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Review Article
Hepatitis A virus – a general overview

*Yong, H. T. and Son, R.

Centre of Excellence for Food Safety Research, Faculty of Food Science and Technology, University Putra Malaysia, 43400 Serdang, Selangor, Malaysia

Abstract: Hepatitis A virus infection occurs globally and is causing a public health concern, primarily in developing countries due to its persistent circulation in the environment. The improved sanitary condition and increase in awareness of personal hygiene have led to the marked reduction of HAV prevalence in industrialized countries during childhood and to a shift of the infection towards adulthood. HAV is an environmentally stable, positive single stranded RNA virus that is primarily transmitted by the fecal-oral route, person to person contact or ingestion of contaminated food and drink. One of the main causes leading to HAV infection is epidemiologically linked to the consumption of raw or undercooked shellfish particularly oysters and clams. Due to their filter-feeding style, these shellfishes readily concentrate viruses from the surrounding water containing municipal sewage, and as a consequence pose a health threat to consumers. Therefore, development of detection techniques possessing the requisite sensitivity and specificity for the practical routine monitoring purposes is of great importance necessary for the protection of shellfish-consuming public. Nucleic acid based method such as reverse transcription PCR has emerged as the popular method of choice in view of its rapidity, accuracy and sensitivity in contrary of the time-consuming conventional cell culture and hybridization techniques. However, detection of hepatitis A virus is firstly hampered by the non-cytophatic effect of wild type HAV strain, secondly, the low concentration of viral genome present in the environmental sample which requires effective isolation and concentration of virions and lastly the labor-extensive purification and thorough removal of the abundance of the PCR inhibitors which will unfavorably reduce the efficiency of PCR detection.

Keywords: Hepatitis A Virus

Introduction

Hepatitis A, a term first introduced in 1967, is known to be a liver infection caused by 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 that belongs to the family of *Picornavaridae* and is the unique member of the genus *Hepatovirus*. Unlike the other members of the family, HAV requires a long adaptation period to grow in cell culture, replicates slowly and rarely produces a cytopathic effect (Cromeans *et al*., 1987; Lemon, 1992). African green monkey kidney cells or fetal rhesus kidney cells are commonly used for culturing the virus, although many different cell types are suitable. The genome of the HAV is approximately 7.5kb, consists of a highly conserved 5' end non-translated region (NTR) and has a covalently linked virus specific protein (Vpg) instead of a cap structure (Weitz *et al*., 1986). Translation occurs in a cap-independent pattern under control of an internal ribosome entry segment within the 5'NTR (Brown *et al*., 1991). The 3'end NTR has a poly-A tail (Cohen *et al*., 1987), and the remainder of the genome is composed of a single open reading frame that will be translated to single large polyprotein. The polyprotein will be subsequently cleaved by a viral protease which results in production of four capsid proteins and some non-structural proteins (Schultheiss *et al*., 1994).

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 exposed to young children and older adults with underlying chronic liver disease (Akriviadis and Redeker, 1989). The likelihood of

*Corresponding author.
Email: *abbieyong84@gmail.com*

clinically apparent disease associated with HAV infection increases with age (Hadler *et al*., 1980). More than 70% of cases of HAV infection occur in children less than 6 years old are asymptomatic, or, if illness occurs, it is not accompanied by jaundice (Hadler *et al*., 1980). However, in older children and adults, HAV infection causes more-severe clinical illness, including jaundice malaise, fever and dark urine, in 70% of cases (Lednar *et al*., 1985).

Epideomologically, HAV is present in a worldwide distribution, the highest prevalence of infection occurring in regions where low standards of sanitation promote transmission of the virus (Hadler, 1991). In areas of high endemicity in Asia, Africa, Latin America, and the Middle East, the prevalence of HAV IgG antibodies reaches 90% in adults, and most children have been infected by 10 years of age. In areas of intermediate endemicity in some countries in Asia and Europe, only 50%–60% of adults and 20%–30% of 10-year-old children have been infected. In some countries of low endemicity, notably in Europe, hygienic improvement and overall rises in socioeconomic status have brought a fall in HAV infection during early childhood. Majority of adults thus remains susceptible to infection, which may lead to the occurrence of hepatitis A outbreaks among the general population (Mackiewicz *et al*., 2005).

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 common reported routes of foodborne associated hepatitis A are shellfish, vegetables and fruits where shellfish consumption would account 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 quantity of water, 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 moth in seawater (Callahan *et al*., 1994), and the protective effect 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 of HAV in water and its resistance to many physical and chemical agents including depuration process (Biziagos *et al*., 1988; Siegl *et al*., 1984).Consumption of raw or partial cooked shellfish and the intestinal tract that does not inactivate HAV has been implicated in many outbreaks. The first documented outbreak of hepatitis A, involving 629 cases associated with the consumption of raw oysters, occurred in Sweden in 1955 (Roos, 1956). Another classic example would be the large scale outbreak of human hepatitis A in Shanghai, China, in 1987 where 300,000 patients were involved. The outbreak was initially spread by virus contaminated clams and by person-person transmission (Halliday *et al*., 1991).

In the view of shellfish which can serve as the potential vector of HAV transmission, the availability of a reliable and widely applicable technique for detection and quantification of HAV would be of interest from a public health point of view. Unfortunately, wild type HAV strains are difficult to propagate *in vitro* and often impaired by its slow growth in cell culture without exhibiting apparent cytopathic effects. Therefore this method would not be practical for preventive detection of HAV shellfish for consumption. Besides, antigen detection and nucleic acid hybridization are not feasible in environmental samples generally containing limited quantity of virus contamination (Jiang *et al*., 1986; Le Guyader *et al*., 1996; Zhou *et al*., 1991). Consequently, nucleic acid based techniques; especially reverse transcription PCR represents the most widely method of choice for the rapid and sensitive detection of HAV RNA which can overcome the difficulty of other available methods. So far, for the shellfish samples, HAV has been successfully isolated by RT-PCR from bivalve mollusks *(*Di Pinto *et al.,* 2004; Sanchez *et al*., 2002), oyster (Desenclos *et al*., 1991; Le Guyader *et al*., 2000; Coelho *et al*., 2003), mussels (Le Guyader *et al*., 2000; Casas and Sunen, 2001; Chironna *et al*., 2002; Croci *et al*., 2005) and clams (Sunen and Sobsey, 1999; Bosch *et al*., 2001; Kingsley *et al*., 2002; Sunen *et al*., 2004).

The crucial point concerning the successful application of RT-PCR resides in the preliminary processing steps of shellfish samples prior to the RNA amplification. Therefore, the choice of a suitable processing method resulted in a low volume, non-cytotoxic extract with sufficient viral recovery and efficient RNA purification is essential for the PCR accuracy and reproducibility. Numerous protocols have been suggested for processing the shellfish samples (Sobsey *et al*., 1978; Yang and Xu, 1993; Lees *et al*., 1994; Atmar *et al*., 1995; Le Guyader *et al*., 1996; Cromeans *et al*., 1997; Traore *et al*., 1998; Sunen and Sobsey, 1999; Croci *et al*., 1999; Kingsley and Richards, 2001; Legeay *et al*., 2000). Two general schemes designated for the virus recovery are extraction-concentration and adsorption-elution-concentration. Viral extraction methods have been described are clarification in the 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. Protocols that purify RNA such as guanidine isothiocyanate-phenol-chloroform, Tri-reagent-chloroform, guanidine isothiocynate-silica based method followed by alcohol precipitation or the use of commercial available RNA extraction kits are being adopted after the extraction of the virus from the tissue sample and before the nucleic acid amplification.

More recently, real-time PCR has been developed for the production and quantification of amplicon using intercalating dyes or fluorescent probes or primers. Emergence of real time-PCR has revolutionized nucleic acid detection by 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 reductions 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 (Tapp *et al*., 2000; Mackay *et al*., 2002; Abd El Galil *et al*., 2004; Costafreda *et al*., 2006) and Taqman (Costa-Mattioli *et al*., 2002; Jothikumar *et al*., 2005; Costafreda *et al*., 2006; Villar *et al*., 2006) based assays are a few of the chemistries based on fluorescently labeled and target specific probes that have been used for real time RT-PCR detection of HAV RNA. On the other hand, 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 harvesting and sales of the shellfish products. However, the regulations only specify bacteriological parameter as an indicator for the safe consumption, where 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; therefore, water containing acceptable levels of faecal coliforms, may contain high levels of enteric viruses (Gerba and Goyal, 1978). In sense of this loophole of the regulations, EU and United States have been working out for solutions to develop a molecular diagnostic method suitable for the routine monitoring of the shellfish or sea water of the shellfish growing area. Malaysia has maintained statistical data on hepatitis A, but we are still lagging behind in the aspect of systematically collating and reporting such data through a disease surveillance system. The actual cases of outbreak might be underestimated in which poor rural people are the main consumers of the wild shellfish and the lack of the disease awareness has driven them not seeking help and bringing attention to the medical and public health officials, and therefore escaping reporting.

Morphology and physiochemical properties of Hepatitis A virus

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

Hepatitis A virus was first identified in 1973 by electron microscope and 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 (Lemon, 1994; Stapleton and Lemon, 1994; Koff, 1998) 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 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).

In common with all enteric viruses, hepatitis A is acid stable and able to retain infectivity below pH3. HAV remains infectious after refrigeration and freezing and is resistant to heating at 60˚C for 30 minutes. Besides, chlorine a component commonly used as a disinfection agent is partially effective in removing the virus where conflicting reports on the efficacy for inactivation of HAV, which may be due to the different experimental condition used, have been reported. However, it is readily inactivated by ionizing radiation, phenol and formaldehyde (Siegl *et al.*, 1984).

Genome and proteins of Hepatitis A virus

Genome of the virion consists of positive-stranded RNA which is approximately 7.5kb in length. There are non-translated regions (NTR) at the both ends of the viral genome. The highly conserved 5'end NTR extending over 10% of the total genome and is covalently attached to the viral protein, VPg (2.5kD) (Brown *et al*., 1991; Melnick, 1992). On the other hand, the 3' end terminates with a poly (A) tail of 40-80 nucleotides. A single open reading frame (ORF) encoding a single polyprotein of 2227 amino acids, is divided into three functional regions termed P1, P2 and P3. P1 encodes for the polypeptides which are posttranslationally processed into the capsid, whereas P2 and P3 encode the non-structural polypeptides which are associated to replication (Totsuka and Moritsugu, 1999). Both the virion capsid proteins VP1 to VP4 and non-structural proteins are generated from the polyprotein by a series of viral protease ($3C^{pro}$) cleavage (Toyoda *et al*., 1986; Sommergruber *et al*., 1989).

Genotype of Hepatitis A virus

There is only one serotype of hepatitis A virus been documented, and the single infection confers lifelong immunity. However the genetic variants of HAV have been identified based on the sequencing selected, short genome regions, including the VP3 C terminus (Jansen et al., 1990), VP1 amino terminus (Robertson *et al*., 1991) and the VP1/P2A junction region (Jansen *et al*., 1990; Robertson *et al*., 1992). The genetic heterogeneity of HAV has enabled further classification into seven different HAV genotypes, designated to I to VII, where I, II, III and VII genotypes have been associated with human disease. Most human HAV strains belong to genotypes I and III, with genotype I being most prevalent, comprising at least 80% of circulating human strains. Genotypes I and III are further divided into subtypes A and B. Genotypes II and VII are represented only by one human strain each, and genotypes IV, V, and VI include strains recovered from simians (Patricia Arauz-Ruiz, 2001). Genotypes were defined by sequences and differ from each other by at least 15%; subtypes differed by 7.0-7.5 % (Robertson *et al*., 1992).

Epidemiology of Hepatitis A virus

Approximately 1.4 million clinical cases of HAV reported annually worldwide and four major patterns of HAV infection can be described based on the age-specific prevalence of antibodies to HAV, which result in characteristic features of hepatitis A epidemiology including disease rates and predominant transmission (Bell, 2002; Tanaka, 2000).

These range from high endemicity to very low, where the levels correlate with hygienic and social economy status of each geographic area. In the high endemicity areas such as parts of Africa, Asia and central and South America, where poor sanitary and unhygienic conditions are found, infection is acquired during early childhood and most infections are asymptomatic, and if symptoms occur, they are mild and non-specific. Reported disease rates in such areas are therefore low and outbreak of disease is rare. However, in such high endemic areas disease rates may be high due to the high level of circulation virus. For example, a population based study conducted in the Amazon basin of Brazil found the incidence of clinical disease among children to be over 100/100000 population (Bensabath *et al*., 1987). The route of transmission is mainly from person to person, and also contaminated food and water source.

Developing countries with translational economies and some regions of industrialized countries where sanitary and socio-economic conditions are improved are defined as intermediate endemicity. Southern and eastern Europe and some regions in Middle East are the examples where the reductions in exposure to the HAV in childhood have been reported. However, the disease rates are high in older children, adolescents and young adults because of the high level of circulating HAV via the food and waterborne transmission that lead to the outbreak.

In the low and very low endemicity, majority of the population remains susceptible throughout adulthood whenever the virus is introduced, but the less opportunity for the exposure of virus contributes to the lower cases of hepatitis A infection. In North America, hepatitis A infection mainly occurs in the community as a whole, mostly affecting young adults and children in lower social-economic classes. Outbreaks have also been reported among men who have sex with men and intravenous drug users, as well as isolated community. In the region of Europe where there is a low local prevalence of HAV, disease is only usually occurs among specific risk group like the international travelers and HAV-infected migrants (Arankalle *et al*., 1995; Beutels *et al*., 1997; Hodges *et al*., 1998; Beran *et al*., 1999; Hau *et al*., 1999; Sawayama *et al*., 1999; David, 2004).

Route of transmission of Hepatitis A virus

Hepatitis A is an enterically transmitted disease, typically via the fecal-oral route either by person-to-person contact or by contaminated water and food, particularly shellfish, fruits and salad (Fiore, 2004).

Transmission via person-to-person contact within the household is the predominant way of spreading the virus. Currently, outbreak across the community

is reported as the main source of HAV infection. Schoolchildren, adolescents, and young adults are in high risk of contracting this disease. Large families, poor education, inadequate waste disposal systems and mixing with other children in the day care centre are linked to the HAV outbreak. Young children aged between 3-5 years are the important transmitters of the infection for others as they develop the asymptomatic or unrecognized symptoms.

Foodborne outbreaks of HAV have been associated with uncooked food such as contaminated bivalve mollusks, salad or fruits, and cooked food which are often contaminated through fecally contaminated water in their growing area or during preparation through contact with fecally contaminated surfaces or infected food handlers (Jaykus, 2000). Hepatitis A outbreaks related with shellfish ingestion accounts for 50% of the total HAV foodborne reported case especially since they are frequently eaten raw or undercooked (Cliver, 1985). In fact, cooking does not guarantee the total inactivation of virus, for example an outbreak was implicated after the consumption of cooked (grilled, stewed or fried) oyster (McDonnell *et al*., 1997). Shellfish is notably identified as an especially high risk factor in HAV transmission is because of their filter feeding characteristic that will bioconcentrate virus in their edible tissue during the natural feeding process (Mitchell *et al*., 1966; Gerba and Goyal, 1978; Metcalf *et al*., 1979; Gaillot *et al.*, 1988). Many of these commercial species breed in inshore estuaries or shallow or drying areas where level of nutrients are high and the waters are sheltered which frequently contaminated with human sewage (Lees, 2000). Besides, the protective effect of high lipid and protein content of shellfish tissue to HAV attributes to the resilience of the virus to the damage of many chemical and physical agents including in the post-harvest depuration process practiced in many countries (Croci *et al*., 1999). In addition, the nature of environmental stability of HAV proves to be able to survive in the seawater for more than a month either in the water column, attaching to particulate matter or accumulating in the sediment (Callahan *et al*., 1994), posing higher chances of contamination to shellfish.

The impact of the shellfish-borne transmitted HAV is substantial. The first outbreak of hepatitis A involving 629 cases was reported in 1955 occurred in Sweden after the consumption of raw oysters. Since then, many outbreaks of hepatitis A have been associated with the consumption of shellfish globally (Rippey, 1994; Divizia *et al*., 1998; Salamina, 1998; Divizia *et al*., 1999). The extent of public hazard is represented by the most classic example of the 1988 common source outbreak in Shanghai, due to the consumption of contaminated raw shellfish which affected over 300,000 persons (Halliday *et al*., 1991). This outbreak ranks as the largest viral food poisoning outbreak reported. The USA has also reported a number of shellfish associated hepatitis A outbreaks (Richards, 1985; Rippey, 1994) as has the UK (Bostock *et al*., 1979; Sockett *et al*., 1993), Japan (Fujiyama *et al*., 1985) and other European countries such as Italy (Mele *et al*., 1989; Malfait *et al*., 1996), France (Apaire-Marchais *et al*., 1995).

The stability of HAV and its abundant shedding in feces (10^6 to 10^{10} virus particles per g) suggest the potential indirect transmission via waterborne (Papaevangelou, 1984). Inadequate sewage management is implicated as the main source of contaminated groundwater obtained from wells or rivers as well as drinking water (Lippy and Waltrip, 1984). Occasional outbreaks of hepatitis A among persons who share small private or community well or swimming pool have been reported (Fiore, 2004).

Non-human primates (chimpanzees, owl monkeys, cynomolgus monkeys, rhesus monkeys, stump tailed monkeys, African green monkeys, tamarins, marmosets and squirrel monkeys) can serve as the link in the chain of infection of man (Hillis, 1961; Schulman *et al*., 1976; Mao *et al*., 1981; Lemon *et al*., 1987;). HAV-induced disease in non-human primates resembles human disease, but is milder and followed by complete recovery. Outbreaks of the HAV infection have been reported among primate handlers and experimenters, and the primates that were HAV infectious were those born in the wild and not those born and raised in captivity (CDC, 1996).

Hepatitis A Virus cultivation

Cell culture is the most common method for the propagation of viruses. Cell culture is prepared by dissociating tissues into single-cell suspension by mechanical disruption and with the aid of proteolytic enzymes treatment. The cells are then suspended in culture medium consisting isotonic solution of salts, glucose, vitamins, coenzymes and amino acids buffered to a pH between 7.2 and 7.4, and incubated in the tissue culture flask under optimum condition. Most culture medium are supplemented with animal serum which contains substances that promote the growth of the cell line. As the cells divide, they cover the flask surface and form the monolayer. Alternatively, cells can be remained in suspension, in which a spinning magnet continuously spins the cells. Virus can be cultivated on the monolayer and some viruses kill the cells in which they replicate giving the cytopathic effect of the infected cells. Cytophatic effects can be exhibited in several manners, such as

rounding up and detachment of cultured cells, cell lysis, swelling of nuclei and these effects can be seen under simple light of phase contrast microscope without staining the cells. However, other cytophatic effects such as development of intracellular masses of virions or assembled viral components in nuclear or cytoplasm require the help of high power microscope for observation. There are some viruses which multiply in cells without causing obvious cytophatic effect; therefore the infectivity of these viruses must be accessed using alternative method (Flint *et al*., 2004).

Hepatitis A virus can be propagated in conventional mammalian cell culture including fetal rhesus monkey kidney cells and African green monkey kidney cells. Wild type HAV replicates slowly in cell culture without any apparent cytophatic signs of infection and host cell damage. More efficient replication of HAV in cell culture often occurs after the extensive adaptation period, and once adapted, HAV produces a persistent infection and becomes attenuated (Feinstone *et al*., 1983). Cytophatic variants of HAV have been observed in certain cell culture system. The replication cycle of these cytophatic viruses is relatively shorter and they yield higher viral load in comparison with the non-cytophatic strains (Cromeans *et al*., 1987).

Molecular diagnostic approaches for Hepatitis A virus detection

Nucleic acid-based detection techniques such as reverse transcription PCR offer an edge over immunoassays and virus infectivity assays for the detection of HAV in environmental samples. It proves to be more sensitive, reliable and time saving which is essential for the timely detection of the wild type HAV strains found in naturally contaminated samples.

RT nested PCR

RT nested PCR is a modification of reverse transcription PCR which aims to eliminate unspecific amplification in the first round RT-PCR due to unexpected primer binding site. RT-PCR itself is a process where RNA is reverse transcribed into DNA and subsequent exponential amplification of the resultant DNA. RT-PCR reaction can be categorized as one-step or two-step. In the one-step reaction, *Thermus thermophilus* (Tth) polymerase with intrinsic RT activity is coupled with bicine buffers containing Mn^{2+} ions that are compatible with both reverse transcription and subsequent PCR amplification (Chiocchia and Smith, 1997). Single tube reaction offers the advantage of minimization of hands-on time and risk of contamination. Alternatively, RT-PCR can be carried out in two stages in two different tubes. In the first strand reaction, RNA is first reverse transcribed into cDNA , and newly synthesized cDNA generated from the single stranded mRNA earlier on is transferred to a new tube to serve as a starting material for the initiation of PCR amplification. Separation of RT and PCR process allows the generating of stable cDNA pool that can be stored indefinitely. Successive run of nested PCR is performed intended to amplify the secondary target within the first-round RT-PCR product with another set of primers to ensure specificity. The logic behind the incorporation of RT-PCR with nested PCR strategy is if the wrong locus were amplified by mistake in the first round RT-PCR due to similar or alternative primer binding sites, it is very unlikely that the unwanted amplicon will be amplified by the second pair of sequence specific primers.

Nested RT-PCR based for detection of hepatitis A in shellfish have been reported extensively (Croci *et al*., 1999; Kingsley and Richards, 2001;De Medici *et al*., 2001; Formiga-Cruz *et al*., 2002; Croci *et al*., 2003; Sunen *et al*., 2004; Croci *et al*., 2005).

Real Time RT-PCR

Real time RT-PCR is a revolutionary breakthrough in the biological investigation; this is because it is perceived as the powerful tool to simultaneously quantify the starting amount of mRNA while the reverse transcription polymerase chain reaction (RT-PCR) is occurring.

The real time RT-PCR system is based on the detection and quantification of fluorescent reporter. This fluorescent signal increases in direct proportion to the amount of PCR product in a reaction. Reactions are characterized by the point in time during cycling when amplification of a PCR product is first detected rather than the amount of PCR product accumulated after a fixed number of cycles. The higher the concentration of starting material, the sooner the significant increase in fluorescence is observed. A significant increase in fluorescence above the baseline (usually 3 to 15 cycles) indicates the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline. The parameter Ct (threshold cycle) reflects the cycle number at which the fluorescence generated within a reaction crosses the threshold (Bustin and Nolan, 2004). Generally, a plot of the log of initial target copy number for a set of standards versus Ct produces a standard curve. Quantification of the amount of target in unknown samples is accomplished by measuring Ct and extrapolating the standard curve to determine starting copy number.

Currently there are various different chemistries, which use fluorescent dyes and combine the processes of amplification and detection of target RNA to allow real time monitoring of PCR reactions. SYBR Green, the cheaper alterative, is the double stranded DNA binding chemistry, which quantifies the amplicon by the use of a non-sequence specific fluorescent intercalating agent (Morrison *et al*., 1998). SYBR Green is a fluorgenic dye that exhibits little fluorescence when in solution, but emits a strong fluorescent signal upon binding to double-stranded DNA (Morrison *et al*., 1998). During the extension phase, more and more SYBR Green will bind to the PCR product, resulting in an increased fluorescence. Consequently, during each subsequent PCR cycle more fluorescence signal will be detected (Bustin, 2000). The drawback of the SYBR is non-specificity where it binds indiscriminately to double-stranded nucleic acid including the non-specific amplification and primer dimmer. Consequently, the requirement of extensive optimization and the follow-up assays such as melting point analysis is strongly recommended (Ririe *et al*., 1997) whereas Taqman analysis which utilizes the 5' nuclease activity of the DNA polymerase to cleave the probe offers an alternative for same precision but increased specificity and sensitivity (Gut *et al*., 1999).

Hydrolysis probes are sequence-specific and dually fluorophore labeled DNA oligonucleotides that bind to the amplicon during the annealing or extension phase of the PCR. The probes contain a fluorescent reporter dye at 5'end, and a quencher at 3'end. When both reporter and quencher are in close proximity, the emission spectrum of reporter dye is absorbed by the quencher. This is an example of fluorescence resonance energy transfer (FRET) where the energy of the reporter is transferred to the quencher. During amplification, 5' nuclease activity of DNA polymerase breaks the oligonucleotide apart, separating the reporter and quencher, allowing the reporter's energy and fluorescent signal liberated (Bustin, 2000). The common examples of reporter dye are FAM, VIC, and NED. On the other hand, quenchers that normally used are TAMRA, DABCYL and BHQ (Wilhelm et al., 2003). Molecular beacons, sunrise primers are other quencher-reporter theme variations that rely on the hybridization of fluorescence-labeled probe to the correct amplicon. There are still much other specific and non-specific fluorescent chemistry available like hybridization probe and light-up probe. In addition, development of new chemistries is continually invented with a focus on increasing sensitivity, precision and cost effectiveness (Valasek and Repa, 2005).

Molecular beacons (Tapp *et al*., 2000; Mackay *et al*., 2002; Abd El Galil *et al*., 2004; Costafreda *et al*., 2006); Taqman (Costa-Mattioli *et al.*, 2002; Jothikumar *et al*., 2005; Costafreda *et al*., 2006; Villar *et al*., 2006) and SYBR (Brooks et al., 2005) are examples of the use of real time RT-PCR for detection of HAV RNA.

Shellfish food safety

One of the most concerning problems related to shellfish safety is the contamination of bivalve mollusks with enteric viruses and bacterial pathogens. Among those enteric viruses most associated with shellfish-related illnesses are hepatitis A virus and norovirus (USDA, 2002). However, to date the European Union legislation on the safety of shellfish for consumption and National Shellfish Sanitation Program (NSSP) of United States only recommend the use of fecal coliform standard (*Salmonella and Escherichia coli*) for the monitoring of the microbial quality of shellfish meat and shellfish growing water respectively (Kingsley and Richards, 2001). These bacteriological indicators only reflect the coliform bacterial count, but do not reveal the presence of Hepatitis A viruses that may persist within the shellfish tissues for a month or longer after the coliform bacterial counts have become to acceptable level (Gerba and Goyal, 1978). It is proven that HAV have been detected in the shellfish that meet the bacteriological standard (Croci *et al*., 2000). In addition, post-harvest depuration process used to eliminate bacteria from shellfish before their distribution and sales to the public consumer is shown to be insufficient to remove the virus although the fecal coliform bacteria are totally eliminated (Sobsey *et al*., 1988; De Medici *et al*., 2001).

Therefore, one of the major emphasis towards the post-harvest shellfish safety component involved the development for a practical test possessing requisite sensitivity and specificity for viral contamination feasible for routine monitoring purpose. Various molecular diagnostic tools using RT-PCR have been developed to address this problem, however, there is no standardized method of reference that can be practiced worldwide. Recently, Kingsley and Richards (2001) have proposed a rapid detection procedure (GPTT method) that readily extracts virus from live shellfish exposed to virus-contaminated sea-water and from wild shellfish implicated in an outbreak. This GPTT virus extraction method has been recognized for its potential utility by the U.S. Food and Drug Administration and the Centers for Disease Control and Prevention but it is still under evaluation of many laboratories (USDA, 2002). Likewise, European Union Reference Laboratory Shellfish Safety Unit is still in the process of working

out a standardized solution method for the detection of hepatitis A virus from the shellfish (CRL, 2008). Besides the development of an analytical tool and the establishment of effective processing techniques to eliminate enteric virus contamination from shellfish to provide the consumer with a virus-free raw product is of clear need to assure the safety of the public shellfish consumers.

Post-harvest safety measures only manage in blocking and controlling the contaminated shellfish from being marketed. And yet in order to curb the shellfish safety problem, the prevention of fecal contamination to the shellfish breeding area is the utmost sustainable route as prevention is generally proven to be better than cure. Most outbreaks of shellfish-associated viral illness appear to be from shellfish contaminated within their natural environment. Authorities can play their role by imposing tighter enforcement of laws restricting dumping of waste in shellfish harvesting areas to reduce the incidence of enteric virus illness. Another precaution to reduce virus levels in shellfish would be to strict monitoring of sewage treatment plants and septic systems, particularly in coastal regions near rivers, lakes, and shellfish-growing areas, thus, preventing the shedding of virus into the environment (Richards, 2006).

The practise of reporting and epidemiological follow-up is essential to understand the magnitude of enteric virus illnesses, and the risk factors associated with its transmission in order to adopt strategies to prevent the outbreaks (Richards, 2006). Such system has been shown to be effectively implemented in Italy where 35 participating local health units link incidence notification with serology and follow-up questionnaires in their surveillance for hepatitis A (Mele *et al*., 1986; Mele *et al*., 1997). In United States, Centres for Disease Control and Prevention is responsible in maintaining statistics on reported cases of hepatitis A (Richards, 2006). In the UK, epidemiological data is collected for England and Wales by the public Health Laboratory Service Communicable Disease Surveillance Centre and published periodically (Sockett *et al*., 1993) .

However, the true incidence of hepatitis A transmitted by bivalve shellfish is far underestimated, as only a few countries have well established surveillance system. Reporting system is poor or non-existent in many countries due to high cost, particularly in developing countries where the endemicity is the highest and asymptomatic infection observed among young population is predominant (Richards, 2006). It is also notable that outbreaks reported normally involve large number of cases. This long incubation period (ranging from 2 to 6 weeks) incubation period of hepatitis A virus makes association with a particular food vehicle in individual or sporadic cases very difficult because of inconclusive consumption histories, unless portion of the food is reserved for the testing. Therefore shellfish consumption with sporadic hepatitis A is probably under-reported (Rippey, 1994).

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