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Research Paper

Quantitative detection of hepatitis: A Virus from canned food

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ABSTRACT

Hepatitis A virus (HAV) is the most common pathogen transmitted by canned soft fruits or vegetables and causes food borne viral illness in these kinds of foods. To improve the microbiological detection and monitoring to increase insights into the contribution of canned fruits and vegetables to foodborne viral transmission, sensitive, reliable and standardized methods are needed. Hence, in this study Real-Time Reverse Transcription-PCR (rt RT-PCR or qRT-PCR) was used for rapid and quantitative detection of HAV-RNA in different types of canned food where controls and internal amplification controls is discussed. It was suggested that the use of rt RT-PCR (quantitative RT-PCR (qRT-PCR) is considered as the most sensitive molecular method for detection of HAV in food samples.

Keywords: Canned food, HAV, extraction of RNA, specific primers rt RT-PCR.

INTRODUCTION

Hepatitis A virus (HAV) is responsible for acute infectious hepatitis, which is endemic worldwide and is a non-enveloped ssRNA virus with a single serovar (Pebody et al., 1998). HAV refers to liver inflammation caused by HAV infection and is one of the several viruses that can cause hepatitis and also one of the three most common hepatitis viruses in the United States (Amon et al., 2005).

The major routes of food contamination include shellfish, fruits and vegetables, which often become contaminated through contaminated water in their growing area or during preparation through contact with fecally contaminated surfaces or infected food handlers (Robertson et al., 2000). Recent food-borne outbreaks of hepatitis A is associated with different types of canned soft fruit like strawberries (Anonymous, 1997) and vegetables like green onions and lettuces (Bidawid et al., 2000).

Recent studies conceded that HAV is one of the most common causes of illness through oral-fecal infection especially in children and old people in poor developing countries (Fan et al., 2006). Real-time PCR is one of the most promising detection methods used to deliver quantitative data due to its sensitivity, specificity, speed and possibility (Abd El Galil et al., 2004). The main routine detection of HAV in food includes the presence of inhibitory substances in the samples and the low concentration of virus recovered (Costa-Mattioli et al., 2002). Immunomagnetic separation (IMS) is one of the sample treatment methods that can address these limitations using RT-PCR for detection of HAV in food (Abd El Galil et al., 2004; Costa-Mattioli et al., 2002).

The aim of this study was to apply a real-time reverse transcription-PCR (rtRT-PCR) assay combined with IMS pre-treatment for quantification of HAV in fresh produce to determine the sensitivity and specificity of this molecular technique. Hence, we used the two methods RT-PCR and rt RT-PCR to determine which one is considered the more sensitive and specified for detection of HAV-RNA and other RNA human viruses in both clinical and environmental samples and study the effects of Immunomagnetic separation (IMS) treatment methods for elimination of the inhibitors of RT-PCR before quantification of RT-PCR amplified (qRT-PCR or rt RT-PCR).

MATERIALS AND METHODS

Collected canned and some fresh food samples from local markets in Egypt were divided into three groups; soft fruits

(strawberry and peach); vegetables (Lettuce and green onion) and meat products (Burger) were extracted raw joss with method described by Anonymous (2013), Wheeler et al. (2005) and Dietrich et al. (2013) respectively.

Ultrafiltration system for concentration of HAV

Several companies were used to provide ultrafiltration spin columns or micro concentrators which are capable of concentrating volumes varying from less than 1 to 80 ml. Depending on the type of microconcentrator, volumes can be increased from 25 to 200 µl performance; platinum-cured L/S 36, L/S 24, and L/S 15 silicon tubing (Masterflex; Cole-Parmer Instrument Co.) was used for each filtration experiment. All tubing connectors and clamps were autoclaved and the brass fitting of the pressure gauge sanitized with 3% hydrogen peroxide and 10% bleach solution (0.6% sodium hypochlorite) and then washed thoroughly with DI water prior to use in the filtration set-up. The UFs and tubing were discarded after each experiment. The hollow-fiber UFs (spin columns or micro concentrators) used were single-use Fresenius F200NR polysulfone dialysis filters with a molecular mass cut-off of approximately 30,000 Da (30 kDa), a surface area of 2.0 m^2, and a fiber inner diameter of 200 µm (Fresenius Medical Care, Lexington, MA). A Cole-Parmer model 7550-30 peristaltic pump was used for all experiments (Anonymous, 2013; Wheeler et al., 2005). Secondary purification was performed to remove interfering substances previously co-purified with viral particles and to reduce again the final volume of viral concentrates (Dietrich et al., 2013).

Extraction of HAV-RNA Nucleic acid

Real-time-qPCR was performed on UF concentrates by filtering 125 ml (representing roughly 30% of the volume of each UF concentrate) through 0.2 m membrane filter and performing a bead-beating procedure, using a one-quarter section from the filter. Food samples were subjected to the extraction protocol of the HAV-RNA as described by Atmar et al. (1995).

To make this lysis buffer, 120 g of guanidine thiocyanate (Boehringer Mannheim, Indianapolis, IN) was added to 100 ml of TEbuffer (5 mMTris, pH 8.0, 0.5 mM EDTA, pH 8.0) prepared with nuclease-freewater (Ambion, Austin, TX). After the guanidine thiocyanate was dissolved, 0.5 g sodium pyrophosphate (Alfa Aesar, Ward Hill, MA), 11 ml of 5 M sodiumchloride (Ambion, Austin, TX), and 11 ml of 3 M sodium acetate, pH 5.5 (Ambion, Austin, TX) were added. Carrier nucleic acid [2.2 ml from a 2 mg/ml stock solution of poly(A) (Sigma, St. Louis, MO)] was added to a final concentration of 20 µg/ml.

To extract nucleic acids from membrane filters, the filters were cut aseptically using a scalpel into four one-quarter sections. One of these one-quarter sections were inserted into a 2 ml screw cap polypropylene tube (National Scientific Supply) containing 100 mg of 106-µm glass beads, 100 mg of 425 to 600 µm glassbeads and 500 µl of diluent buffer (Dulbecco's PBS with 0.01% Tween 80 and 0.001% antifoam A).

The tube was shaken for 3 min at a high-speed setting in a bead beater (Biospec, Bartlesville, OK). After the bead-beating step, the sample was centrifuged for 30 s at 14,000 × *g* and the supernatant transferred to a 1.6 ml microcentrifuge tube containing 500 µl lysis buffer. The sample was vortexed with the lysis buffer for 30 s and then passed through a silica spin column (QIAGEN, Valencia, CA) by centrifugation at 14,000 × *g* for 30 s.

The column was loaded twice to process the entire sample volume (1 ml, including 500 µl lysis buffer and 500 µl sample). The column was washed once with 500 µl of 100% ethanol (and centrifuged at 14,000 × *g* for 1 min) and twice with 75% ethanol (and spun at 14,000 × *g* for 1 min).

The centrifuge column was again used to remove any excess ethanol and thereafter transferred to a clean microcentrifuge tube and nucleic acid eluted by adding 200 µl TE buffer and the column centrifuged for 1 min at 14,000 × *g*.

The second purification of the concentrated HAV-RNA nucleic acid sample was performed using a Microcon YM-100 (AmiconInc., Beverly, MA) microconcentrator by adding the sample (200 µl) to the Microcon YM-100 reservoir containing 200 µl TE buffer. The mixture (400 µl) was filtered by centrifugation at 14,000 × *g* for 1 min. The filter was then inverted and inserted into a clean microcentrifuge tube to recover the nucleic acid by centrifugation at 4,500 × *g* for 30 s. Approximately, 160 µl DNA was collected (samples with less volume were adjusted to a 160 µl final volume with TE buffer).

To extract nucleic acids from Centricon concentrates (for MS2 analysis), 1 mloflysis buffer was amended with 5 µl of 10% sodium dodecyl sulfate.

This amended lysis buffer was added to 500 µl Centricon concentrate samples at a ratio of 1:1. If less than 500 µl was available, nuclease-free water is added to the sample to effectively increase the volume to 500 µl. The sample was vortexed with the lysis buffer for 30 s, centrifuged at 14,000 × *g* for 30 s, transferred to a silica spin column (QIAGEN, Valencia, CA), and processed as earlier described for membrane filters.

The effect of food quality on real-time RT-qPCR was evaluated by using HAV- RNA as an external control; HAV - RNA was extracted from a stock of HAV strain HM-175, clone 24A (Dubois et al., 2006) using the lysis buffer and silica column as previously described.

The difference in the CP values indicated the magnitude of RT-PCR inhibition associated with a food samples. A relative difference of 3.3 CP values was used as a minimum value for indicating PCR or RT-qPCR inhibition based on the ideal slope for a real-time qPCR standard curve of 3.3 CP

Table 1: Primers employed in this study for the detection of hepatitis A virus in food samples.

Primer	Sequence	Reference
HAVp3	5′-GGAAATGTCTCAGGTACTTTCTTTG-3′	(Atmar et al., 1995)
HAVp4	5′-GTTTTGCTCCTCTTTATCATGCTATG-3	
HAV240	5′-GGAGAGCCCTGGAAGAAAGA-3′	(Borchardt et al., 2013)
HAV68	5′-TCACCGCCGTTTGCCTAG-3′	
HAV1	5′-TTGGAACGTCACCTTGCAGTG-3′	(Jingyuan et al., 2006)
HAV2	5′-CTGACTACCTCAGAGGCAAAC-3′	

values per 10-fold difference in target amounts present in a reaction mix (Julie et al., 2009).

Real-time quantification PCR(RT-qPCR)

Amplification of HAV-RNA targets was performed using an applied thermo cyclerreal-time PCR detection system (Applied, USA). Extracted HAV-RNA (10 µl) was subjected to RT–PCR and performed in a 96-well plate format. Table 1 provides the sequence data for primers and TaqMan probes used for the assay and a final concentration of 100 nM and the primers were used at a final concentration of 250 nM. Reverse transcription was performed with the TaqmanReverse Transcription reagents kit (Applied Biosystems, Branchburg, NJ, USA) with 10 µl of virus RNA, 11 µl of $MgCl_2$, 5 µl of buffer 10X, 10 µl of dNTP at 10 mM, 2.5 µl of hexamers (at 1: 10 dilution), 1.25 µl of Multiscribe and1 µl of RNase inhibitor in a 50-µl final volume. The cyclingprogram was 25°C for 10 min, 48°C for 30 min and 95°C for 5 min. The PCR reaction adapted from a previously described protocol was carried out with the qPCR mastermix-No Rox (Eurogentec, Angers, France) in a 50-µl volume on the Stratagene MX3005P QPCR system (Agilent Technologies) with 10 µl of cDNA, 25 µl of Mastermix, 1 µl of each primer (10 mM) and 0.4 µl of probe (10 mM). The cyclingprogram was 50°C for 2 min, 95°C for 10 min, 45 cycles at 95°C for 15 s and 60°C for 1 min (Koopmans et al., 2002).

Internal control

Nucleic acid extraction, reverse transcription RT-PCR (rt RT-PCR or qRT-PCR)were monitored by using a quantitated solution of bacteriophage MS2, 10 µl of which was spiked into the 200 µl of. PCR detection of MS2 bacteriophage was performedwith the same Mastermix kit on the same cycler with thesame cycling program in a 15-µl final volume including 3 µl of cDNA, 7.5 µl of mastermix, 0.3 µl of each primer (10 mM), and 0.15 µl of probe (10 mM). Primer and probesequences are available upon request from the correspondingauthor. For each sample, validation of the run was performedby comparison with the result obtained for MS2 detection. Briefly, a negative MS2 result or an MS2 Ct valuehigher than one standard deviation (as compared with themean Ct value observed on the whole PCR plate) indicatedeither a technical problem in any of the steps or the presenceof inhibitors in the clinical sample. Such a result washighly suitable in clinical cases .A similar approach has been described previously (Michael et al., 2005; Julie et al., 2009; Koopmans et al., 2002).

Sensitivity of the primer sets

To evaluate the sensitivity of the RT–PCR with the different primer sets (Table 1), serial dilutions of the HAV was prepared in sterile distilled water. using the Superscript™ One-Step RT–PCR System (Life Technologies, Barcelona, Spain) in Applied-thermocyclar (Eppendorf, Hamburg, Germany) (Borchardt et al., 2013).

RT–PCR amplifications of HAV- RNA were performed using the primer pairs HAVp3/HAVp4 (Atmar et al., 1995) targeting the VP1 gene, HAV240/HAV68 (Borchardt et al., 2013) which amplifies a fragment of 174 bp within the 5′ non-coding region and HAV1/HAV2 (Jingyuan et al., 2009). RT–PCR conditions for primers HAV240/HAV68 and HAV1/HAV2 were fixed as previously described by Borchardt et al. (2013) and Jingyuan et al. (2006) respectively.

The program conditions utilized for the primers HAVp3/HAVp4 were those reported by Atmar et al. (1995) with minor modifications. Briefly, after an RT step (45°C, 1 h) and an initial heat denaturation (94°C, 5 min), 40 cycles of template denaturation (94°C, 1 min), primer annealing (48°C, 80 s) and primer extension (72°C, 50 s) were performed followed by a final extension (72°C, 15 min). RNA obtained from FRhK-4 cells infected with HAV strain HM-175 was employed as positive control in all the RT–PCR assays.

Negative controls containing water instead of RNA extract were included in all reactions. 10 µl of the RT–PCR products were analyzed by electrophoresis on 1.2%(w/v) agarose gels with TAE (0.04 M Tris-acetate, 1 mM EDTA) electrophoresis buffer.

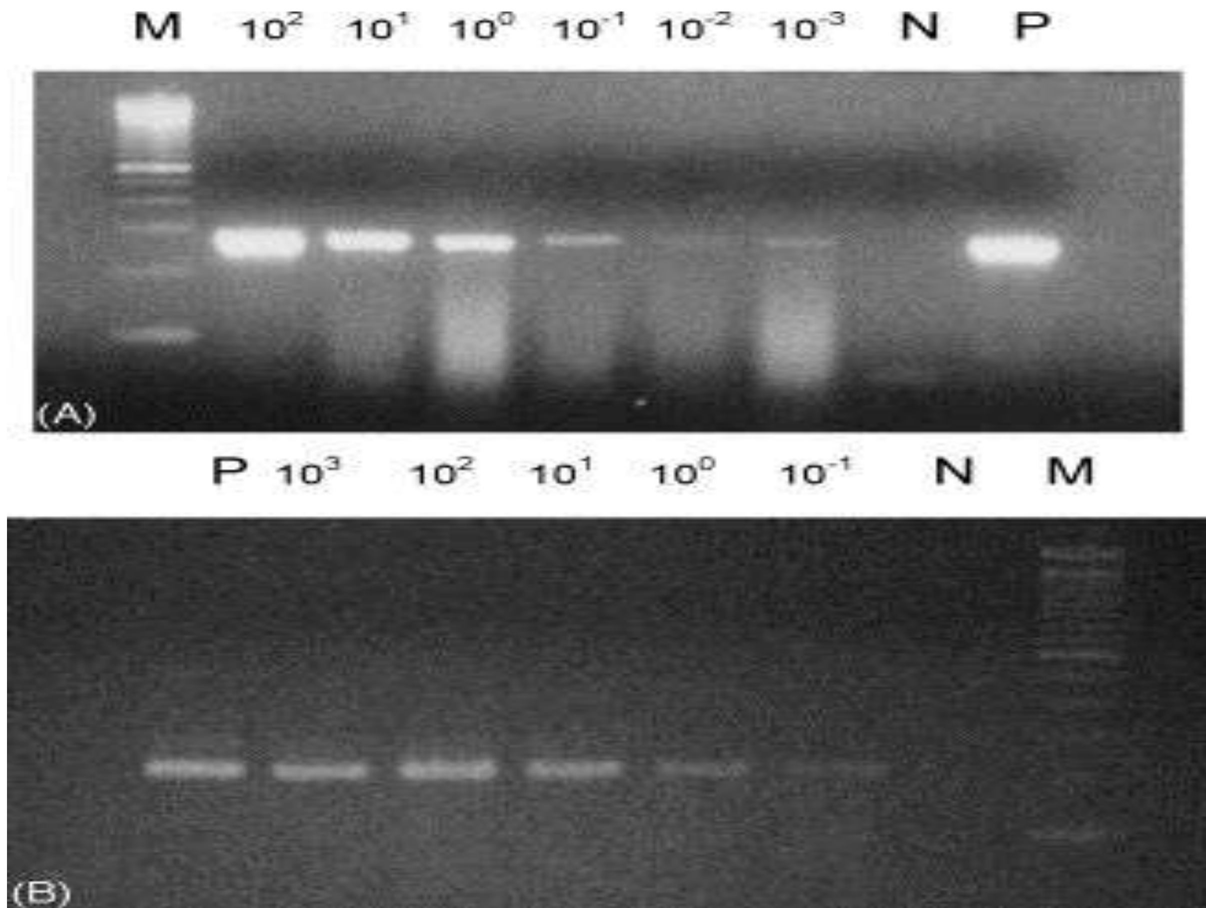

Figure 1: Detection of RT-PCR fragment corresponding to HAV 226-bp with deferent dilutions (A and B) on Ethediumbromied dyed 2% agarose M: molecular ladder; N: negative control; P: positive control.

Statistical analysis

Odds ratios (ORs) were used to measure the strength of associations in the case-control studies, and 95% confidence intervals (CIs) were calculated with the SAS software, using the Mantel–Haenszel statistic. *P*-values of < 0.05 were deemed to be statistically significant of variance and Fisher's protected least significant differences (LSD) test.

RESULTS

All samples were confirmed by 2% agros gel electrophoresis as extra step to detect the sensitivity of amplification of HAV RNA by using primer sets HAV240/HAV68 and HAVp3/HAVp4.

Quantification with RT-PCR assay

To evaluate the quantitative real-time PCR assay for HAV, serial 10-fold dilutions of the HAV external standards were amplified by real-time RT-PCR to generate a standard curve. The standard curve was linear over the range from 10^6 to 10^1 copies, with a slope of -3.41 (R^2 = 0.996). The real-time RT-PCR assay (rt RT-PCR) could distinguish as few as 100 copies of the HAV-RNA with a high degree of confidence, as indicated by the low coefficient of variation (CV; <10%) (Figure 1 and Table 2). Since the CV increased to 173% at low standard RNA copy number, the linear slope between 10^2 and 10^6 RNA copies was used for quantification of HAV.

Effects of food components on real-time RT-PCR assay

The numbers of viral RNA copies detected in green onion and strawberry rinses seeded with 10^4 and 10^3 PFU/ml of HAV were significantly lower than those in PBS controls (Table 3). End point detection results showed that real-time RT-PCR could detect 100 PFU/ml HAV (0.5 PFU per PCR) in all seeded food samples (lettuces, green onion, strawberry, peach and Burger) but only one-third of the samples seeded with 10 PFU/ml HAV were detectable, suggesting a 10-fold-lower sensitivity (Table 4). These results confirmed the

Table 2: Detection of HAV-RNA standards in serial 10-fold dilutions using real-time RT-PCR.

Log dilution	No. of copies		CV (%)[a]
	Mean	SD	
0[b]	900.335	25.425	2.8
1	82.503	5.704	6.9
2	8.114	692	8.5
3	1.236	121	9.9
4	111	7	5.5
5	7	3	41.0
6	0.4	0.6	173.3

a: The CV of a set of data was determined by dividing the standard deviation by the arithmetic mean of the measured values; *b*: HAV RNA standard was 9.8 : 105 copies.

Table 3: Detected HAV-RNA copies in seeded buffer and samples using real-time RT-PCR.

Sample	RNA copies at HAV dilution (PFU/ml) of:			
	10^5	10^4	10^3	10^2
Lettuces	2.029[Aa]	366[A]	28[Ab]	4[A]
Green onion	1.802[A]	147[B]	9[B]	2[A]
Strawberry	1.879[A]	153[B]	10[B]	2[A]
Peach	2.023[Aa]	364[A]	26[Ab]	3[A]
Burger	1.877[A]	151[B]	10[B]	1[A]

a Means in the same column followed by different superscript capital letters are significantly different by Fisher's protected LSD test ($P \leq 0.05$); *b*: Data below 100 copies were semi-quantitative and calculated by extrapolation from a standard curve. Confirmation of the HAV-RNA amplified with different dilutions by passing on 2% agarose gel.

Table 4: Detection of HAV seeded in samples by real-time RT-PCR.

Sample	No. positive/no. samples tested									
	Real-time RT-PCR at log virus titer (PFU/ml) of:					IMS/real-time RT-PCR at log virus titer (PFU/ml) of:				
	5	4	3	2	1	5	4	3	2	1
Lettuces	7/7	7/7	7/7	7/7	5/7	2/2	2/2	2/2	2/2	2/2
Green onion[a]	5/5	2/2	2/2	5/5	2/3	5/5	2/2	2/2	5/5	2/2
Strawberry[a]	5/5	2/2	2/2	5/5	1/3	5/5	2/2	2/2	5/5	3/3
peach	7/7	7/7	7/7	7/7	4/5	2/2	2/2	5/5	2/2	2/2
Burger	5/5	2/2	2/2	5/5	2/3	5/5	2/2	2/2	5/5	2/2

a: Green onion and strawberry rinses were seeded with a known titer of HAV.

need for additional sample treatment prior to quantification to remove PCR inhibitors (Hayden et al., 2013).

Remove PCR-inhibitory by IMS/real-time RT-PCR

The ability of IMS to remove PCR-inhibitory materials from the samples and concentrate virus for PCR analysis and, thus, improve the sensitivity of detection was reported in previous studies (Pebody et al., 1998; Robertson et al., 2000; Anonymous, 1997; Abd El Galil et al., 2004; Dubois et al., 2006). In the present study, the sensitivity of the IMS/real-time PCR assay on food samples was determined in fresh produce rinses seeded with HAV. In both green onion and strawberry samples, the sensitivity attained with IMS treatment was 10 PFU/ml, which was 10-fold higher than in samples without IMS treatment (Table 3). Using quantification in 1 ml strawberry rinse seeded with 10^5 PFU of HAV, IMS-treated samples captured more than 20

Table 5: Quantification of HAV RNA in green onion and strawberry rinses by direct real-time RT-PCR and IMS/real-time RT-PCR.

Sample	Virus titer (PFU/ml)	No. of HAV RNA copies		Ratio of IMS/PCR to PCR
		Direct real-time RT-PCR	IMS/real-time RT-PCR	
Green onion rinse[a]	10^2	2[b]	3	1.5
	10^5	2.836[Ac]	7.053[B]	2.5
Strawberry rinse	10^2	0.2	2	10
	10^5	1.879[A]	38.540[B]	20.5

a Green onion and strawberry rinses were seeded with a known titer of HAV; *b* Means in the same row followed by different superscript capital letters are significantly different by Fisher's protected LSD test ($P \leq 0.05$); *c* Data below 100 copies were semi-quantitative and were calculated by extrapolation from a standard curve.

Table 6: Sensitivity of primer sets evaluated in the amplification of HAV from viral crude extracts and from food samples.

Primer set	Sensitivity (range obtained from four independent experiments)	
	Viral extracts (PFU/µl)	food samples (PFU/g)
Hepatitis A virus		
HAV240/HAV68	0.02–0.1	0.02–0.1
HAVp3/HAVp	4 0.1–1	0.2–0.1
HAV1/HAV2	>104	>104

times more HAV particles than those without IMS treatments (Table 5).

DISCUSSION

Reverse transcription polymerase chain reaction (RT-PCR) is one of the many variants of polymerase chain reaction (PCR). This technique is commonly used in molecular biology to detect RNA expression; RT-PCR is often confused with real-time polymerase chain reaction (qPCR). However, they are separate and distinct techniques, while RT-PCR is used to qualitatively detect gene expression through creation of complementary DNA (cDNA) transcripts from RNA, qPCR is used to quantitatively measure the amplification of DNA using fluorescent probes. qPCR is also referred to as quantitative PCR, quantitative real-time PCR and real-time quantitative PCR (Michael et al., 2005).

Although RT-PCR and the traditional PCR both produce multiple copies of particular DNA isolates through amplification, the applications of the two techniques are fundamentally different. Traditional PCR is used to exponentially amplify target DNA sequences. RT-PCR is used to clone expressed genes by reverse transcribing the RNA of interest into its DNA complement through the use of reverse transcriptase. Subsequently, the newly synthesized cDNA is amplified using traditional PCR (Julie et al., 2009).

The fact that canned food can serve as vectors of important viral human pathogens, including HAV has led to a widely recognized need for improvement of the sanitary control measures of these marine products. Therefore, in recent years the development of RT–PCR techniques for rapid and reliable detection of these viral pathogens has become an important research goal (Pebody et al., 1998; Amon et al., 2005; Anonymous, 2013; Dietrich et al., 2013).

It is clear that the RT–PCR detection of HAV in canned food is the most sensitive method available at present. However, the primer selection constitutes a critical step in order to obtain the required characteristics of sensitivity and specificity (Atmar et al., 1995; Dubois et al., 2006; Michael et al., 2005). Results obtained in this work showed great differences in the effectiveness of amplification in relation to the primer pair used. The sensitivity was higher by 1-log than that obtained with primer set HAVp3/HAVp4 (Table 6 and Figure 2).

On the other hand, no positive amplifications were obtained when primer set HAV1/HAV2 was utilized, which implied a detection limit of >104 PFU/µl (Table 6). However, this set of primers is usually employed in a nested-PCR protocol (Jingyuan et al., 2006), hence, its use in a one-step RT–PCR procedure could have been the reason for lack of amplification products. In addition, the diverse primer pairs amplify different regions of the HAV genome, which may have some effect on their sensitivity. As in the case of HAV, sensitivity could be affected by the genome region amplified by each primer set (Borchardt et al., 2013; Jingyuan et al., 2009; Di et al., 2010; Greening, 2006).

In conclusion, the results obtained that selection of primer sets for the routine detection of HAV in monitoring laboratories is a critical point in the design of RT–PCR

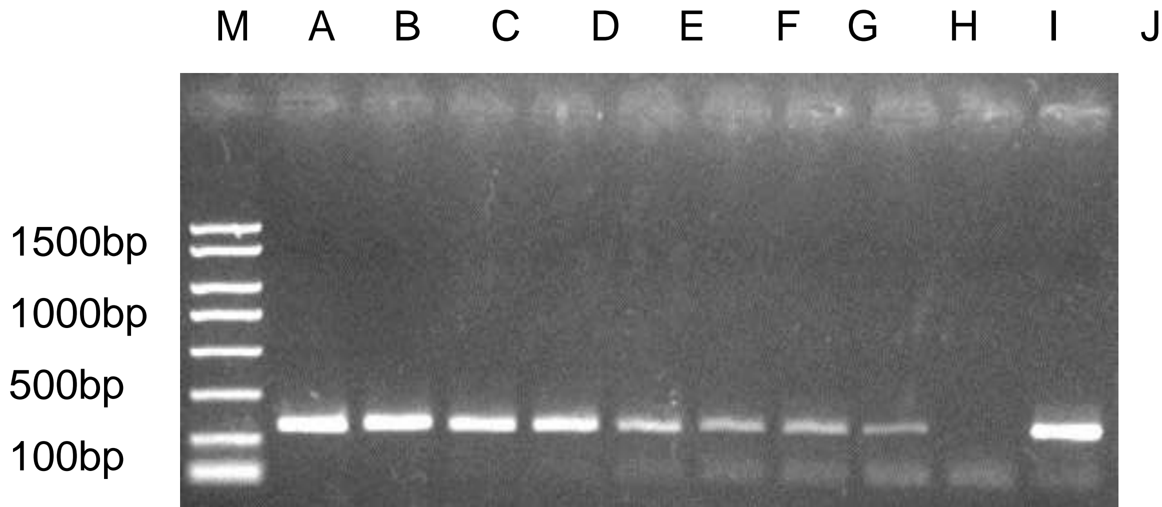

Figure 2: Sensitivity achieved for the amplification of HAV RNA using the primer sets HAV240/HAV68 (A) and HAVp3/HAVp4 (B) [Lanes: A, molecular size marker (PCR marker 50–2,000 bp, Sigma); B to I, amplification of RNA extracted from dilutions of the HAV strain at concentrations of 10^6, 10^5, 10^4, 10^3, 10^2, 10, 1, and 0.1 PFU/μL respectively; J, negative control (no RNA); K, positive control (HAV RNA from undiluted viral stock).

protocols. Primers have to be carefully selected for both sensitivity and specificity and their use has to be standardized among laboratories, before recommendation for their inclusion in a routine detection method (Borchardt et al., 2013; Jingyuan et al., 2009; Di et al., 2010; Greening, 2006). This is useful in the application for routine surveillance of HAV in fresh produce and environmental samples (Greening, 2006; Shan et al., 2005).

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