV-A71 and CV-A16 have shown disease symptoms like those in humans, making gerbils a valuable model for studying these viruses. Yao et al. [36] employed intraperitoneal inoculation to experimentally infect 21-day-old Mongolian gerbils (*Meriones unguiculatus*) with a clinically isolated strain of EV-A71 (EV-A71/58301, C4 genotype). Infected animals exhibited neurological disorders and histopathological abnormalities like those reported in mouse models. A 2015 paper by Xu et al. [37] demonstrated the manifestation of neurological symptoms associated with neuropathology in gerbils infected with EV-A71, including hind limb paralysis, ataxia, and lethargy. In their report, they confirmed that gerbils aged 7 to 21 days, infected with EV-A71 via intraperitoneal or intramuscular routes, exhibited severe lung lesions a pathology not observed in normal Balb/c mice. Moreover, they provided evidence that passive transfer of specific EV-A71 antisera after a lethal EV-A71 challenge can prevent EV-A71-induced lung lesions. Consequently, the gerbil EV-A71 model has exhibited its potential as an animal model for studying the pathogenesis of EV-A71-mediated pulmonary diseases and vaccine study.

Recently, Sun et al. [38] and Yi et al. [39] established a vaccine efficacy model using Mongolian gerbils. In the study by Yi et al. [39], 3-week-old Mongolian gerbils were immunized with EV-A71 and CV-A16 inactivated at 1 and 2 weeks of age and then infected with the viruses at 3 weeks of age. Mongolian gerbils infected with EV-A71 C4a or CV-A16 exhibited high mortality, severe morbidity, histopathological damage, and elevated viral replication within tissues. In contrast, the vaccinated group displayed significantly reduced symptoms and virus proliferation. These research findings suggest that Mongolian gerbils can serve as a valuable animal model for the development of HFMD vaccines [39].

Immunologically Modified Mouse Model

Given the essential role of the host immune system in suppressing viral infections [40], innate immunity such as interferon (IFN) responses are imperative for preventing EV-A71 infection and disease onset as well [41,42]. Therefore, immune-deficient mice such as IFN knock out (KO) can facilitate infections caused by clinical isolates of EV-A71, relying on mouse-adapted lineages, even in the absence of human receptors required for viral entry. Immunologically modified mouse, AG129, with dual knockout of IFN α/β receptors and IFN γ receptors, is employed for the analysis of EV-A71 pathogenicity [43]. AG129 mice exhibit heightened susceptibility to EV-A71 compared to wild-type mice and are readily infected up to the age of 2 weeks. The virus primarily replicates in skeletal muscles and subsequently reaches the CNS, inducing neurological symptoms such as flaccid paralysis. Research involving the AG129 mouse model and EV-A71 mouse-adapted strains, established through serial passages in rodent cells or animals, is conducted. This approach can extend the sensitivity window for more than 6 weeks [27,32,44]. Experimental successes have demonstrated infection with non-mouse adapted EV-A71 strains in AG129 mice [43] and the virus exhibited marked neurotropism and induced neurological symptoms upon intraperitoneal and oral administration routes.

Stat-1, a key transcription factor in host cells, plays a crucial

role in the signaling cascade of IFNs. It has been shown that Stat-1 KO mice can be effectively infected by both genetic subtypes B and C clinical isolates of EV-A71 [45,46]. One of the chemokines highly expressed during EV-A71 infection is IFN gamma-inducible protein 10 (IP-10) [47]. IP-10 KO mice infected with mouse-adapted EV-A71 exhibited a higher mortality rate compared to wild-type mice, indicating a protective role of IP-10 in EV-A71 infection [48]. These research findings underscore the crucial role of IFN signaling in protecting animals from EV-A71 infection.

Cellular Receptor Transgenic Mouse Model

Currently, cellular receptors have been suggested for mediating the entry of EV-A71 virus into host cells. Human PSGL-1 (CD162) [5] and human SCARB2 [3], have been identified as specific receptors for EVs. PSGL-1 is a sialomucin membrane protein expressed exclusively on myeloid and lymphoid leukocytes as well as platelets, playing a pivotal role in the early stages of inflammation. SCARB2, also known as lysosomal integral membrane protein II or CD36b-like-2, is primarily confined to lysosome, and is widely expressed in numerous human tissues and cell types. Additionally, nucleolin and annexin II have been reported as putative receptors for EV-A71 [49].

Recently, transgenic (Tg) mice carrying human SCARB2 have been generated [50,51]. hSCARB2 Tg mice exhibit age-dependent susceptibility up to 3 weeks postnatal, primarily displaying characteristics like previously reported wild-type mouse models, including viral replication in muscle and CNS regions. A 3-week-old hSCARB2-Tg mice infected with EV-A71 via intravenous, intraperitoneal, and oral routes demonstrated symptoms of motor impairment, paralysis, and fatality [51,52]. The pathological features of these mice were reminiscent of human EV-A71 encephalitis. hSCARB2-Tg mice older than 6 weeks of age are readily susceptible to infection by clinical isolates of EV-A71 and CV-A16, utilizing SCARB2 as a receptor, following intracerebral, intravenous, intraperitoneal, and oral administration. These mice exhibit neurotropism, neuropathology, and clinical features akin to those manifested in humans, primates, and wild-type mice, namely EV-A71's neurotropism, neuropathology, and characteristics such as motor impairment, paralysis, and fatality. Unfortunately, however, none of the mouse models exhibited the induction of pulmonary edema or the subsequent rapid onset of cardiopulmonary failure, which are the causes of death following EV-A71 infection. The absence of pulmonary edema limits the applicability of these mouse models to the study of EV-A71's disease mechanism [51].

Liu et al. [50] established a Tg mouse expressing the human PSGL-1 gene. However, these animals were only susceptible to EV-A71 strains adapted to mouse muscles and lacked susceptibility to clinical isolates of EV-A71. The expression of human PSGL-1 facilitated virus replication and symptom severity; however, this effect was limited to the early stages of infection. These results indicate that human PSGL-1 alone is not sufficient to mediate EV-A71 infection, but it can function as a supplementary factor in the early stages of viral infection in mice.

Non-human Primate; Cynomolgus Macaque, Rhesus, Green Monkey

Early studies showed that non-human primates, including cynomolgus, rhesus and green monkeys, are susceptible to EV-A71 infection [53-55]. In 2002, Nagata et al. [56] established a monkey model as an EV-A71-infected primates through intraspinal injections in cynomolgus monkeys. In this experiment, infected animals exhibited neurological symptoms within 1 to 6 days after virus inoculation. Furthermore, viral replication was observed in various organs including the spinal cord, brainstem, cerebellar cortex, and cerebral hemispheres [56]. Zhang et al. [57] reported that in adult rhesus macaques, administration of clinical isolate EV-A71/FY-23 via intracerebral, oral, or intratracheal routes results in CNS infection and lung tissue damage. Furthermore, the infected animals do not exhibit vesicular lesions on the skin, decreased muscle tone in the limbs, or typical neurological symptoms. These findings suggest that beyond neurotropism, EV-A71 induces respiratory tropism in rhesus macaques. These observations contrast with observations based on cynomolgus monkeys and mice. A neonatal primate model that exhibits symptoms more closely resembling human infections has also been established. Liu et al. [58] utilized clinically isolated C4 EV-A71 strain to infect neonatal monkeys aged 4 to 6 weeks, resulting in the observation of vesicle-like formations on their hands and mouths, closely resembling humans. So far, the established primate models have primarily encompassed cynomolgus monkeys and macaques, allowing for the prediction of clinical protective efficacy of experimental vaccines [58-61]. However, due to financial and ethical constraints, this model has not been widely employed.

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Conclusion

Over the past 2 decades, the prevalence and frequency of outbreaks of EV-A71 and CV-A16 in the Asia-Pacific region have escalated, giving rise to significant public health concerns. An inactivated EV-A71 vaccine has been approved and used in China. However, due to the ongoing emergence of variant viruses and the substantial proliferation of circulating genotypes, there is a notable increase in demand for multivalent vaccines. In general, mice serve as the most extensively employed experimental animals for evaluating the efficacy of vaccine candidates. However, mice infected with EV-A71 or CV-A16 do not exhibit symptoms, thus rendering them unsuitable as experimental animal models. At present, the most appropriate strategy for vaccine development involves utilizing mouse-adapted viruses in genetically modified mice expressing human receptors, which mimic human susceptibility to the virus. The Mongolian gerbil model recently attempted has demonstrated favorable outcomes and has substantial potential for future implementation. However, to provide a more effective framework for the development of vaccines aimed at suppressing rapidly evolving and diverse circulating viruses, it remains imperative to continue the development and utilization of appropriate animal models that possess comprehensive validity.

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