Tropical Biomedicine 26(3): 352–359 (2009)

Molecular characterization of Hepatitis A virus causing an outbreak among Thai navy recruits

Theamboonlers, A.[1], Rianthavorn, P.[1], Jiamsiri, S.[2], Kumthong, S.[2], Silaporn, P.[2], Thongmee, C.[1] and Poovorawan, Y.[1*]

[1] Center of Excellence in Viral Hepatitis, Paediatric Department, Faculty of Medicine, Chulalongkorn University and Hospital, Bangkok, Thailand

[2] Field Epidemiology Training Program (FETP), Bureau of Epidemiology, Department of Diseases Control, Ministry of Public Health, Thailand

Corresponding author email: Yong.P@chula.ac.th

Received 18 September 2009; received in revised form 27 September 2009; accepted 27 September 2009

Abstract. Hepatitis A virus (HAV) infection is a communicable disease, typically transmitted by faecal-oral contamination. HAV outbreaks usually occur in endemic areas. We report an outbreak of HAV from June to July, 2008 among Thai navy recruits who had received training at the Sattahip Navy Base, Chonburi province, Thailand. Upon conclusion of the training, the recruits were deployed to serve at several navy bases across the country. Secondary cases of HAV infection were reported among military personnel from these navy bases. To elucidate origin and distribution of these outbreaks, we characterized the genome and genotype of HAV isolated from the different navy bases. Sera and stool from the subjects were tested for antiHAV IgM, antiHAV IgG and HAV RNA. Subsequently, molecular characterization of HAV was performed by nucleotide sequencing of the VP1-P2A region, BLAST/FASTA and phylogenetic analysis. HAV RNA was detected in specimens obtained from different areas. All isolated strains clustered in the same lineage and belonged to genotype 1A. They shared nearly 100% genome homology indicating a single point source of this outbreak. This study provides essential baseline data as a reference for genetic analysis of HAV strains causing future outbreaks. Early detection of HAV infection and identification of the source by using molecular characterization and prompt preventive measures will hopefully prevent further outbreaks.

INTRODUCTION

Hepatitis A virus (HAV) is classified as Enterovirus type 72. It belongs to the genus Hepatovirus in the *Picornavirus* family (Yokosuka, 2000). The HAV genome comprises 7.5 kb single stranded RNA and is divided into three functional regions, P1, P2 and P3 (Rueckert *et al.*, 1984). The P1 region encodes 4 capsid polypeptides (VP1, VP2, VP3 and putative VP4). The P2 and P3 regions encode non-structural proteins necessary for various essential functions including viral replication. HAV is divided into at least 7 genotypes based on the VP1-P2A region (Robertson *et al.*, 1991; Robertson *et al.*, 1992; Taylor *et al.*, 1997). However; genotype VII was recently reclassified as a sub-genotype of genotype II (Costa-Mattioli *et al.*, 2002; Lu *et al.*, 2004). HAV genotypes I, II, III and VII were isolated from humans, whereas genotypes IV, V and VI were isolated from several simian species. Most human HAV strains belong to genotypes I and III. HAV subtype IA is responsible for the majority of HAV infections worldwide including cases from Thailand (Theamboonlers *et al.*, 2002; Poovorawan *et al.*, 2005). HAV subtype IB is endemic in the Mediterranian region (Nainan *et al.*, 2006; Pintó *et al.*, 2007). Phylogenetic analysis of various human HAV genomes suggested an association between nucleotide sequence homology and geographic distribution of HAV. (Jansen *et al.*,1990; Robertson *et al.*, 1991). Hence,

characterization of HAV genotypes by partial genome sequencing can determine both source and evolution of the virus and may be used when comparing among future outbreaks of HAV.

The incidence of HAV infection is higher in developing countries than in developed countries. Faecal-oral contamination transmits HAV, causing sporadic cases or epidemics of acute infectious hepatitis (Lemon *et al.*, 1985; Cuthbert, 2001). In developing countries where HAV infection is endemic, most of the people get infected and become immune to HAV during childhood. Improvement of public sanitation and hygiene reduces the HAV infection rate in children and increases the number of adults susceptible to HAV infection (Poovorawan *et al.*, 2000; Poovorawan *et al.*, 2002). This leads to a decline of anti-HAV immunity in the community, thus facilitating HAV outbreaks in these developing countries. (Sinlaparatsamee *et al.*, 1995; Poovorawan *et al.*, 2000; Theamboonlers *et al.*, 2002; Poovorawan *et al.*, 2005; Barameechai *et al.*, 2008). In Thailand, there has been a significant decrease of hepatitis A seroprevalence, from high to intermediate and low endemicity over the past 25 years (Chatproedprai *et al.*, 2007).

This study has been conducted to determine the molecular characterization of HAV during the 2008 outbreak among navy recruits. The outbreak occurred among navy recruits who had undergone training at the Sattahip Navy Base, Chonburi province, Thailand.

MATERIALS AND METHODS

During June and July 2008, an HAV outbreak occurred among the navy recruits who were trained at the Sattahip Navy Base, Chonburi province, 165 km east of Bangkok, the capital city of Thailand. Upon conclusion of their training, the recruits were deployed to serve at several navy bases all over the country including the center (Bangkok), northeast (Nongkhai), east (Chonburi and Chantaburi) and south (Chumphon, Naratiwat and Songkhla) of Thailand (Fig. 1). Those navy bases experienced HAV outbreaks 2-10 weeks after primary cases had been reported.

Outbreak investigation

In June 2008, the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health (MOPH), Thailand was notified from Songkhla Provincial Health Officer of a hepatitis outbreak among the navy recruits who had undergone training at the Sattahip Navy Base, Chonburi province. Outbreak investigation was conducted by a review of medical records from Somdejphrapinklao Hospital. Criteria for index cases included navy recruits who had undergone training at the Sattahip Navy Base with jaundice and at least 2 of the following symptoms from the followings: fever, anorexia, right upper quadrant abdominal pain, vomiting, and dark urine. Secondary cases were defined as any military personnels who had been in contact with index cases and developed the previously mentioned symptoms or signs within 15-65 days after the onset of illness in index cases.

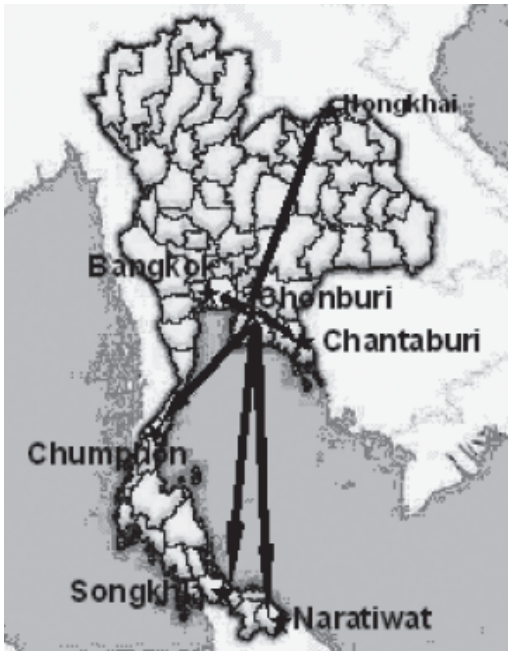

Figure 1. The map of provinces that navy bases experienced HAV outbreaks after training.

Table 1. The number and location of sera and stool collection, HAV IgG, HAV IgM, HAV-RNA and number of random sequencing

Location of Outbreak	Sample tested No.	AntiHAV IgG positive No.	%	AntiHAVIgM positive No.	%	RT-PCR positive No.	%	No. of random Sequence
Sera								
Bangkok	134	100	74.6	79	58.9	66	83.5	6
Chonburi	26	18	69.2	14	53.8	11	100	4
Narathiwat	6	5	83.3	6	100	3	50	3
Songkhla	1	1	100	1	100	1	100	1
Stool								
Bangkok	5	–		–		3		0
Chantaburee	5	–		–		3		1
Songkhla	4	–		–		1		1

Specimen collection

To investigate the outbreak and take control measures, we obtained the serum and stool samples from the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health. Number and location of the specimens are shown in the Table 1. Each specimen was labeled and sent to the Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University with a code that did not provide any personal information of the subjects. The specimens were kept at –70°C until tested.

Laboratory Methods

Serological test for anti-HAV IgM and IgG

Sera were tested for anti-HAV IgM by using commercially available ELISA kits (HAVAB-M, ARCHITECT, Abbott Laboratories, Wiesbarden, Germany). For anti-HAV IgG testing. To determine anti-HAV IgG, Murex anti-HAV (total) kits (HAVAB-G, ARCHITECT, Abbott Laboratories, Dartford, UK.) were used. The cut-off levels for anti-HAV IgM and IgG titers were calculated as recommended by the manufacturer.

Detection of HAV-RNA by nested RT-PCR

Sera and the supernatant of faecal specimens diluted 1:10 with PBS (Phosphate buffered saline) were used to analyze HAV-RNA by nested RT-PCR. RNA was extracted from 50-µl samples applying the guanidinium-isothiocyanate method as described elsewhere (Theamboonlers *et al.*, 2002). The RNA was then reverse transcribed into cDNA using primer BR-9b 5'-AGTCACACCTCTCCAGGAAAACTT-3' (nt 3310-3286). The cDNA was amplified by nested PCR using primer BR-5b 5'-TTGTCTGTCACAGAACA ATCAG 3' (nt 2950-2972) as the outer sense primer, and BR-9b as the outer antisense primer. RJ-3c 5'-TCCCAGAGC TCCATTGAA-3' (nt2984-3002) was used as the inner sense primer and Br-6b 5'-AGGAGGTGGAAGCACTTCATTTGA-3' (3217-3193) as the inner antisense primer (Robertson *et al.*, 1994). Both amplification reactions were performed under the following conditions: initial denaturation at 95°C for 3 minutes followed by 35 cycles comprising denaturation at 95°C for 1 minute, primer annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. The amplification reactions were concluded by a final extension step at 72°C for 7 minutes. After preparation of the PCR products and subsequent electrophoresis in a 2% agarose gel stained with ethidium bromide, the expected 234 bp-band was examined on a UV trans-illuminator (Gel Doc 1000, BIO-RAD, CA).

HAV sequencing and genotype characterization

The PCR products were purified for sequencing by using the Perfectprep Gel Cleanup Kit (Eppendorf, Westbuty, NY) and subsequently subjected to 2% agarose gel electrophoresis in order to ascertain their purity. The concentration of the amplified DNA was determined by measuring each sample's absorption at 260 nm in a Bio-Photometer (Eppendorf, Hamburg, Germany). The DNA concentration was calculated based on the conversion of 1 OD 260 being equivalent to 50 µg of double-stranded DNA. Between 10 and 30 ng/µl (3-6 µl) of each DNA sample were subjected to cycle sequencing by a thermocycler (9600 Perkin Elmer Cetus, Norwalk, CT) using 8 µl of dye terminator from the DNA Sequencing kit (Big Dye Terminator V.3.1 Cycle Sequencing Ready Reaction, Fostercity, CA) and 3.2 pmole of specific primer in a final reaction volume of 20 µl. This round of amplifications was performed according to the manufacturer's specifications, using primers BR-6b and RJ-3C (to reconfirm the sequence) to amplify the specific DNA strand of interest for further sequencing. The extension products were subsequently purified from excess unincorporated dye terminators by ethanol precipitation according to the manufacturer's specifications (ABI Sequencing kit, ABI, Foster City, CA) and subjected to sequence analysis by the ABI Prism 310 Genetic Analyser (ABI, Foster City, CA).For all subsequent steps, we referred to the ABI Prism 310 Genetic Analyser user manual.

Hepatitis A virus genotype and phylogenetic analyses

For characterization of the HAV strains from this outbreak, the BLAST/FASTA program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Phylogenetic analysis were used. The sequences were edited by using programs CHROMASLITE v.2.0 (http://www.mb.mahidol.ac.th) and SeqMan (DNASTAR, Madison, WI). To investigate the relationship between HAV strains, the un-rooted tree topology based on multiple alignments of the VP1-2A nucleotide sequences with those of known genotype from Genbank (genotype 1-7) was obtained by the neighbour-joining method calculated with MEGA 3.1 (http://www.megasoftware.net). Consistency of branching was tested with a bootstrap analysis with 1000 re-samplings of the data using MEGA 3.1. Multiple protein translation and sequence alignments were generated with BioEdit version 7.0.1 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). All HAV strains from this study were grouped and compared with L07693, L077297, L07691, AJ299465, L07732, L07731, AF543492, AF543493, L07703, EF207355, EF207326, AF503454, AF544395, AY148804, EF207350, AY352216, EF207344, AY150858 serving as reference strains.

RESULTS

The mean age (range) of patients in this outbreak was 22 (21-26) years. During the HAV outbreak from June to July 2008, 134 sera were obtained from the navy recruits serving in Bangkok. Anti HAV IgM, anti HAV IgG and HAV-RNA were detected in 79, 100 and 66 sera, respectively. In Chonburi, positive results for anti-HAV IgM, anti HAV IgG and HAV-RNA were 14, 18 and 11 out of 26 sera, respectively; in Narathiwat, 6, 5 and 3, respectively; in Songkhla 1 serum was positive for all parameters tested and from 4 stool samples, 1 was positive for HAV-RNA; of the 5 stool samples from Bangkok, 3 were positive; and of the 5 stool samples from Chantaburi, 3 were positive for HAV-RNA. The serological test was not performed on all stool samples (Table 1).

Sixteen strains of HAV from sera and faecal specimens that were positive for HAV RNA were randomly subjected to direct sequencing. Subsequently, the 168- bp products of the VP1-P2a region were analyzed and compared with those of the 19 strains previously published. We found that all 16 strains of HAV from this outbreak clustered in the same lineage and belonged to genotype 1A (Fig. 2). Based on BLAST/FASTA analysis, these 16 strains shared 99.2-100% genome homology. In comparison, HAV genome homology previously published in

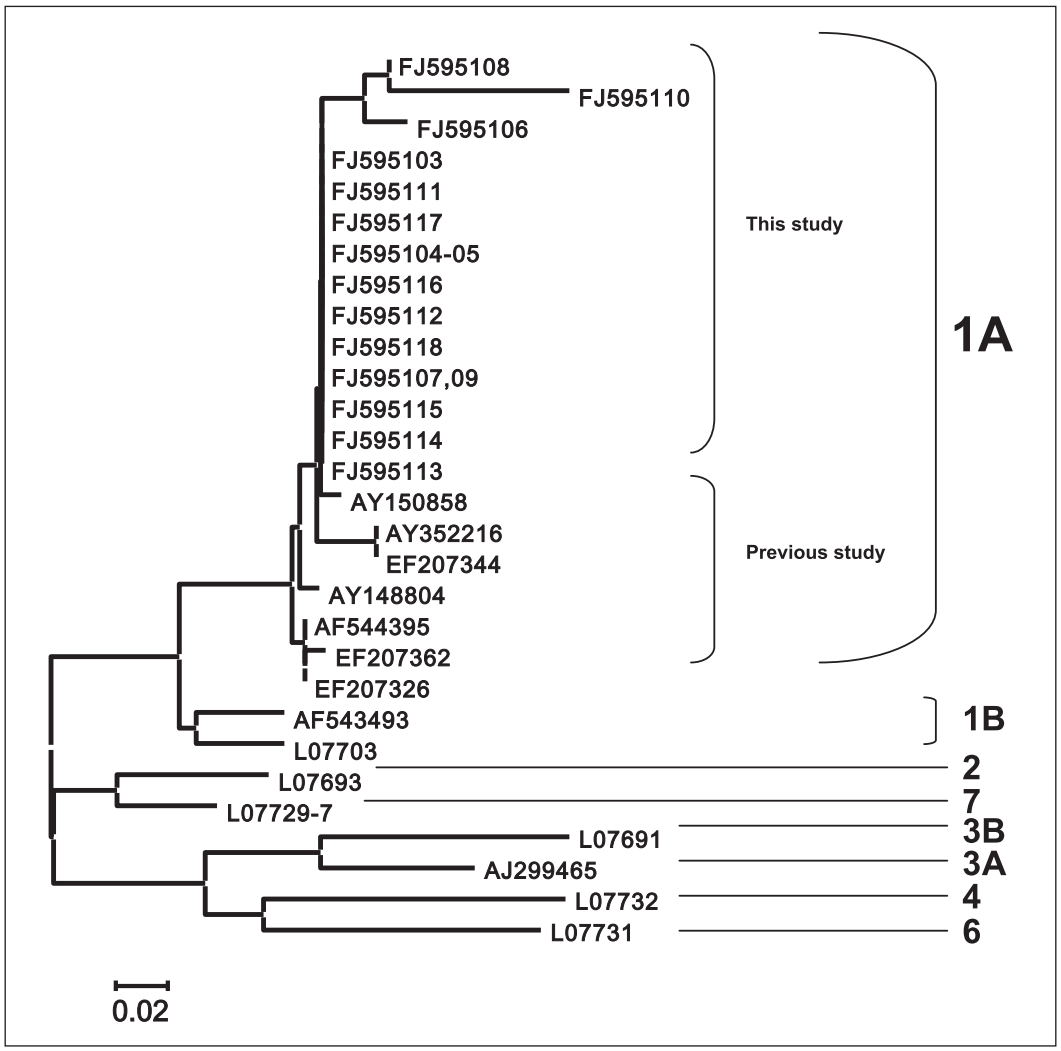

Figure 2. Phylogenetic tree which showed the relationship between HAV strains by using un-rooted tree topology based on multiple alignments of the VP1-2A nucleotide sequences with those of known genotype from Genbank (genotype 1-7) was obtained by the neighbour-joining method calculated with MEGA3.1

Thailand only amounted to 97.5-98.3% (Barameechai *et al*., 2008).

DISCUSSION

In developing countries where HAV infection is endemic, majority of persons are infected during early childhood and all adults are immune. Over the past 25 years, there has been a significant shift in epidemiology of hepatitis A in Thailand, from high to intermediate to low endemicity. Hepatitis A seroprevalence in Thailand continues to decline due to the significant improvements in sanitation and personal hygiene (Chatproedprai *et al*., 2007). This has resulted in a growing population of susceptible adolescents and adults, the groups most likely to be symptomatic when infected with HAV, and can lead to outbreaks of the disease.

Between 2001and 2005, several outbreaks of HAV occurred in Thailand and the main subgenotype has been identified as 1A (Theamboonlers *et al*., 2002; Barameechai

et al., 2008). Our results concurred with findings from previous studies in that HAV strains from Thailand belonged to genotype 1A. (Barameechai *et al.*, 2008). We could isolate only one HAV subtype from the specimens indicating one single source causing this outbreak. The mean age of subjects was 22 years, confirming that immunity against HAV is declining among the young generation in Thailand.

Unfortunately, we could not detect the first index case. This particular individual might have had mild symptoms and spread the disease within the training camp. He might have contracted hepatitis A virus during his home visit and upon his return to the training Navy Base, might have transmitted it to his fellow recruits. Alternatively, the virus might have originated from the food supply at the Navy Base. As the incubation period of the disease amounts to up to four weeks, the navy recruits had by then completed their training and been transferred to different Navy bases throughout the country. Thus, they disseminated the disease within their respective Navy base.

Prior to discovery of the VP1/P2A junction as more variable and more suitable for detection and characterization of HAV RNA (Jansen *et al.*, 1990 Robertson *et al.*, 1991) the carboxy-terminal region of VP3 had been used for PCR and genotype determination of HAV (Jansen *et al.*, 1990). Recent analysis of HAV RNA in sera provided new evidence that, besides the faecal-oral route, HAV outbreaks may have been prompted by HAV contaminated clotting factor transfusion (Mannucci *et al.*, 1994; Robertson *et al.*, 1994; Chudy *et al.*, 1999). Other reported sources of infection included improper chlorination of the water supply (Sinlaparatsamee *et al.*, 1995; Lee *et al.*, 2008; Sowmyanarayanan *et al.*, 2008).

Besides better sanitation, basic precautions when handling food and increased public awareness of HAV, vaccination of individuals susceptible to HAV infection is the cornerstone of outbreak control. From the previous study, Poovorawan *et al.* demonstrated that the HAV outbreak affecting 61 radiology technician students in Thailand was successfully controlled by vaccination of the contact cases (Poovorawan *et al.*, 1994). From cost-benefit analysis of hepatitis A vaccination, the benefit from universally vaccinating young adult population with 2 doses of hepatitis A vaccine does not justify the expense encountered (Teppakdee *et al.*, 2002). However; only one single dose of hepatitis A vaccine was proven to induce enough antibody levels to prevent infection but the reason for administering the second dose is to sustain the long term immunity (Poovorawan *et al.*, 1996). These data suggested that during an outbreak, administration of one single dose of hepatitis A vaccine to seronegative subjects who may be in contact with the index case can effectively control the outbreak.

Further research aimed at HAV genotype characterization should be performed in other parts of Thailand to identify the genotype most common in the country. To prevent future outbreaks, better sanitation, HAV vaccination of susceptible groups, early detection of HAV infection and prompt identification of the source of infection will be urgently required. For future outbreaks, this study provides essential baseline data as a reference for genetic analysis of HAV strains.

Acknowledgements. We are grateful to the Center of Excellence in Clinical Virology Fund, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Thai Red Cross Society. Commission on Higher Education, Ministry of Education, Chulalongkorn University, CU Centernary Academic Development Project. Also, we would like to express our gratitude to the entire staff of the Center of Excellence in Viral Hepatitis, Chulalongkorn University and the staff from the Bureau of Epidemiology, Department of Disease Control, Epidemiological Investigation and Surveillance Section , Epidemiology section, Bangkok Naval Base, Epidemiology section, Somdejphrapinklao Hospital, and the officer of Bangkok Naval Base for their efforts in

collecting specimens. We also would like to thank Ms Petra Hirsch for reviewing the manuscript.

REFERENCES

Barameechai, K., Sa-Nguanmoo, P., Suwannakarn, K., Thongmee, C., Payungporn, S. & Chongsrisawat, V. (2008). Molecular characterisation of the hepatitis A virus circulating in the 2001-2005 outbreaks in Thailand. *Annals of Tropical Medicine Parasitology* **102**: 247-57.

Chatproedprai, S., Chongsrisawat, V., Chatchatee, P., Theamboonlers, A., Yoocharoen, P. & Warinsathien, P. (2007). Declining trend in the seroprevalence of infection with hepatitis A virus in Thailand. *Annals Tropical Medicine and Parasitolology* **101**: 61-8.

Chudy, M., Budek, I., Keller-Stanislawski, B., McCaustland, K.A., Neidhold, S. & Robertson, B.H. (1999). A new cluster of hepatitis A infection in hemophiliacs traced to a contaminated plasma pool. *Journal of Medical Virology* **57**: 91-9.

Costa-Mattioli, M., Cristina, J., Romero, H., Perez-Bercof, R., Casane, D., Colina, R. & Garcia, L. (2002). Molecular evolution of hepatitis A virus: a new classification based on the complete VP1 protein. *Journal of Virology* **76**: 9516-25.

Cuthbert, J.A. (2001). Hepatitis A: old and new. *Clincal Microbiology Review* **14**: 38-58.

Jansen, R.W., Siegl, G. & Lemon, S.M. (1990). Molecular epidemiology of human hepatitis A virus defined by an antigen-capture polymerase chain reaction method. *Proceedings of the National Academy of Sciences of the United States of America* **87**: 2867-71.

Lee, C.S., Lee, J.H. & Kwon, K.S. (2008). Outbreak of hepatitis a in korean military personnel. *Japanese Journal of Infectious Diseases* **61**: 239-41.

Lemon, S.M. (1985). Type A viral hepatitis. New developments in an old disease. *New England Journal of Medicine* **313**: 1059-67.

Lu, L., Ching, K.Z., Paula, V.S., Nakano, T., Siegl, G. & Weitz, M. (2004). Characterization of the complete genomic sequence of genotype II hepatitis A virus (CF53/Berne isolate). *Journal of Genral Virology* **85**: 2943-52.

Mannucci, P.M., Santagostino, E., Di Bona, E., Gentili, G., Ghirardini, A. & Schiavoni, M. (1994). The outbreak of hepatitis A in Italian patients with hemophilia: facts and fancies. *Vox Sanguinis* **67**: 31-5.

Nainan, O.V., Xia, G., Vaughan, G. & Margolis, H.S. (2006). Diagnosis of hepatitis a virus infection: a molecular approach. *Clinical Microbiology Review* **19**: 63-79.

Pintó, R.M., Alegre, D., Domínguez, A., El-Senousy, W.M., Sánchez, G. & Villena, C. (2007). Hepatitis A virus in urban sewage from two Mediterranean countries. *Epidemiology and Infection* **135**: 270-3.

Poovorawan, Y., Tieamboonlers, A., Chumdermpadetsuk, S., Glück, R. & Cryz, S.J.Jr. (1994). Control of a hepatitis A outbreak by active immunization of high-risk susceptible subjects. *Journal of Infectious Disease* **169**: 228-9.

Poovorawan, Y., Theamboonlers, A. & Safary, A. (1996). Single-dose hepatitis A vaccination: comparison of different dose levels in adolescents. *Vaccine* **14**: 1092-4.

Poovorawan, Y., Theamboonlers, A., Sinlaparatsamee, S., Chaiear, K., Siraprapasiri, T. & Khwanjaipanich, S. (2000). Increasing susceptibility to HAV among members of the young generation in Thailand. *Asian Pacific Journal of Allergyand Immunology* **18**: 249-53.

Poovorawan, Y., Chatchatee, P. & Chongsrisawat, V. (2002). Epidemiology and prophylaxis of viral hepatitis: a global perspective. *Journal of Gastroenterology and Hepatology* **17**: S155-66.

Poovorawan, Y., Theamboonlers, A., Chongsrisawat, V., Jantaradsamee, P., Chutsirimongkol, S. & Tangkijvanich, P. (2005). Clinical features and molecular characterization of hepatitis A virus outbreak in a child care center in Thailand. *Journal Clinical Virology* **32**: 24-8.

Robertson, B.H., Friedberg, D., Normann, A., Graff, J., Flehmig, B. & Shouval, D. (1994). Sequence variability of hepatitis A virus and factor VIII associated hepatitis A infections in hemophilia patients in Europe. *Vox sanguinis* **67**: 39-45.

Robertson, B.H., Jansen, R.W., Khanna, B., Totsuka, A., Nainan, O.V., Siegl, G., Widell, A., Margolis, H.S., Isomura, S. & Ito, K. (1992). Genetic relatedness of hepatitis A virus strains recovered from different geographical regions. *Journal General Virology* **73**: 1365-77.

Robertson, B.H., Khanna, B., Nainan, O.V. & Margolis, H.S. (1991). Epidemiologic patterns of wild-type hepatitis A virus determined by genetic variation. *Journal of Infecious Disease* **163**: 286-92.

Rueckert, R.R. & Wimmer, E. (1984). Systematic nomenclature of picornavirus proteins. *Journal of* Virology **50**: 957-9.

Sinlaparatsamee, S., Nuniem, J., Kankao, J., Theamboonlers, A., Chumdermpadetsuk, S. & Poovorawan, Y. (1995). An outbreak of hepatitis A in school children at Nakhon Si Thammarat, southern Thailand. *Southeast Asian Journal Tropical Medicine and Public Health* **26**: 104-8.

Sowmyanarayanan, T.V., Mukhopadhya, A., Gladstone, B.P., Sarkar, R. & Kang, G. (2008). Investigation of a hepatitis A outbreak in children in an urban slum in Vellore, Tamil Nadu, using geographic information systems. *Indian Journal Medical Research* **128**: 32-7.

Taylor, M.B. (1997). Molecular epidemiology of South African strains of hepatitis A virus: 1982-1996. *Journal Medical Virology* **51**: 273-9.

Teppakdee, A., Tangwitoon, A., Khemasuwan, D., Tangdhanakanond, K., Suramaethakul, N. & Sriratanaban, J. (2002). Cost-benefit analysis of hepatitis a vaccination in Thailand. *Southeast Asian Journal Tropical Medicine and Public Health* **33**:118-27.

Theamboonlers, A., Jantaradsamee, P., Chatchatee, P., Chongsrisawat, V., Mokmula, M. & Poovorawan, Y. (2002). Molecular characterization of hepatitis-A-virus infections, in the context of two outbreaks in southern Thailand. *Annals of Tropical Medicine Parasitology* **96**: 727-34.

Yokosuka, O. (2000). Molecular biology of hepatitis A virus: significance of various substitutions in the hepatitis A virus genome. *Journal of Gastroenterol Hepatol* ogy **15** Suppl: D91-7.