

Flavivirus Research at the Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand

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Abstract

The Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand was established in 1960. The Department of Virology has a long tradition of flavivirus research that started with studies on dengue haemorrhagic fever (DHF). AFRIMS has contributed to flavivirus research with observations on the epidemiology of dengue and Japanese encephalitis (JE) and the development of key assays to test for these viral pathogens. Notable achievements were the development of the plaque reduction neutralisation assay and the IgM/IgG enzyme immuno-assay for JE and dengue as well as the JE vaccine trial in Thai children. The Department of Virology, AFRIMS, continues this strong tradition of flavivirus research initiated in the 1960s. Today, scientific protocols are being performed with both Thai and international collaborators on the immunology of DHF, the pathogenesis of asymptomatic dengue disease, the long-term circulation of dengue serotypes in Thailand and vaccine development of a tetravalent dengue vaccine. Japanese encephalitis is an additional important focus of our flavivirus research. Active research protocols are ongoing on the epidemiology and clinical manifestations of JE, the occurrence of natural JE infection and immunologic boosting in children vaccinated with the killed JE vaccine and the development of new second generation JE vaccines. The Department maintains a high level of expertise and is a resource on flavivirus diagnostics, the epidemiology and immunology of flavivirus diseases in Southeast Asia, and flavivirus vaccine development.

THE UNITED States Army Medical Component, Armed Forces Research Institute of Medical Sciences (USAMC–AFRIMS) has a long and distinguished history of research in tropical infectious diseases. Established in 1958 as part of the Southeast Asian Treaty Organization (SEATO), USAMC–AFRIMS has been instrumental in documenting and reporting the emergence of dengue and dengue haemorrhagic fever, Japanese encephalitis, chikungunya, hepatitis A, hepatitis E, cholera, antibiotic-resistant enteric organisms, drug-resistant malaria, drug-resistant scrub typhus and HIV virus (subtype E). Its current extensive network of field sites throughout Southeast and Southwest Asia continues to provide essential information on these diseases as well as potential emerging new infectious diseases.

Epidemiology of Dengue in Thailand

Dengue virus is the causative agent of dengue fever (DF) and dengue haemorrhagic fever (DHF) and has emerged in the last 50 years as one of the most important global health problems of the 20th century. In Thailand, the reported number of cases of dengue disease has increased since 1958 to more than 50 000 cases of DHF and DF per year, with periodic outbreaks of more than 100 000 cases. Little is known about the long-term circulation of dengue serotypes and their association with epidemics and severe dengue disease in a country hyperendemic for dengue.

A longitudinal diagnostic study of children with suspected dengue at the Queen Sirikit National Institute of Child Health (QSNICH, formally known as the Bangkok Children's Hospital) was conducted in Bangkok, Thailand from 1973 to 1998 (Nisalek et al. in prep.). During this period of observation 17 277 children were admitted with suspected severe dengue disease, of whom 14 680 were diagnosed

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with acute dengue infection: 2276 serologically consistent with primary dengue and 12 206 with secondary dengue. Dengue virus was isolated from 4881 patients and identified as den-1 in 1241 children (25% of all isolates), den-2 in 1575 (32%), den-3 in 1597 (33%) and den-4 in 468 (10%).

The predominant isolated dengue virus serotype by year was: den-2 from 1973 to 1986; den-3 in 1987; den-2 in 1988 and 1989; den-1 in 1990 to 1992; den-4 in 1993 and 1994; and den-3 from 1995 to 1998. No association was observed between specific dengue serotypes and severe dengue years.

In one country hyperendemic for dengue, at least three dengue serotypes can be isolated in any given year. One predominant serotype emerges and re-emerges as the cause of the yearly epidemic over time without specific dengue serotypes associated with large epidemics.

The Current State of Dengue Diagnosis

A rapid immunochromatographic test for IgM and IgG antibodies to dengue viruses was evaluated. The rapid test demonstrated 100% sensitivity in the serologic detection of dengue virus infection and was able to distinguish between primary and secondary dengue virus infections through the detection of IgM and/or IgG antibody. The specificity of the test for non-flavivirus infections was 88% (3 of 26 positive), while for JE virus infections the specificity of the test was only 50% (10 of 20). The rapid test demonstrated a good correlation with reference laboratory assays and may be useful for the rapid diagnosis of dengue virus infection (Vaughn et al. 1998).

Understanding the Immune Response in Dengue Disease

Fever, headache, eye pain and severe muscle and joint pain classically characterise the acute illness caused by dengue virus infection and described as dengue fever. Shock and haemorrhage associated with dengue infection, dengue haemorrhagic fever (DHF), was first noted in Manila in 1953 and recognized in Bangkok, Thailand as a clinical entity in 1958 (Gubler 1997). In 1958, there were 2158 reported cases of DHF in Thailand with 300 reported deaths.

Since 1958, Thailand has experienced a dramatic rise in the annual reported cases of dengue and is now considered hyperendemic for dengue with periodic large outbreaks of disease occurring every three to five years. The reported annual incidence of DHF increased from 9 per 100 000 in 1958 to 189 per 100 000 in 1998.

The largest reported outbreak of DHF in Thailand occurred in 1987 with 174 285 reported cases of DHF with an incidence rate of 325 per 100 000 (Nisalak et al. 1999). Plasma leakage is the hallmark of DHF though little is known about the pathophysiology of severe dengue disease.

Our group has ongoing studies that are demonstrating several important viral and host determinants of disease severity in dengue haemorrhagic fever. The association of viral factors such as certain dengue specific serotypes with more severe dengue disease and the demonstration that peak virus titre correlates with clinical severity have been recently demonstrated in our studies on dengue (Nisalak et al. 1999, Vaughn et al. 1999). T lymphocyte activation, increased cytokine levels and apoptosis also correlate with early immune activation in acute dengue illness and is related to the development of plasma leakage and disease severity (Green et al.).

Conclusion

The Department of Virology, AFRIMS maintains a high level of expertise in the field of arbovirology and flaviviruses. It is a resource for information on regional viral disease threats, diagnostic assays, product development, vaccine field site development, and phase III vaccine trials.

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Serological Responses to Japanese Encephalitis in Thai Swine

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Abstract

Studies of Japanese encephalitis virus (JEV) in Thailand have contributed to our understanding of the transmission cycle of JEV between *Culex* mosquitoes, swine and humans. Studies performed in the Chiangmai Valley, Northern Thailand in 1970 revealed that JEV infections in sentinel and indigenous swine occur every month in Thailand except during January and February. Most infections occur in a 2–3 month period, from May to July. The role of vector density and JEV transmission was studied in three suburban communities in Bangkok from January to June 1987. All three communities demonstrated comparable rates of transmission of JEV to sentinel swine despite one community having a fraction of the mosquito density of the other communities. Peak seroconversion rates in swine preceded the peak mosquito density. In 1985, a field study of JEV demonstrated the isolation of virus in mosquito pools at the same time that infections were noted in sentinel swine. Of 64 seronegative swine placed in five villages, 58 seroconverted and JEV was isolated in four swine. Serologic studies of swine in 1993 demonstrated that JE-specific IgM is detected first followed by haemagglutination inhibition and IgG antibody. Both virologic and serologic data demonstrated that 35%, 80% and 100% of sentinel swine became infected by study days 8, 17 and 57 respectively. Understanding this transmission cycle and the serologic response of swine to JEV infection as sentinels for human disease will further our understanding of the pathogenesis of this disease.

JAPANESE encephalitis (JE) virus is a mosquito-borne flavivirus first isolated in Japan in 1935 from the brain tissue of a fatal encephalitis case. Early epidemiological studies demonstrated the seasonal occurrence of this disease in Japan and suggested that an insect vector may be transmitting the virus. In 1938, the virus was isolated from *Culex tritaeniorhynchus* mosquitoes and subsequently shown to be its principal vector. Since the first clinical descriptions of this disease, much has been learned about the epidemiology of JE. High JE seroprevalence rates has been well documented among pigs, horses, and in the bird population in Japan and several other countries. Other vertebrates including cattle, sheep, dogs, and monkeys have also been found to have appreciable JE seroprevalence rates. Pigs are considered to be the primary amplifying host of JEV. The peak seasonal occurrence of JE varies geographically. In Thailand,

epidemic peaks occur between late June and early August or September. In China, Japan, and Korea, epidemic peaks tend to be in August.

Studies at AFRIMS

In 1969, an epidemic of Japanese encephalitis occurred in the Chiangmai Valley and other areas of northern Thailand. Several epidemiological studies of JEV in Chiangmai Valley were conducted by AFRIMS from 1970 to 1971. These studies examined the relationship of rainfall, *Culex* mosquito density, human encephalitis cases and JEV infections of sentinel pigs. The studies demonstrated that JEV transmission to pigs and humans is highly endemic in Northern Thailand and occurs throughout the year. Peak incidence of cases occurs during the rainy season and correlates with the vector density and infection rate in swine.

Studies by AFRIMS from 1985 to 1987 explored the relationship of JE and vector density during an

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urban outbreak of this disease. In 1985, an unexpected outbreak of Japanese encephalitis occurred in Bangkok. An epidemiological survey was initiated in three suburban areas. *Culex tritaeniorhynchus* and *Cx Gelidus* comprised 71–96% of all mosquitoes collected by CO₂-baited CDC traps at the three sites. JEV was isolated in both species and the minimal infection rate (MIR) was comparable in both species.

The proportion of sentinel pigs that had JE antibodies increased proportionately and correlated with vector abundance at each site. Vector abundance was highest during the monsoon (May–October), moderate during the transition season (March–April and November–December), and lowest during the dry (January–February) seasons.

Mosquitoes collected during the monsoon yielded the greatest amount of JE isolates. Swine seroconversions were greater during the monsoon and transition seasons than in dry seasons. Indices of JE transmission activity (vector abundance, pig seroconversions, and MIRs) increased proportionately with rainfall.

In 1985, AFRIMS conducted a field trial study using an inactivated Japanese encephalitis vaccine (Biken) in 65 224 school children in Kamphangphet province, Northern Thailand. Studies of the transmission of JEV were included in the vaccine trial. Two sentinel pigs, seronegative for JEV, were placed in each village throughout the province every two weeks during the vaccine trial. Mosquitoes were

collected at each site and swine tested for seroconversion to JEV every two weeks.

Results of the survey demonstrated that transmission of JEV was widespread. JEV was isolated from mosquito pools (the first sample was collected in the last week of May), and new anti-JE antibody was detected in a serum sample collected from a sentinel pig on 24 May, 1985. Of 54 seronegative pigs placed in five villages, 48 seroconverted. JEV was recovered from the blood of four pigs.

In 1993, studies conducted at AFRIMS examined swine as a potential animal model for JEV vaccine testing. Antibody analysis after acute JEV infection demonstrated that JE-specific IgM is detected first followed by haemagglutination and IgG antibody. Both virologic and serologic data demonstrated that 35%, 80% and 100% of sentinel swine became infected by study days 8, 17 and 57 respectively.

Conclusion

Pigs are important in the transmission cycle of JEV. Virtually all-domestic swine that became infected develop viraemia capable of infecting mosquitoes. Our studies with sentinel swine demonstrated that they became infected within one week of entering an endemic region with infection occurring throughout most of the year. Serologic data demonstrated that anti-JEV IgM is detected first followed by haemagglutination-inhibition and IgG antibodies.

Genetic Variations in Chinese Field Strains of Hog Cholera Virus

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Abstract

RT-PCR was employed to amplify the portion of the E2 gene encoding major immunogenic sites at the N terminal of the hog cholera virus E2 glycoprotein. This fragment was amplified directly from 23 field strains from hog cholera (HC) tissue samples, which had been responsible for serial HC outbreaks in nine geographically distinct provinces in China. Computer-based phylogenetic relationships among these strains and reference strains were obtained by analysing nucleotide sequence data. This resulted in classification of the 23 strains into two major groups. Nineteen belong to Group 2 and were further subdivided into Subgroups 1, 2 and 3. The remaining four strains, together with the Chinese reference Shimen and attenuated vaccine C strains, belong to Group 1. These findings reveal, surprisingly, that HCV field strains prevalent in China in recent years are genetically divergent from the Shimen and vaccine C strains, indicating a different origin for this virus in China.

HOG CHOLERA (HC), caused by the hog cholera virus, a pestivirus belonging to the Flaviviridae family, is a highly contagious domestic animal disease worldwide, having important economic ramifications. The disease first occurred in the 1920s in China, where now there are two standard HCV strains, a virulent Shimen strain and an attenuated vaccine strain. The latter, appearing in 1957, was derived from serial passages of virulent Shimen strain through rabbits, and so is now known as hog cholera lapinised virus (HCLV). Since that time, HCLV has been used in China as a unique vaccine strain to prevent HC in pigs (Yin et al. 1997).

After spreading gradually worldwide, HCLV became known as 'C' strain. Because of a nationwide immunisation policy of twice-yearly inoculations of pigs, in spring and autumn, hog cholera is well controlled in China, with large-scale outbreaks rarely seen. However, sporadic onsets can be found each year.

It is noteworthy that a mild and atypical form of hog cholera, having a long duration, atypical clinical and pathological symptoms, and relatively low morbidity and mortality, has been observed often since the late 1970s, and that a certain proportion of vaccinated pigs contract it. The reason for this remains unknown, but is assumed to be due either to insufficient immunisation caused by non-rationalised vaccination procedures, or to a genetically variant, less virulent HCV.

Although serologic investigation has not shown different serotypes, studies using monoclonal antibody typing (Lowings et al. 1996), gene sequence analysis (Lowings et al. 1994, 1996; Vilcek et al. 1996) and restriction enzyme mapping (Lowings et al. 1996) have shown distinct HCV genetic groups, thus furthering understanding of HCV molecular epidemiology and evolution. However, this kind of work has not been carried out in China, a geographically huge country, where exist factors likely to influence virus variation and evolution. Such factors include a complex ecology, the coexistence of various animal husbandry methods, a vaccination-based disease eradication policy, and highly mobile commercial herds.

In order to understand HCV's genetic history in China, we have analysed E2 gene variation in

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23 HCV field strains by nucleotide sequencing and phylogenetic analysis. The 23 strains cover 23 sites in nine provinces, almost one-third of the country. Sequence data obtained from this fraction of the country reveal the existence of at least two distinct HCV groups in China. Most of the strains prevalent in recent years are clustered in Group 2 and are surprisingly divergent from the classical Shimen and vaccine HCLV strains, both of which belong to Group 1. Thus, a different origin of HCV in China has been proposed. This investigation has yielded results similar to those of previous studies (Hofmann et al. 1994; Lowings et al. 1996; Vilcek et al. 1996).

Materials and Methods

Viruses

All 23 HCV field strains used were from spleen or lymph node tissue samples from pigs that died from clinically-diagnosed HC. Each of the 23 samples was collected from a different site in nine Chinese provinces; thus, each strain was taken from a single site. The area involved in the investigation comprises about one-third of China.

RT-PCR and sequencing

One set of degenerate primers were chemically synthesised based on a previously published study (Lowings et al. 1996). Sense primer is 5' TC(GA)(AT) CAACCAA (TC)GAGATAGGG3' corresponding to Alfort position 2467-2487. Antisense primer is 5' CA CAG(CT)CC(AG)AA(TC)CC(AG)AAGTCATC 3' corresponding to Alfort position 2738-2716 (Meyers et al. 1989).

Total RNA, prepared directly from collected tissue samples, using TRIzol reagent according to the manufacturer's (Gibco/BRL) instructions, was used to amplify a region encoding predominant neutralising epitopes at the E2 N terminus by previously established RT-PCR protocol (Li et al. 1998). Utilising ABI PRISM 377 DNA Sequencer, PCR products were gel-purified and automatically sequenced without cloning.

Phylogenetic analysis

DNAsis computer software (HITACHI Software Co. Ltd) was used to construct a phylogenetic tree of HCV strains. In addition to the 23 nucleotide sequences and their derived amino acid sequences obtained in this study, six reference sequences representing two groups and four subgroups were retrieved from the GenBank database: Shimen (Accession No. U72047, Li et al. 1998) and HCLV or C (Accession No. 72048, Li et al. 1998), Alfort

(Accession No. J04358, Meyers et al. 1989), Brescia (Accession No. M31768, Moormann et al. 1990), CIW (Accession No. L36164, Lowings et al. 1994) and ALD (Accession No. D49532, Ishikawa et al. 1995).

Results and Discussion

Sequence-based phylogenetic analysis is a powerful tool widely used for molecular epidemiology and viral evolution. The current study shows that the region encoding predominant neutralising epitopes on the E2 gene 5' is not highly variable, but variable enough (van Rijn 1993) to distinguish HCV groups. Analysis of this region is highly consistent with the results for the 5' NCR region, NS5B region, and even monoclonal antibody typing (Lowings et al. 1996).

Utilising sequence data analysis, extensive study of phylogenetic relationships among HCV isolates from more than 200 samples revealed HCV's worldwide molecular epidemiology history (Hofmann et al. 1994; Geerts et al. 1995; Lowings et al. 1996, 1999; Stadejek et al. 1996; Vilcek et al. 1996; Vilcek and Paton 1998; Harasawa and Giangaspero 1999; Widjoatmodjo et al. 1999). However, there exists no relevant information for China.

China has veterinary world importance, not only because it is geographically huge, but also for the following reasons. HC has been endemic in China for more than half a century; outbreaks were dominated by typical and acute infection of pigs at all ages with high morbidity and mortality before the 1970s, then featured atypical and chronic infection and sporadic onset with relative low morbidity and mortality since the 1980s.

In recent years, infection of piglets has been seen much more frequently than of young and adult pigs. In addition, China has pigs numbering 475 million, the largest in the world. This, coupled with China's vaccination-based control policy and the high mobility of commercial herds, maximises opportunities for HCV transmission.

In order to understand the background of HCV molecular epidemiology in China, and to make this kind of analysis comparable to a previous study (Lowings et al. 1996), the exact same primer set was used to sequence the partial E2 gene of field strains collected in China so far. Nucleotide sequences of 23 Chinese field strains, along with six reference sequences, were aligned and compared to analyse variation and determine the phylogenetic relationship among the strains (see Figure 1). It was determined that the 23 field strains could be divided into two major groups with a nucleotide homology of less than 80%. Most, 19 of the 23, were genetically

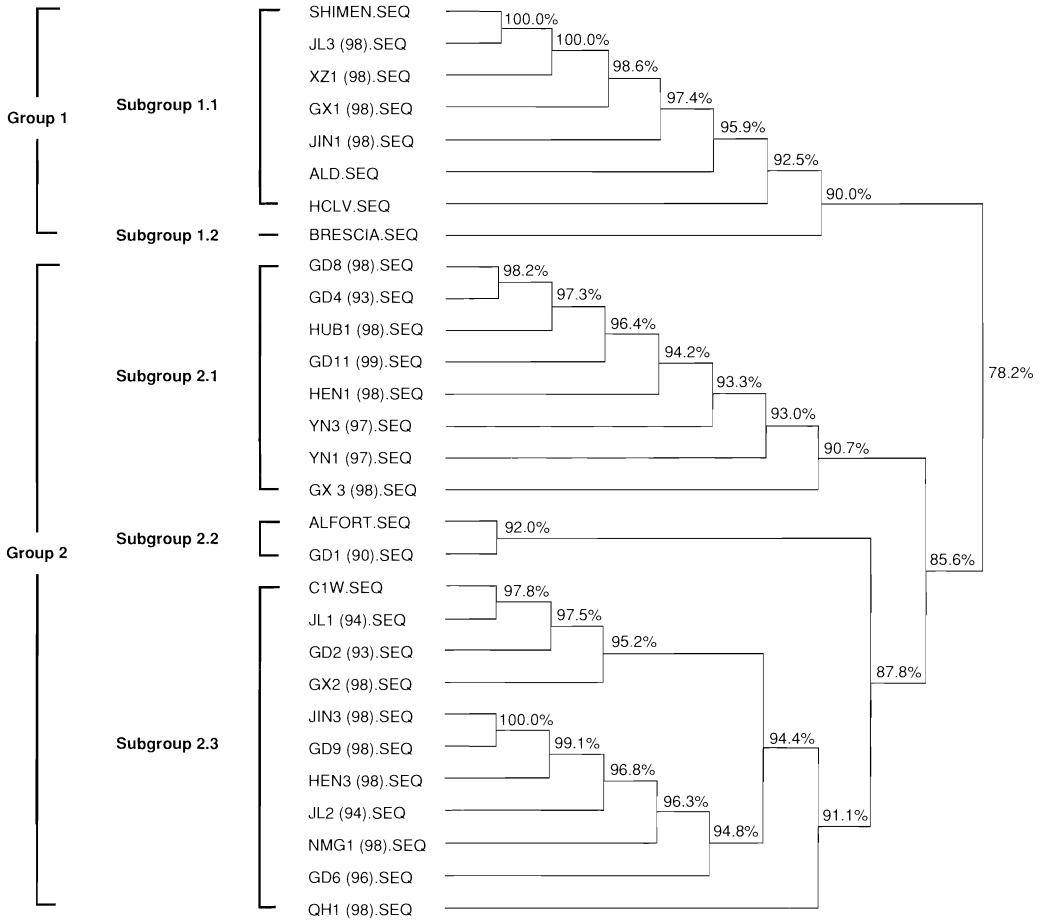


Figure 1. Key to field-strain designation: Capital letters indicate the provincial location of the virus strain, i.e. JL: JiLin; XZ: XiZang; GX: GuangXi; GD: GuangDong; HUB: HuBei; HEN: HeNan; YN: YunNan; NMG: NeiMongGu; and QH: QingHai. The numbers following the letters indicate the order in which the strains were collected. Parenthesised numbers indicate the year that the virus strain was collected. Reference strains are identified by their original names from previous publications. Percentages represent the homology between two lineages.

divergent from the classical Shimen strain (isolated in the 1950s and now a Chinese reference strain), their variation, compared with Shimen and HCLV, existing in the E2 gene. The 19 strains, together with Alfort and C1W (an Italian field strain isolated in 1985), belong to Group 2 and could be further divided into three subgroups. The remaining four strains, together with Brescia, ALD, HCLV and Shimen, belong to Group 1. This would indicate that our classification is comparable to those published previously (Lowings et al. 1996; Stadejek et al. 1996; Vilcek et al. 1996).

The phylogenetic tree shows that all seven strains from GuangDong province (GD strains), belong to Group 2 and could be further divided into three subgroups, indicating HCV variety in the province. Located at extreme south of China, GuangDong is one of the most economically developed of the Chinese provinces. It has had heavy pig trading, with most of its pig populations having been introduced from overseas, as well as from inner Chinese provinces. The high pig herd mobility would suggest cross-transmission of divergent HCV strains in that area. In addition, an evolutionary relationship might

be deduced from the fact those seven GD strains have spanned 10 years, from 1990 to 1999, implying a possible relationship with Alfort, since the oldest strain, GD1, was closest to it. This finding would explain how the European HCV strain was transmitted to China. HCV variety was also found in neighbouring GuangXi Province (GX strains), where two groups of HCV exist: strain GX1 belongs to Group 1, whereas strains GX 2 and 3 belong to Group 2.

Of six references, four virulent strains, Alfort, Brescia, C1W and ALD, were previously clustered into four subgroups (Lowings et al. 1996) and were taken in this study as lineage references. These four reference sequences confirmed the reliability of our grouping of 23 Chinese field strains into two major groups and four subgroups. Moreover, three of the reference strains, though not Brescia, revealed close counterparts to Chinese strains in their lineage, indicating evolutionary origins for Chinese HCVs other than the one from which Shimen derived.

This is a first report on HCV molecular epidemiology in China. Although the data obtained so far are limited, thorough investigation is under way to determine nationwide HCV variation.

Acknowledgments

The authors thank all those who provided HC tissue samples. We thank Susan Mazzocchi, Center for Advanced Biotechnology and Medicine, NJ, United States of America, for her critical review. This project was supported by the National Natural Science Foundation of China as a grant to Z. Yin and C. Tu (No. 39893290).

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DNA-mediated Protection Against Hog Cholera Virus

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Abstract

Four DNA fragments encoding hog cholera virus (HCV) E2 glycoprotein with different function motifs were obtained by PCR amplification. Four corresponding eukaryotic expression plasmids were constructed and designated as: (a) pcDST containing the entire E2 gene insertion with the signal sequence at 5' end and the transmembrane one at 3' end; (b) pcDSW containing the E2 gene insertion with the signal, but not the transmembrane sequence; (c) pcWT containing the E2 gene insertion with transmembrane, but not the signal sequence; and (d) pcDWW containing the E2 gene insertion without both the signal and the transmembrane sequences. All four plasmids have been readily transfected in BHK cells with pcDST and pcDSW capable of secreting E2 antigen. Plasmids pcDST and pcDSW were shown to induce humoral immune response against HCV in mice when administered intramuscularly, but no immune responses were detected with either pcDWT or pcDWW. The antibody level elicited by pcDSW was higher than that induced by pcDST. The results showed the different function motif of E2 gene exerted a significant influence on DNA-mediated immune response. When the pcDSW was used to immunise rabbits and pigs, both rabbits and pigs were shown to be protected from the virulent challenge of HCV (hog cholera lapinised virus for rabbits and Shimen strain for pigs).

HOG CHOLERA (HC), characterised by symptoms of haemorrhagic fever and immune depression, is a contagious swine disease usually leading to substantial economic losses. The pathogen is HC virus (HCV), a member of pestivirus genus, Flaviviridae family, and also known as classical swine fever virus (CSFV). The hog cholera lapinised virus (HCLV) vaccine, developed in China in the 1950s, has played a key role in the control of the pandemic of hog cholera. However, an atypical form of HC and immunisation failure has emerged in China since the 1970s, especially in recent years. Infection of piglets has been seen much more frequently than in young and adult pigs. In some countries such as Germany and The Netherlands where HCV had been eradicated, the epidemic of HC emerged again. It is

important to develop a safer and more efficacious vaccine with a new type of HC strain in control of hog cholera.

DNA vaccine can be developed as an alternative to the traditional vaccines. Vaccination of DNA constructs encoding prominent immunogens of viruses has been shown to induce antiviral immunity in various animal models. For example, HIV (MacGregor et al. 1998; Ugen et al. 1998; Boyer et al. 1999), HBV (Yuen et al. 1999) and malaria (Wang et al. 1998) in humans; BHV1 (Hurk et al. 1997; Lewis et al. 1999) and BRSV (Schrijver et al. 1997, 1998) in bovine and FMDV (Huang et al. 1999; Ward et al. 1997), PRV (Gerdtts et al. 1999; Haagmans et al. 1998), PRRSV (Pirzadeh and Dea 1998) and *E. coli* (Turnes et al. 1999) in swine. Although some host animal trials have been shown to be promising in protection against the viral challenge, much more remains to be done to enhance the efficacy of the naked DNA vaccination. The objective of this study was to determine the HCV-specific immune responses elicited from the DNA constructs and the animal protection from a virulent challenge.

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Materials and Methods

Construction of eukaryotic expression plasmids of E2 gene of HCV

There is a signal peptide preceding the mature E1 protein and three transmembrane domains at its C terminus. Four E2 gene fragments were amplified by PCR using plasmid pHCE2 as template (Li 1998) to contain various combinations with or without the signal sequence and the sequences of the transmembrane domains. The primers were chemically synthesised as shown in Table 1.

The underlined sequences of the primers are corresponding to the E2 gene sequences. The remaining sequences are for restriction enzyme sites for cloning purposes. The CCACCATG sequence has also been added to enhance the translation level of E2 gene according to the Kozak rules (for forward primers). Forward primer Ps contains the signal sequence and Pws not. Reverse primer Pt contains the transmembrane sequence and Pwt not. Random combination of forward and reverse primers would lead to the PCR amplification of four different E2 gene fragments. The PCR procedure was as follows: 95• 60S, 60• 70S, 72• 60S, 30 cycles. In order to construct HCV DNA vaccine plasmid, each of the four amplified E2 gene fragments was digested with Bam HI and Eco RI and then cloned into the same-enzyme digested plasmic pcDNA₃ (Invitrogen). The in-frame sequence of E2 gene fragment in each plasmid was verified by auto-sequencing using ABI PRISM™ 377 DNA sequencer.

Bacterial transformation and plasmid preparation

Transformation of *E. coli* and the DNA plasmid preparation were done according to the method as described (Wicks et al. 1995). The concentration and purity of plasmid DNA were assayed by measuring the absorbency at 260 nm and 280 nm with Gene-Quant (Pharmacia).

Evaluation of eukaryotic E2 expression plasmids in vitro

The expression efficiency of the E2 gene of the four plasmids was analysed by in vitro transfection of BHK-21 cells followed by direct ELISA detection of the cells and culture supernatants. Transfection was

performed with the LIPOFECTIN reagent (Life Technologies) according to the manufacturer's instructions with the following modification. Briefly, the mixture of DNA and LIPOFECTIN Reagent was placed on 40• 60% confluent monolayer of BHK-21 cells for 12 hours. The suspension was replaced by Gibco RPMI 1640 containing 10% newborn calf serum and 40 µg/mL of G₄₁₈. The cells were incubated at 37• for 10 days to let the transfected cells be confluent and the untransfected control cells be dead. Then the G₄₁₈ resistance cells were passaged one more time by repeating the above procedure. Forty-eight hours later, the G₄₁₈ resistance cells and the culture supernatant were harvested for assaying the E2 expression by direct ELISA. The pig anti-HCV antibody was prepared as described by Tsai Chun Lin (1969). Peroxidase-conjugated anti-HCV antibody was made by the method similar to that described by Wilson et al. (1978).

Immunisation of mice

Female 18• 20 g KM mice were purchased from the Laboratory Animals Center of Changchun Institute of Biological Products. Prior to the vaccination, the mice were anaesthetised by i.p. injection of 0.2 mL saline containing ketamine and xylazine (100• g/g and 10• g/g body weight, respectively). The DNA vaccine was prepared by diluting the purified DNA preps to 1µg/µl in PBS. Approximately 100• g of the DNA vaccine plasmid were given to each mouse with 50 µg DNA injected to the tibias anterior (TA) area of each hind leg. A total of six mice was inoculated with each of the plasmid preparations. Mice inoculated with the same amount of blank vector pcDNA₃ were used as control. The mice were immunised at weeks 0, 2 and 4 and the sera were collected by tail bleeds at weeks 3, 4, 5, 6, and 7.

Rabbit immunisation and challenge

Six New Zealand white rabbits (about 2 kg) were purchased from the Laboratory Animal Center of the university. They were divided into three groups (pcDST, pcDSW and pcDNA₃ as control). Two rabbits were given for each group. The rabbits were injected intramuscularly at the TA areas of two legs with 0.5 mg per leg of the vaccine DNA preparation and intradermally (i.d.) at 10 skin sites on the back

Table 1. The primer sequences used to amplify the different E2 gene fragments.

Name	Primers	Primers location in the E2 gene
Ps	5'•CGGGATCCGCCACCATGGTATTAAGAGGACAGGTCGTGC•3'	-63•-42nt
Pws	5'•CGGGATCCGCCACCATGGGCCGGCTAGCCTGCAAG•3'	-3• 15nt
Pt	5'•CGGAATTCCTACTGTAGACCAGCGGCGAGCTGTTC•3'	1102• 1125nt
Pwt	5'•CGGAATTCCTAGTCAAACCACTGATACCTGCC•3'	966• 984nt

(20 µg per site). The inoculation was repeated three times at two-week intervals. All immunised rabbits were challenged with a dose of 10 MID HCLV two weeks after the last immunisation.

Immunisation of pigs

Eight pigs at two months of age were purchased from a HCV-free farm on the outskirts of Changchun City. The pigs and their sows had never been vaccinated with HCV vaccine and their sera were tested to be HC-negative by indirect ELISA (Yu et al. 1999). The immunisation protocol was the same as described for the rabbits. Six pigs were immunised with pcDSW and two with pcDNA₃ as control.

Antibody assays

Anti-HCV antibodies were detected by an indirect ELISA using the recombinant E2 as antigen according to the method described previously (Yu et al. 1999). Briefly, each well of Nunc immunosorb plate was coated with 1.2 µg recombinant E2 antigen in carbonate buffer saline (CBS, 0.05M pH9.6) for overnight at 4°C and then blocked for 1 hour at 37°C with 3% gelatin in PBS. Serum samples were diluted in PBS at 1:200 prior to the addition to the appropriate wells. After 2 hours of incubation at 37°C the plates were washed three times with PBS/0.05% Tween-20 and then added with anti-mouse (or pig) peroxidase-conjugate (Sigma). After repeating the above incubation and washes, the plates were added with 0.045% H₂O₂ and OPD (0.4 mg/mL) in phosphate-citrate buffer (0.1 M citric acid, 0.2 M sodium phosphate dibasic). Allow the reaction for 30 minutes and add 2 M H₂SO₄ to stop the reaction. Absorbency at 490 nm was read on a DG3022A ELISA-Reader.

Results

HCV E2 expression in vitro cells

In order to determine whether the signal sequence and the transmembrane domain of E2 gene of HCV exert any influence on the immune response of DNA vaccine, four different plasmids, pcDST, pcDSW, pcDWT and pcDWW, were constructed and then used to transfect BHK-21 cells. Each plasmid-transfected cell and their culture supernatant were assayed respectively by ELISA for HCV E2 expression. The results showed that E2 products could be

detected from the cells transfected with plasmids pcDST and pcDSW, but not with pcDWW and pcDWT. Moreover, E2 expression was detected in the culture pellets of cells transfected by both pcDST and pcDSW and in the culture supernatant of pcDSW-transfected cells as well, but not in the supernatant of pcDST-transfected cells based on the ELISA assay (data shown in Table 1). These results suggested that E2 glycoprotein could be anchored on the cell membrane by the expression of transmembrane domain of E2 gene and could only be secreted into the culture supernatant as the transmembrane domain was deleted.

Comparison of mouse immune responses induced by four different plasmids

Four plasmids were used to immunise the four mouse groups with six mice in each group. The collected sera were diluted 200-fold and their antibody responses against HCV were evaluated by an indirect-ELISA using the recombinant E2 as antigen (Yu et al. 1999). Serum conversion was judged by the ratio P/N = 2.0.

The results showed that the mice immunised with pcDST and pcDSW exhibited serum conversion at week 4 (i.e. 2 weeks after the second immunisation). The antibody responses were increased gradually to week 7, the end of bleed. The specific antibody level induced by pcDSW was slightly higher than that induced by pcDST (Table 3). However, immune responses were not detected for either pcDWT or pcDWW (data not shown).

Protection of rabbits against HCLV challenge

Plasmids pcDST and pcDSW were used to vaccinate rabbits. All immunised rabbits were challenged with a dose of 10 MID HCLV two weeks after the last immunisation. The body temperatures of the rabbits were measured twice a day for 3 days before challenge and for 2 days after challenge, and then 4 times a day for 2 weeks 2 days after challenge.

After challenge, pcDNA₃ controls were deemed not to yield immunity against HCLV infection since a significant increase of body temperature over 1°C was observed at 84–96 hours after challenge and lasted for about 36 hours. In pcDST group a mild increase of body temperature over 0.5°C was observed lasting a short time about 12 hours. However, no body temperature increase was observed in pcDSW

Table 2. The expression of HCV DNA vaccine plasmids in BHK-21 (direct-ELISA, OD₄₉₀).

Groups	pcDST	pcDSW	pcDWT	pcDWW	pcDNA ₃ control
Supernatants	0.070	0.656	0.025	0.037	0.014
Transfected cell pellets	0.521	0.510	0.213	0.175	0.200

Table 3. The antiHCV IgG levels induced by pcDST and pcDSW (indirect-ELISA).

Groups	OD ₄₉₀				
	3w	4w	5w	6w	7w
pcDSW	0.086±0.021*	0.139±0.023	0.167±0.032	0.240±0.020	0.283±0.032
pcDST	0.073±0.021	0.135±0.016	0.163±0.025	0.212±0.015	0.267±0.038
pcDNA ₃	0.061±0.032	0.060±0.018	0.069±0.020	0.058±0.030	0.070±0.021

*the mean value from 6 immunised mice.

Table 4. Antibody responses against HCV in pigs immunised with DNA vaccine (indirect ELISA).

Groups	OD ₄₉₀					
	Days after immunisation				Days after challenge	
	0	15	30	45	4	8
pcDNA ₃	0.12±0.01	0.14±0.01	0.13±0.01	0.13±0.02	0.13±0.01	0.14±0.01
pcDSW	0.12±0.01	0.13±0.02	0.22±0.05	0.30±0.03	0.40±0.04	0.45±0.03

group which, thereby, was deemed to yield an efficient immunity against HCV infection. These results probably suggest that pcDSW was likely to provoke stronger immune response than pcDST, although both were effective in eliciting the HCV immune response in rabbits.

Protection of pigs against HCV challenge

Plasmid, pcDSW seems to be the most effective construct in eliciting an immunity against HCV based on the mouse and rabbit studies and so was chosen to be used to immunise pigs. Two weeks after last inoculation all six pcDSW-immunised pigs completely resisted the challenge with a lethal dose of HCV Shimen strain as determined by the observation of clinical signs, visible pathological inspection and antigen detection of HCV. Two of them just showed a slight fever without HC symptoms and pathological lesions and recovered next day. The two pcDNA₃ control pigs suffered severe HC after challenge, which showed typically acute HC symptoms and pathological lesions. One died 10 days later and another was killed at moribund stage. The titre of HCV-specific antibodies elicited by DNA vaccine increased with the time and the recalling reaction of immune response after challenge was observed for immunised pigs but not for controls (as shown in Table 4).

Discussion

In this study, four different functional HCV E2 fragments were amplified by PCR and subcloned into eukaryotic expression vector pcDNA₃ for the

construction of the four E2 expression plasmids. Since E2 gene is one partial sequence of a large open reading frame and does not contain the initiative code, the sequence CCACCATG, which is compatible with Kozak's rule, was added to the 5' end of E2 to enhance the efficiency of translation initiation.

A signal peptide precedes the N terminus of mature E2 protein and three transmembrane domains locate at its C terminus (Rumenape et al. 1993). In order to understand whether these two function motifs exert any influence on the immune response level, four different E2 gene expression plasmids with or without the signal sequence and the transmembrane sequences were constructed. Immunisation of mice with these plasmids showed that pcDST and pcDSW (both contain signal sequence preceding E2) could elicit an immune response whereas pcDWT and pcDWW (both do not contain signal sequence) could not. The result was different from that obtained by Haddad et al. (1997). The reason may be that E2 is a glycoprotein and cannot be processed to be a mature protein without a signal sequence.

Zijl et al. (1991) has found that the presence of the transmembrane domains at the C terminus of E2 is required to obtain complete protection mediated by the recombinant pseudorabies virus (PRV) expressing HCV E2. However, it might be different for the HCV DNA vaccine. The mouse serology experiment in this study showed that the level of immune response elicited by the E2 DNA plasmid without transmembrane domains was higher than that with transmembrane domains when administered intramuscularly.

The reason is unclear. It can be speculated that muscles are the major vaccine injection sites and there are few AP cells (APC) in muscles. Muscle cells do not possess the function of antigen presentation (AP). Therefore, if the antigen expressed by muscle cell with the transmembrane domain, it would be associated with the membrane and thus the antigen would not be transported properly. When the transmembrane domain is omitted, the E2 antigen could be secreted out of the cell and delivered elsewhere through tissue liquids, lymph liquids and even blood and thus the antigen message could be presented properly to the immune system.

It is further evidence that pcDSW could completely protect immunised rabbits from the HCLV challenge but pcDST only provided limited protection. All six pigs immunised with pcDSW were protected from the challenge of virulent HCV Shimen strain. The results showed pcDSW could induce a strong immune response.

Acknowledgment

This work was supported by the Ministry of Science and Technology, China (Grant 96C01-04-01 to C. Tu).

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