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厦门大学

博 士 学 位 论 文

# 人肠道病毒 Echo25 型的病毒学及免疫学特征与柯萨奇 A16 型动物感染模型研究

# Virological and Immunological Characteristics of Human Echovirus 25 and a Murine Model of Coxsackievirus A16 Infection

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# 目录

# Context

# 摘要

由人肠道病毒感染引起的手足口病（Hand, Foot and Mouth Disease，HFMD）多发生于婴幼儿，在我国法定丙类传染性疾病中发病率和死亡率最高，严重危害婴幼儿的生命和健康。2014 年我国手足口病的发病率超过 2012 年和 2013 年同期的发病率，当前尚无投入实际应用的疫苗或特效药物。目前我国大陆地区的 HFMD 疫情主要与肠道病毒 71 型（EV71）和柯萨奇病毒 A16 型（CA16）有关，但由于肠道病毒种类多样，一些较少被关注的肠道病毒同样也可导致较为严重的疫情，近期在美国发生的肠道病毒 D68 型（EV-D68）疫情就是一个典型的案例。因此，开展相关的病原体流行病学监控及对不同肠道病毒的基础性研究对于及时发现和发展有效防控手段具有十分重要的意义。本研究开展了厦门地区 HFMD 的分子流行病学调查研究，分析了解厦门地区肠道病毒的流行趋势，同时开展病毒流行株分离鉴定，对一种来自厦门的并在我国有多地报告的肠道病毒 Echo25 型开展了病毒学和免疫学研究，进行了病毒全基因组的克隆方法、免疫学检测方法以及疫苗开发可行性的研究。本研究还建立了 CA16 动物感染模型，为相关预防性疫苗和治疗性抗体研究提供支持。

本研究首先对厦门地区 2012 至 2014 年的 HFMD 患者临床标本开展了肠道病毒分子流行病学调查。结果显示，厦门地区报告的 HFMD 病例均主要集中在每一年度的第 2 季度和第 3 季度，其中主要以 4 至 8 月份为主。患者的年龄分布主要为 1 至 3 周岁。通过提取标本的病毒核酸进行 RT-PCR 检测发现，厦门地区 2012 至 2014 年的 HFMD 患者的病原体主要为 EV71 和 CA16，分别占 32%和 16%，但从连续 3 年的检测结果分析，EV71 和 CA16 在 HFMD 患者中的检出比例呈现下降趋势，而非 EV71 和 CA16 的肠道病毒的检出比例呈现上升趋势。该现象与其它一些地区报道的肠道病毒流行情况有一定相似，即在一定时期呈现不同型别病毒交替出现。这种现象可为不同肠道病毒的重组和进化等提供合适的机会，也加大了发展通用 HFMD 疫苗的难度。因此，更全面地开展对不同肠道病毒的研究对于提升应对 HFMD 及相关疾病的能力是很有必要的。

本研究进一步对上述部分非 EV71 和 CA16 感染的 HFMD 患者标本在 MRC-5、RD 和 Vero 细胞中开展了肠道病毒分离的研究，分离获得 1 株 Echo25 病毒和 7

株CB5病毒。其中，该株Echo25病毒为厦门地区首次分离获得的流行株（XM0927株）。本研究对该 Echo25 毒株进行了全基因组克隆及测序（GenBank No. KP099941）。通过对获得的病毒基因组序列开展系统进化分析显示，该毒株与我国山东地区和河南地区分离的Echo25毒株GQ246503和HM031191同属D亚型。通过进一步开展基因组重组分析显示，该Echo25毒株的5'-UTR区与EV77病毒可能存在重组的现象。本研究进一步成功构建了 Echo25 病毒的首个全基因组感染性克隆，并在细胞水平证明其可高效产生具有感染活力的病毒，其增殖能力与原始分离株无显著性差异。该感染性克隆可为进一步开展 Echo25 病毒的基因功能、中和表位和反向疫苗学等研究提供重要条件。本研究同时开展了 Echo25 病毒的制备和纯化研究，获得了较好纯度的病毒颗粒，并开展了透射电镜检测。通过将该 Echo25 病毒免疫 BALB/c 小鼠，获得多抗血清，并进一步筛选获得了 7 株可特异性针对 Echo25 毒株的单克隆抗体。通过对上述 7 株单抗的亲和能力和特异性进行评价，结果显示，单抗 5B9 具有较优的亲和力和反应灵敏度。本研究进一步制备了辣根过氧化物酶标记的 5B9（5B9-HRP）建立 Echo25 感染细胞的酶联免疫斑点检测方法，并成功建立了 Echo25 中和抗体酶联免疫斑点检测方法。应用该方法，本研究筛选获得 3 株抗 Echo25 中和性单克隆抗体，其中 2E9 具有较优的中和能力，其 $IC_{50}$ 为 4.1 ng/mL。对 Echo25 病毒免疫小鼠后的多抗血清进行抗其它不同肠道病毒的中和能力检测显示，Echo25 病毒免疫血清不能中和本研究中使用的其它 4 种肠道病毒，包括 CA16、EV71、CB3、Echo30；同时 EV71 与 CA16 病毒的免疫后血清也不能阻断 Echo25 的感染，说明当前研制的 EV71 或 CA16 疫苗无法阻断 Echo25 的感染。本研究进一步对厦门地区 2010 年收集的部分 EV71 或 CA16 感染确诊患者的血清进行 Echo25 中和抗体检测，发现其中部分血清具有阻断 Echo25 感染细胞的能力，这表明厦门地区可能存在 Echo25 与 EV71 或 CA16 共感染和共流行的情况，由于受限于检测手段而不为人所知。本研究也初步探索了基于该 Echo25 流行株建立新生小鼠感染模型的可行性，但结果显示 Echo25 对 BALB/c 新生鼠的毒性极低，需要开展进一步的研究。

疫苗在进入临床试验之前，必须要在动物水平上评价其免疫原性和保护性。因此，建立相关的评价模型至关重要，特别是动物攻毒保护模型。为支持 CA16 疫苗的研究工作，本研究同时开展了 CA16 动物感染模型的研究。在已有工作基

础上，本研究进一步验证了 CA16 强毒株 4479 和 Z49 对新生小鼠的毒性，并通过对攻毒方式、实验小鼠品系和攻毒株稳定性等方面的摸索，建立了 CA16 动物感染模型，同时建立了被感染小鼠的组织病理检测方法。根据 CA16 病毒免疫母鼠产生的中和抗体可通过血胎屏障传递给乳鼠，并对其产生保护效果的原理，本研究建立了基于母传抗体的 CA16 动物保护模型，并用于 CA16 疫苗的动物模型保护效果评价研究。

**关键词：**肠道病毒；埃可病毒 25 型；感染性克隆；中和抗体；柯萨奇病毒 A16 型动物模型

# Abstract

Hand, foot and mouth disease (HFMD) is a common infectious disease in infants caused by enterovirus, with high morbidity and mortality in Class-C communicable disease. In 2014, the incidence of HFMD remains higher than 2012 and 2013. Currently, effective chemoprophylaxis or vaccination approaches for dealing with HFMD are still not available. The most common strains causing HFMD are coxsackievirus A16 (CA16) and enterovirus 71 (EV71) . Besides EV71 and CA16, numerous outbreaks of HFMD have occurred throughout the world by other HEVs. For example, the largest outbreak of enterovirus D68 (EV-D68) is currently occurring in the United States (US), causing substantial hospitalisation of children with severe respiratory disease. Therefore, the program to prevent this disease concentrates on monitoring and epidemiological characteristics is of great importance. In this study, clinical samples from HFMD patients were investigated in order to identify the virus serotypes outbreak in Xiamen City. The predominant enterovirus serotypes were analyzed. The virological and immunological studies of identified strains Echo25 were performed. In addition, the construction of the infectious cDNA clone, immunological detection methods and vaccine studies were also performed. We also explored the CA16 animal infection models, which is important for the research of vaccines and therapeutic antibody.

In this study, clinical samples from HFMD patients were investigated in order to conduct an analysis on epidemiological characteristics in Xiamen during the period from 2012-2014. Monthly changes in the number of cases showed HFMD cases were detected throughout the year. A sharp peak in the number of cases occurred in April-August. The greatest number of cases was in the age group between zero and three years. Overall, the main pathogens causing HFMD in Xiamen during the period from 2012-2014 were enterovirus 71 (EV71) and coxsackievirus A16 (CA16), which accounted for 32% and 32% of the total causative viruses by real-time RT-PCR. It is important to note that the proportion of EV71 and CA16 showed a significance

declining trend over the years. In some cases, the proportion of other HEVs has been even higher. Besides EV71 and CA16, the co-circulation of other HEVs has also been reported. This phenomenon may provide suitable opportunities for other enterovirus recombination and evolution, but also increased the difficulty of developing common HFMD vaccine. Therefore, a more comprehensive research on the other enteroviruses for preventing HFMD is necessary.

Furthermore, isolation of enterovirus was carried out from HFMD patients infected by non- EV71 or non-CA16. Finally, one Echo25 virus and seven CB5 virus were isolated from MRC-5, RD and Vero cell lines. Interestingly, Echo25 virus which named XM0927 strain was the first time isolated in Xiamen. Firstly, genome of Echo25 had been extracted and sequenced (GeneBank No. KP099941). Based on phylogenetic analysis of the sequence, we found that XM0927 strain was subtype D, which was similar to GQ246503 strain isolated from Shandong province and HM031191 strain isolated from Henan province. And as genome recombination analysis shown, 5'-UTR of Echo25 might be recombinant with EV77. Then infectious cDNA clone of Echo25 was first time constructed. And it had been demonstrated that infectious cDNA clone of Echo25 could be efficiently used to produce recombinant virus in cell model. Just like with original isolated Echo25 virus, recombinant virus also could prolife in cells. It was suggested that infectious clone and recombinant virus could be used for function, neutralizing epitope and reverse vaccinology research of Echo25. Then, Echo25 had been purified and detected by EM. Subsequently, the purified virus were used to administrate in mice. And the screening of monoclonal antibodies against Echo25 were carried out with ELISA. 7 mAbs cells were isolated, and 7 mAbs were harvested. Among them, 5B9 had higher reactivity. It was worth mentioning that 5B9 was conjugated with HRP, and a novel Elispot method with 5B9-HRP was constructed to detect neutralizing activity of mAbs against Echo25. Then 7 mAbs were evaluated with novel neutralizing model. And it was verified that 3 mAbs could block Echo25 infection. Among them, 2E9 had higher neutralizing activity, which $IC_{50}$ was 4.1 ng/ml. At the same time, serum from Echo25-immunized mice had been evaluated to neutralize other enterovirus. The

results showed that it could not cross-neutralizing CA16, EV71, CB3 and Echo30. Similarly, serum from EV71 and CA16 administrated mice also could not cross-neutralizing Echo25. It was predicted that EV71 and CA16 vaccine might not be that efficient to Echo25 infection. To further verify if EV71 and CA16 vaccine could protect Echo25 infection from human, serum of EV71 and CA16 infected patients were evaluated to neutralize Echo25. Serum were collected in 2010 at Xiamen City, and were detected by Elispot. Importantly, it was observed that few serum could block Echo25. It was suggested that patients might be infected by Echo25, EV71 and CA16 at the same time. However, it was not verified due to the limit detection method. Also, we try to construct Echo25 infected mouse model. Unfortunately, BLAB/c mouse was not sensitive enough for Echo25 infection. It was worth to take further research.

As we known, evaluation of immunogenicity and protective on animal model was important to vaccine clinical trial. Specially, it's necessary to construct virus infected animal model. In order to evaluate CA16 vaccine, we also tried to construct CA16 infected animal model. We had isolated high infectious virus strain 4479 and Z49, which were evaluated in newborn mouse. In our studies, the stain of mouse, stability of the virus, and administrated methods of virus were also optimized. At last, CA16 infected mouse model had been constructed. Also histopathological detection method was established. In previous studies, it was reported that neutralizing antibodies of pregnant mouse immunized with CA16 virus could transferred to newborn mouse due to the fetal blood barrier. Therefore, we try to construct novel model to evaluate CA16 vaccine based on neutralizing antibodies obtained from mother.

**Keywords:** Enterovirus; Echo25; Infectious cDNA clone; Neutralizing antibody; CA16 animal infection models

# 缩略词

Amp：Ampicillin，氨苄青霉素

bp：base pair，碱基对

CA16：Coxsackievirus A16，柯萨奇病毒 A 组 16 型

CFT：complement fixation test，补体结合实验

CPE：cytopathic effect，致细胞病变效应

Da：Dalton，道尔顿

DNA：Deoxyribonucleic Acid，脱氧核糖核酸

DMSO: Dimethyl Sulfoxide, 二甲亚砜

Echo25：Echovires 25，埃可病毒 25 型

ELISA：Enzyme-linked ImmunoSorbant Assay，酶联免疫吸附测定

Elispot：enzyme linked immunospot assay，酶联免疫斑点法

EM：Electron Microscopy，电子显微镜

EV：enterovirus，肠道病毒

EV71：enterovirus 71，肠道病毒 71 型

FBS：Fetal Bovine Serum，胎牛血清

HE：hematoxylin and eosin Staining，苏木素伊红染色

HFMD：Hand, foot and mouth disease，手足口病

HRP：Horseradish Peroxidase，辣根过氧化物酶

$IC_{50}$：50% Inhibitory concentration，半数有效抑制浓度

IFA：Immuno fluorescence Assay，免疫荧光实验

IgG：Immunoglobulin G，IgG 抗体

IgM：Immunoglobulin M，IgM 抗体

IHC：Immunohistochemical detection，免疫组织化学检测

Kan：Kanamycin，卡那霉素

kb：kilo base pair，千碱基对

kD：kilo Daltons，千道尔顿

mAb：monoclonal antibody，单克隆抗体