UNIVERSITI PUTRA MALAYSIA

COMPARISON OF EXTRACTION METHODS FOR DETECTING HEPATITIS A VIRUS IN SHELLFISH (*MYSTILUS GALLOPROVINCIALIS*) USING TISSUE CULTURE TITRATION, RT-NESTED PCR AND REAL TIME RT-PCR

PARVANEH HAJEB

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By

YONG HAN TEK

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

June 2009

Specially dedicated to my beloved family and dear friends

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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June 2009

Chairman: **Professor Son Radu, PhD**

Faculty: **Food Science and Technology**

Thirty commercial shellfish samples (22 cockles and 8 surf cockles) were collected from several locations around Serdang and tested for the presence of hepatitis A virus (HAV) using a modification of the glycine, polyethylene glycol, Tri-reagent, poly dT bead (GPTT) extraction protocol. None of the 30 tested samples yielded positive results for HAV contamination. Subsequently, detection of HAV in artificially spiked shellfish sample was performed using the modified GPTT method to ensure the negative results were not due to methodological limitations. A parallel comparison between the modified GPTT method and an alternative method, proteinase K-miniMAG in terms of virus recovery and RNA purification efficiency was performed simultaneously. For the first stage of comparison of virus recovery rate, shellfish extract digested by proteinase K resulted in higher recovery when 1 x 10^4 $TCID_{50}$/ml of HAV was recovered in contrast to only 4 x 10^2 $TCID_{50/}$ ml by the glycine-PEG method. The second stage of comparison was conducted to determine

the efficiency of three combinations of virus recovery and RNA purification methods: glycine-PEG-Tri-reagent (M1), glycine-PEG-miniMAG (M2) and proteinase K-miniMAG (M3). Undiluted and serially diluted RNA samples extracted by each method were subjected to RT-nested-PCR. Samples from M1 and M2 were only positive when the samples were undiluted with M2 producing a higher intensity band compared to M1. Samples from M3 were detectable even when diluted up to 100 times indicating that proteinase K digestion was more effective in recovering HAV from shellfish matrix than glycine-PEG and miniMAG was more effective in purifying viral RNA than conventional Tri-reagent. To further investigate the precise efficiency differences among the three methods, a comparison of the Ct values generated by real time RT-PCR was conducted. M3 was again proven to be the most efficient by showing on average the lowest Ct values among the three methods. Lastly, 34 shellfish samples collected in Naples, Italy which were processed using the proteinase K-miniMAG method and analyzed using real time PCR were all found to be negative for HAV. Collectively, these results indicate that a combination of proteinase K with miniMAG that is capable of recovering higher numbers of virus and of yielding higher quantities of intact RNA than the glycine-PEG-Tri-reagent method which was used previously to type the 30 Malaysian samples. Hence this method combination should serve as the method of choice for future detection of HAV from shellfish samples in Malaysia.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PERBANDINGAN KAEDAH PENGEKSTRAKAN UNTUK PENGESANAN KEHADIRAN VIRUS HEPATITIS A DALAM CENGKERANG (*MYSTILUS GALLOPROVINCIALIS*) MENGGUNAKAN TITRASI TISU KULTUR, RT-NESTED PCR DAN REAL TIME RT-PCR

Oleh

YONG HAN TEK

Jun 2009

Pengerusi : Profesor Son Radu, PhD

Fakulti : Sains dan Teknologi Makanan

Tiga puluh sampel cengkerang kommersial (22 kerang dan 8 lala) telah dikutip dari beberapa lokasi di Serdang untuk pengesanan kehadiran virus hepatitis A (HAV) dengan menggunakan kaedah pemprosesan GPTT yang telah diubahsuai. Kaedah asal menggunakan glycine, polyethylene glycol, Tri-reagent dan poly d(T) untuk pengestrakan RNA diubah melalui pengecualian langkah poly d(T). Semua sampel negatif untuk kehadiran HAV. Seterusnya, sampel yang dijangkiti dengan HAV secara artifisial telah diproses dengan modifikasi kaedah GPTT untuk memastikan keputusan negatif bukan disebabkan ketidaksesuaian kaedah. Pada masa yang sama, experimen perbandingan antara modifikasi kaedah GPTT dan kaedah alternatif, proteinase-K-miniMAG dari segi keberkesanan dalam pemulihan virus dan pemencilan RNA dijalankan serentak. Dalam perbandingan peringkat pertama, ekstrak cengkerang yang dicernakan oleh proteinase K menghasilkan kadar

pemulihan yang tertinggi di mana 1 x 10^4 $TCID_{50}$/ml virus telah dipulihkan berbanding dengan 4 x 10^2 $TCID_{50}$/ml menggunakan cara glycine-PEG. Peringkat kedua perbandingan adalah bertujuan untuk menentukan keberkesanan tiga kombinasi kaedah untuk memulihkan virus and pengekstrakan RNA virus: glycine-PEG-Tri-reagent (M1), glycine-PEG-miniMAG (M2) dan proteinase K-miniMAG (M3). Sampel asal dan sampel yang dicairkan dari setiap kaedah diperiksa dengan RT-nested-PCR. M1 dan M2 hanya positif untuk sampel asal tanpa pencairan dengan M2 mempamerkan jalur yang lebih tinggi intensitinya. M3 mampu menghasilkan jalur walupun dicairkan seratus kali memnunjukkan percernaan menggunakan proteinase K lebih berkesan untuk pemulihan HAV dari sampel cengkerang berbanding glycine-PEG dan miniMAG lebih sesuai untuk pengestrakan RNA berbanding Tri-reagent. Untuk menyelidiki kejituan antara ketiga-tiga kaedah, perbandingan *Ct value* melalui real time RT-PCR telah dijalankan. M3 sekali lagi menghasilkan *Ct value* yang terendah antara ketiga-tiga kaedah. Akhirnya, 34 sampel cengkerang telah dikumpul dari Naples, Italy dan telah diproses dengan kaedah proteinase K-miniMAG dan dianalisis dengan real-time RT-PCR dan kesemua sampel adalah negatif. Kesimpulannya, kesemua keputusan memaparkan bahawa kombinasi kaedah proteinase K disusuli miniMAG mampu memulihkan jumlah virus yang tinggi dari sampel dan menghasilkan RNA dan kaedah ini berpotensi untuk dijadikan kaedah pilihan untuk pengesanan HAV dari sampel cengkerang di Malaysia.

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I certify that an Examination Committee has met on date of viva voce to conduct the final examination of Yong Han Tek on her Master of Science thesis entitled "Comparison of extraction methods for the detection of hepatitis A virus in shellfish by using tissue culture titration, RT-nested PCR and real time RT-PCR." in accordance with Universiti Putra Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Fatimah Abu Bakar, PhD
Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairman)

Nazamid Saari, PhD
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Internal Examiner)

Tan Soon Guan, PhD
Professor
Faculty of Biotechnology and Biomolecular Science
Universiti Putra Malaysia
(Internal Examiner)

Wan Kiew Lian, PhD
Associate Professor
Faculty of Science and Technology
University of Kebangsaan Malaysia
Malaysia
(External Examiner)

_____
HASANAH MOHAMAD GHAZALI, PhD
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Son Radu, PhD
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairman)

Farinazleen Mohammad Ghazali, PhD
Senior Lecturer
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Member)

Luciana Croci, PhD
Research Scientist
Istituto Superiore di Sanita
Rome, Italy
(Member)

_____
HASANAH MOHD GHAZALI, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 11 September 2009

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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YONG HAN TEK

Date: 20 August 2009

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
CDC	Centers for Disease Control and Prevention
DNA	deoxyribonucleic acid
cDNA	complementary deoxyribonucleic acid
CRL	Community Reference Laboratory
dNTPs	deoxy nucleotide triphosphate (PCR nucleotide mix containing dATP, dTTP, dGTP and dCTP
EC	European Commission
EU	European Union
FDA	Food Drug Administration
G	gram
HAV	hepatitis A virus
IMR	Institute of Medical Research
kb	kilo basepair
MEM	minimal essential medium
NSPP	National Shellfish Sanitation Program
PCR	polymerase chain reaction
PEG	polyethlyneglycol
RNA	ribonucleic acid
RNase	ribonuclease
RT-PCR	reverse transcription PCR
RT-nested PCR	reverse transcription nested polymerase chain reaction

Real Time RT-PCR	real time reverse transcription polymerase chain reaction
rpm	revolution per minute
SDS	sodium dodecyl sulphate
TBE	tris borate EDTA electrophoresis buffer
TCID	tissue culture infectivity dose
UV	ultra violet
US	United States
USDA	United States Department of Agriculture
WHO	World Health Organization
x g	unit gravity

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Hepatitis A, a term first introduced in 1967, is a liver infection caused by the hepatitis A virus (HAV) whose primary replication site is in the hepatocytes (Krugman et al., 1967). HAV is a positive-sense, single stranded RNA virus of the *Picornavaridae* family, and is the sole member of the genus *Hepatovirus*.

Infection by HAV confers life-long immunity and can produce effects that range from asymptomatic to fulminant hepatic failure; which, in some cases, can cause death (Ross and Anderson, 1991). However, the fatality rate in HAV infections is lower than 0.1%, and the higher risk is usually limited to young children and older adults with underlying chronic liver disease (Akriviadis and Redeker, 1989). The likelihood of clinically apparent disease associated with HAV infection increases with age (Hadler et al., 1980). More than 70% of cases of HAV infection that occur in children less than 6 years old are asymptomatic, or, if illness occurs, it is not accompanied by jaundice (Hadler et al., 1980).

Epideomologically, HAV is present worldwide; the highest prevalence of infection occurring in regions where low standards of sanitation promote transmission of the virus (Hadler, 1991).

Most cases of hepatitis A can be explained by fecal-oral transmission of the virus. Infection is usually acquired by person to person contact or through ingestion of contaminated food or water. The most commonly reported vectors of foodborne associated hepatitis A are shellfish, vegetables and fruits; where shellfish consumption accounts for 50% of the food transmitted hepatitis A cases (Cliver, 1985). Bivalve shellfish are readily contaminated with HAV present in the water, because they are known to filter large quantities of water, and retain and concentrate the viruses during the natural feeding process (Gaillot et al., 1988; Gerba and Goyal, 1978; Metcalf et al., 1979; Mitchell et al., 1966). Furthermore, HAV has been shown to survive for more than one month in seawater (Callahan et al., 1994), and the protective effects of the shellfish meat on virus stability from the natural environmental temperature changes further enhance the survival of HAV in less favorable conditions (Croci et al., 1999). This is in agreement with all the experiments and data concerning long term survival ability of HAV in water; and its resistance to many physical and chemical agents, as used in the depuration process (Biziagos et al., 1988; Siegl et al., 1984b).

As shellfish serve as a leading vector of HAV transmission, the availability of a reliable and widely applicable technique for the detection and quantification of HAV in shellfish would be of interest to the public health community. Reverse transcription PCR (which can overcome the shortcomings of other available methods) represents the method of choice for the rapid and sensitive detection of HAV RNA. So far, for shellfish contamination, HAV has been successfully isolated by RT-PCR from bivalve mollusks (Di Pinto et al., 2004; Sanchez et al., 2002),

oyster (Coelho et al., 2003; Desenclos et al., 1991; Le Guyader et al., 2000), mussels (Casas and Sunen, 2001; Chironna et al., 2002; Croci et al., 2005; Le Guyader et al., 2000) and clams (Bosch et al., 2001; Kingsley et al., 2002; Sunen et al., 2004; Sunen and Sobsey, 1999).

The crux of the successful application of RT-PCR resides in the preliminary processing of shellfish samples prior to the RNA amplification. Therefore, the choice of a suitable processing method which results in a low volume, non-cytotoxic extract with sufficient viral recovery and efficient RNA purification is essential for PCR accuracy and reproducibility. Numerous protocols have been suggested for processing shellfish samples (Atmar et al., 1995; Croci et al., 1999; Cromeans et al., 1997; Kingsley and Richards, 2001; Le Guyader et al., 1996; Lees et al., 1994; Legeay et al., 2000; Sobsey et al., 1978; Sunen and Sobsey, 1999; Traore et al., 1998; Yang and Xu, 1993). Two general schemes designated for the virus recovery are extraction-concentration and adsorption-elution-concentration. The viral extraction methods that have been described are clarification in presence of different buffers, flocculation using polyelectrolytes or extraction by organic solvents. On the other hand, the adsorption-elution schemes are largely based on the alteration of pH and the ionic condition of the buffer (Legeay et al., 2000). Similarly, concentration techniques that have been used are precipitation by PEG or in acidic condition, ultracentrifugation, and also organic flocculation.

More recently, real-time PCR has been developed for the production and quantification of amplicons using intercalating dyes, or fluorescent probes or

primers. Emergence of real time-PCR has revolutionized nucleic acid detection with its high speed, sensitivity, reproducibility, and minimization of contamination. The incorporation of reverse transcription followed by hot start real-time PCR allows the detection and enumeration of RNA viruses simultaneously. The increased speed of real time PCR is mainly due to the reduction in amplification cycles and elimination of the post-PCR detection procedures (Mackay et al., 2002). Real-time data collection is achieved using fluorescent chemistries that provide a strong correlation between fluorescence intensity and PCR product quantity. Molecular beacons (Abd El Galil et al., 2004; Costafreda et al., 2006; Mackay et al., 2002; Tapp et al., 2000) and Taqman (Costa-Mattioli et al., 2002; Costafreda et al., 2006; Jothikumar et al., 2005; Villar et al., 2006) based assays are a few of the chemistries that use fluorescently labeled, target specific probes for real time RT-PCR detection of HAV RNA. Alternatively , SYBR (Brooks et al., 2005) , a DNA binding fluorophore has been widely used because of its simplicity and ability to detect highly variable genome regions (Karlsen et al., 1995; Kiltie and Ryan, 1997; Morrison et al., 1998), although it is less specific and sensitive compared to probe-based assays.

The Unites States and the European Union have regulated the criteria for the harvesting and sale of shellfish products. However, the regulations only specify bacteriological parameters as an indicator for safe consumption, which is inadequate as the absence of bacteria does not guarantee the virological quality of shellfish (Kingsley and Richards, 2001). This is because viruses tend to be more resilient than bacteria to the effects of sewage treatment processes and environmental stressors. Thus, water while containing acceptable levels of faecal coliforms, may contain high

levels of enteric viruses (Gerba and Goyal, 1978). Due to this loophole of the current regulations, the EU and The United States have been trying to develop a molecular diagnostic method suitable for the routine monitoring of shellfish (or the sea water of the shellfish growing area) for contamination by either bacteriological or viral contaminants.

Malaysia has maintained statistical data on hepatitis A, but has not instigated a systematic disease surveillance system for collating and reporting data. Consequently, the actual number of outbreaks associated with shellfish consumption is unknown and may have been underestimated because in many developing countries, poor rural people are the main consumers of wild shellfish (Rehnstam-Holm and Henroth, 2005). In addition, the lack of disease awareness has caused them not to seek help from medical and public health officials. Thus, such viral disease outbreaks are not often reported and brought to public attention.

The focus of this study was a comparison of extraction methods for the detection of HAV in shellfish samples. The findings of this study are significant as the determination of the most efficient method could facilitate the implementation of routine monitoring of commercial shellfish contamination levels and the initiation of a shellfish surveillance system in Malaysia.

1.2 Objective

The purpose of the study is as follows:

To compare glycine-PEG-Tri-reagent and proteinaseK-miniMAG methods of viral recovery and RNA purification for viral recovery rate and RNA purification efficiency by infectivity assay, RT-nested PCR and also real time RT-PCR

CHAPTER 2

LITERATURE REVIEW

2.1 Morphology and Physiochemical Properties of HAV

HAV was formerly classified as *Enterovirus* (serotype 72) within the *Picornavaridae* family. However, due to its unique structural composition, stability characteristics, tissue tropism and genetic distance from other members of picornaviruses genera, it is now classified as a separate genus, *Hepatovirus* (Melnick, 1992; Rueckert and Wimmer, 1984).

HAV, first identified in 1973 by electron microscope, is one of the smallest and structurally simplest RNA animal viruses. The viral particle is non lipid enveloped, therefore resistant to ether, chloroform and alcohol. Morphologically, HAV is an isometric particle with a diameter of 27-32nm and composed entirely of 70% viral protein and 30% ribonucleic acid (Koff, 1998; Lemon, 1994; Stapleton and Lemon, 1994) and it appears as a featureless sphere under the electron microscope. The buoyant density of the full viral particles is 1.32-1.34g/cm^3 in CsCl and has a sedimentation coefficient of 156-160 S in neutral sucrose solutions. During early infection, empty capsids, collected in feces, band at 1.20 and 1.29-1.31g/cm^{3,} with sedimentation coefficient ranging from 50 S to 90 S, predominantly 70 S (Koff, 1998).