Food Environ Virol (2017) 9:103–113
DOI 10.1007/s12560-016-9267-z

ORIGINAL PAPER

Preliminary Source Tracking of Male-Specific (F^+) RNA Coliphage on Lettuce as a Surrogate of Enteric Viruses Using Reverse Transcription-PCR

Mojgan Yazdi[1] · Masoud Yavarmanesh[1] · Masumeh Bahreini[2] · Mohebbat Mohebbi[1]

Received: 12 March 2016 / Accepted: 19 October 2016 / Published online: 4 November 2016

Abstract The aim of this research was to preliminary track fecal source male-specific F^+RNA coliphages including human and animals in lettuce. At first, two published virus extraction procedures of ultracentrifugation and PEG precipitation were compared using DAL assay for determining the recovery efficiency in lettuce spiked artificially with three concentrations (10^2, 10^4, 10^6 pfu/100 ml) of MS2 coliphage. The results showed that PEG precipitation had the highest recovery in which the recovery efficiency at the spiked level of 10^6 pfu/100 ml was 16.63 %. Aqueous phase obtained from the final step of PEG method was applied for enumeration of coliphage and viral RNA extraction in naturally contaminated lettuce samples ($N = 30$) collected from two sources (market and farm). The samples were then analyzed based on (I, II, III, and IV primer sets) using RT-PCR method. Coliphages were detected in 9 (60 %) and 12 (80 %) out of 15 market and farm samples, respectively, using DAL assay, whereas male-specific F^+RNA coliphages were detected using the RT-PCR method in 9 (60 %) and 13 (86.6 %) out of 15 samples of market and farm, respectively. Based on the results, only genotype I of male-specific F^+RNA coliphages was detected in lettuce samples and no sample tested was positive for other genotypes (II, III, and IV).

Keywords Microbial source tracking · Lettuce · PEG precipitation · Ultracentrifugation · Male-specific F^+RNA coliphage · Recovery efficiency

Introduction

Soft fruits and vegetables consumed raw are sources of a fairly large and growing number of viral infections in various countries (Seymour and Appleton 2001). Raw fruits and vegetables have been known as human disease carriers for at least a century. These products can be contaminated with microorganisms capable of causing foodborne outbreaks, while still on the plants in the farm or orchards, or during harvesting, transporting, processing, distribution, marketing, or at home. Pollution can occur through contact with human/animal feces, untreated irrigation water, surface water, or by coming into contact with the hands of people such as in food preparation places, through street vendors, food service institutions, or at home (Cliver 1997).

Few studies have been carried out for the presence of viruses or parasites on the surface of raw fruits and vegetables, largely because of the lack of sensitive methods in the plant material (Hedberg and MacDonald 1994). Various studies have dealt with the development of standardized methods for the detection of enteric viruses in food (Le Guyader et al. 2004b). However, a single, detailed, and internationally validated protocol may not be suitable for all fruits and vegetables. Various modifications of a basic protocol for groups of fruits and vegetables are thought to be needed to take account of the differences in the morphology and hydrophobic interactions of fruit and vegetable surfaces, differences in tissue compositions, and the processing conditions to which the produce was subjected

✉ Masoud Yavarmanesh
yavarmanesh@um.ac.ir

[1] Department of Food Science and Technology, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

[2] Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran

to before they were sampled. On the other hand, human enteric viruses responsible for gastroenteritis and hepatitis following shellfish, poultry, and salad vegetable consumption cannot be cultured by conventional techniques. Although molecular techniques for detection of NLVs and hepatitis virus are now available, these methods are currently too expensive and time-consuming for routine screening of different food stuffs (Koopmans 2004).

Detection and tracking of microbial fecal contamination affecting the beasts, is one of the major challenges in environmental microbiology. An effort to identify sources of fecal pollution (human and animal feces) is defined as microbial source tracking that has been developed during the last 12 years (Feachem 1975; Kasper et al. 1990). One of the major advantages of the microbial source tracking is that by recognizing the origin of the contamination, the contamination could be controlled better. Typically, the selection of a valid microbial indicator is one of the most important parts of the MST technique (Yan et al. 2007). Common indicator microorganisms that are used as indicator for MST in laboratory include fecal coliforms (Enterobacteriaceae family), enterococci species for bacterial fecal contamination, somatic bacteriophages, male-specific F^+RNA coliphage, and bacteroides fragilis phages as an indicator or model for enteric viruses (Berger and Oshiro 2002; Leclerc et al. 2000). The F-specific RNA bacteriophages (F^+RNA bacteriophages) are a group of single-stranded RNA viruses with simple cubic capsids that are 24–27 nm in diameter. The genomic and physical properties of these phages are similar to that of NLVs and hepatitis A virus. The slow elimination kinetics of F^+RNA bacteriophages appears to be representative of the elimination kinetics of human enteric viruses (Doré et al. 2000). The abundance of these phages in sewage and the ease of working in the laboratory, being low cost, and low risk to human health make them attractive indicators of viral contamination in the environment (Havelaar and Hogeboom 1984; Grabow 1998, 2004; Jofre et al. 2011; Mesquita and Emelko 2012). Based on serological and phylogenetic analyses, this group of phages has been divided into four different genotypes. The genotypes II, III, and I, IV are associated with human feces and animal feces, respectively. Therefore, one of the valuable functions of F^+RNA coliphages is to discriminate human from non-human fecal contamination in microbial source tracking studies that can be carried out by serotyping or genotyping of F^+RNA coliphage isolates (Furuse et al. 1981; Hsu et al. 1995).

The presence of F^+RNA coliphages has been reported not only in polluted water but also in different food stuffs such as shellfish, poultry, vegetable, and lettuce (Hsu et al. 2002; Endley et al. 2003; Williams 2005; Kirs and Smith 2007; Wolf et al. 2008). Nowadays, for applying MST most researchers pay attention to the F^+RNA phages. The aim of this study was to apply a simple, novel, and cost-effective method for enumeration and detection of genotypes of F^+RNA coliphages using a molecular technique (RT-PCR) to preliminarily identify sources of fecal pollution in naturally contaminated lettuce samples obtained from farm and market.

Materials and Methods

Sample Collection

30 lettuce samples were collected from three suspected farms (15 samples) and markets (15 samples) located in the city of Mashhad, North East of Iran, from April to July 2013 (spring to summer). All collected samples were stored in sterile plastic packages at 4 °C and analyzed within 24 h.

Spiking of Lettuce Samples

MS2 coliphage (ATCC 15597-B1) was selected as surrogate for male-specific F^+RNA coliphages to analyze phage recovery. Titration of MS2 stocks was performed using double agar layer (DAL) method (US EPA 2001). The stock was diluted in phosphate-buffered solution (pH 7.2) to achieve the following concentrations: 10^6, 10^4, 10^2 pfu/ml. Coliphage preparation containing 10^6, 10^4, 10^2 pfu/ml in 100 µl was distributed on 20 spots on the surface of three mixtures of lettuce samples. Each sample was placed in a polypropylene filter bag (Interscience, France) and the samples were kept under a laminar hood at room temperature until the spots dried. Uninoculated lettuce sample was used as a negative control in each test.

Evaluation of Extraction and Concentration Methods for Phage Recovery

Polyethylene Glycol Precipitation

The methods described by Butot et al. (2007), Dubois et al. (2002), and Scherer et al. (2010) were combined with some modifications for PEG precipitation method. The steps of this method are in detail in Fig. 1. Briefly, 10 g of lettuce samples were weighed and rinsed with 40 ml of TGBE elution buffer (Tris–HCL 100 mM, glycine 50 mM, 1 % beef extract, pH 9/5) in a plastic bag for 1 h on a rotary shaker (200 rpm) at room temperature. The rinse fluid was removed via bag filtration and centrifuged at 10,000×*g* for 30 min at 4 °C to sediment residual food particles.

Supernatant was transferred to a clean container and the pH was adjusted to 7.2 ± 0.2 by the addition of 5 N HCl.

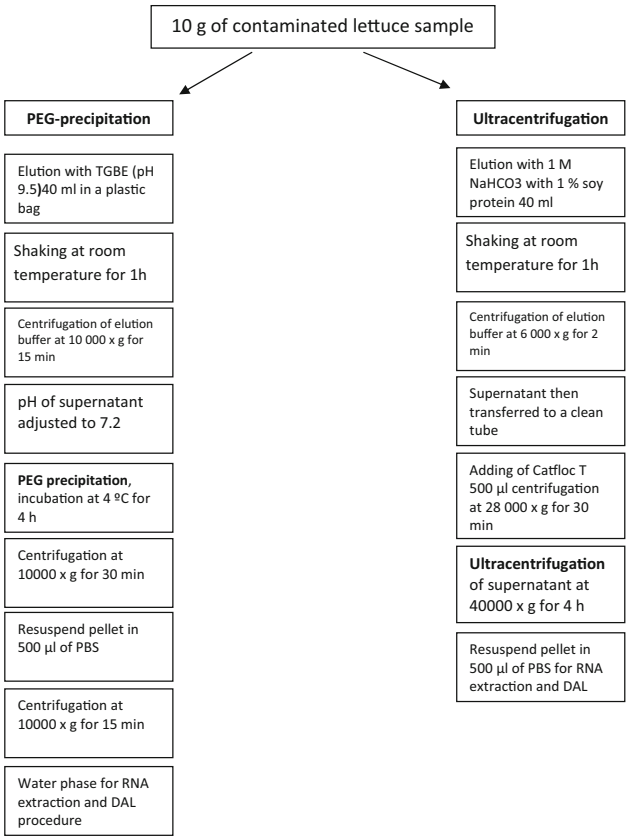

Fig. 1 Flowchart for recovery, concentration, and detection of F^{+}RNA coliphages in lettuce samples

The neutralized fluid was supplemented with 0.25 volumes of a 50 % polyethylene glycol 8000 (PEG; Sigma-Aldrich, Germany) solution in 1.5 M NaCl and incubated for 4 h at 4 °C. Phages were then concentrated by centrifugation at 10,000×*g* for 15 min at 4 °C. Supernatant was discarded and pellet was dissolved in 500 µl of phosphate buffer (PBS). The final solution was used for enumeration of infectious male-specific phages by plaque assay (DAL). Each experiment was performed in triplicate and each series of experiments included a negative control (uninoculated lettuce samples) and positive control (viral suspensions) [modified method of Butot et al. (2007); Dubois et al. (2002); Scherer et al. (2010)].

Ultracentrifugation Method

The methods described by Rzezutka et al. (2005) and Summa et al. (2012) were combined with some modifications for ultracentrifugation method. The steps of this method are in detail in Fig. 1. Briefly, 10 g of lettuce samples was weighed and rinsed with 40 ml of elution buffer 1 M $NaHCO_3$ with 1 % soy protein powder (90 % soy protein), 500 µl of Catfloc T (Calgon Corp., USA), which was diluted to 34.5 % solution in Tris–glycine (100 mM Tris, 50 mM glycine) and the buffer was added to each sample. Ultracentrifugation (Sigma 3K30) was performed in 45 ml centrifuge tubes at 40,000×*g* for 4 h. The pellet was resuspended in PBS after the supernatant was decanted. Each experiment was performed in triplicate and each series of experiments included a negative control (uninoculated lettuce samples) and a positive control (viral suspensions) (modified method of Summa et al. 2012; Rzezutka et al. 2005).

Enumeration of Male-Specific Coliphages

Suspension made from the two aforementioned extraction and concentration protocols was assayed for coliphage enumeration by double agar layer (DAL) technique (US EPA 2001). *Escherichia coli* F_{amp} (ATCC 700891) was used as host bacteria for male-specific coliphages. Briefly, in this technique, 500 µl of extracted and concentrated suspension followed by 100 µl host suspension were added to 5 ml TSA (Tryptic Soy Agar) containing 0.7 % agar and 50 µl antibiotic (ampicillin and streptomycin) as top agar layer then poured onto bottom TSA plate. Each sample was analyzed in duplicate. Also, each inoculation experiment was tested in triplicate. The plates were incubated at 37 °C overnight, and after that, clear zones (plaques) were counted. Positive and negative controls were included in each assay. The numbers of plaques per plate were recorded to determine the percentage of coliphage recovery for each method used to extract and concentrate the coliphages and choose the best one for blind samples. For blind lettuce samples collected from various farms and markets, the DAL assay was carried out and plaques were also counted. Then, plaques were dissolved in 5 ml of sterilized PBS (pH 7.4) that poured on the surface of culture plates for 60 min and after filtration (pore size 0.2 µm), the solution was stored at 4 °C.

Statistical Analysis

Results were reported as the average of three replications; all treatments were evaluated in three batches. Statistical analysis was performed to calculate the differences between the two virus detection methods regarding the different inoculation concentrations. Statistical tests were conducted using the Mstat C Software. Duncan's tests were used to study the statistical differences of the means with 95 % confidence.

Coliphage Enrichment

The phage solution which was prepared in the previous step was enriched to obtain better phage identification in the molecular technique. The phage solution was

centrifuged at 8000×*g*, for 10 min and the clarified supernatant passed through a 0.22 μm filter to remove any endogenous bacteria. Then, 1 ml of filtered sample was added to 4 ml of sterile broth medium (TSB) in a sterilized tube, inoculated with 100 μl of a fresh overnight host culture (*E. coli* Famp). Enrichment culture was incubated at 37 °C overnight and then stored at 4 °C until genotyped (Center for Phage Technology Texas 2011).

RNA Extraction

A heat-release procedure was applied for coliphage RNA extraction. At first, 10 μl of the final solution was heated in 50 μl capacity microtubes for 5 min at 98 °C and then chilled on ice for 2 min (Schwab et al. 1997; Vinjé et al. 2004). Aliquots of 5 μl were immediately placed into RT-PCR mixture. Isolated viral RNA from representative coliphage strains was used as a positive control of male-specific coliphages RNA.

Primers

Primers are depicted in Table 1 (Dryden et al. 2006). All primers are from Macrogen Company (Macrogen, Korea) and were used in RT-PCR for detection of four groups of F^+RNA coliphages.

RT-PCR

Extracted RNA was transcribed into cDNA via RT kit (Fermentas, Germany) in two steps according to the manufacturer's instruction: 5 μl RT Dilution Buffer, 5 μl extracted RNA, and 1 μl of a 10 PM reverse primer were used for reverse transcription. RT conditions were as follows: 45 °C for 60 min 80 °C for 10 min.

To each 25 μl reaction volume there were 1 μl of synthesized cDNA, 0.5 μl of a 10 PM forward, 0.5 μl of a 10 PM reverse primer, and 10.5 μl of RNase-free sterilized water that were added to 12.5 μl of Master mix red solution containing PCR reaction buffer, $MgCl_2$, dNTP, and DNA polymerase (Taq) enzyme.

The PCR reaction was performed using the Thermocycler device (Sensequest, Germany) with these conditions: 95 °C for 15 min followed by 40 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min with a final extension of 72 °C for 10 min. Amplicons were separated by gel electrophoresis in 2 % agarose, stained with DNA green viewer and visualized under UV light (Geldoc, Sony, Japan). For each reaction, a positive (specified template RT-PCR for F^+RNA coliphages) and negative control (no template RT-PCR) was prepared. To avoid contamination, PCR master mixes, amplification, electrophoresis, and template and/or viral preparations were conducted in separate rooms. Sequencing of PCR products was carried out by Macrogen Company (Macrogen, Korea).

Results

Comparing of MS2 Coliphage Recovery Efficiency (PEG Precipitation Method Vs Ultracentrifugation Method)

To compare the MS2 coliphage recovery efficiency, three spiking levels (10^2, 10^4, 10^6 pfu/ml) were used for each method and recoveries were carried out using plaque assay. Results showed that both methods were capable of MS2 coliphage recovery at three spiking levels (10^2, 10^4, 10^6 pfu/ml). Two spiked concentrations of 10^4 and 10^6 pfu/ml were consistently identified in both methods in triplicate. The concentration of 10^2 pfu/ml was identified in just one sample using the ultracentrifugation method and two samples in PEG precipitation method (Table 2).

Also, a statistically significant difference was found between two methods in two spiked concentrations (10^4

Table 1 Sequence of designed primers for F^+RNA coliphages

Coliphage	Primer	Sequence	Temperature (°C)	Amplicon (bp)	Source
MS2	1F	5′-AATCTTCGTAAAACGTTCGTGTC-3′	53.7	204	Group I (non-human)
	1R	5′-GAGCCGTACCCACACCTTATAG-3′	56.8		
GA	6F	5′-CGTACTTAGCGGTATACTCAAGACC-3′	56.3	240	Group II (human)
	6R	5′-GTTTCCTGCATATAAGCATACCA-3′	52.9		
Qbeta	3F	5′-CTACTGCTGGTAATCTCTGGC-3′	62.2	795	Group II (human)
	3R	5′-CAACRCCGTTRGTGGGATTTAC-3′	61.3		
SP	2F	5′-TTAAACTAATTGGCGAGTCTGTACC-3′	54.9	236	Group IV (non-human)
	2R	5′-AACAGTGACTGCTTTATTTGAAGTG-3′	54.1		

and 10^6 pfu/ml) with 95 % confidence (Table 3). The PEG precipitation method showed higher average recovery rates of 0.83 for 10^2 pfu/ml, 15.02 for 10^4 pfu/ml, and 16.63 for 106 pfu/ml than the ultracentrifugation method for all the three spiked concentrations (Table 3). Therefore PEG precipitation method was chosen for evaluating lettuce samples.

Enumeration of Male-Specific Coliphages Using PEG Precipitation Method by DAL Procedure from the Surface of Naturally Contaminated Lettuce Samples

The enumeration of male-specific coliphages by plaque assay in three regions of farm and market is shown in Table 4 separately. Among thirty lettuce samples collected from different farms and markets only 21 samples (9 out of 15 from farm and 12 out of 15 from market) were positive for male-specific coliphages (Table 4). Concentration of coliphage was <50 pfu/10 g and <200 pfu/10 g in 33 and 40 % of farm and market samples, respectively. A statistically significant difference was found in enumeration of coliphages in market samples than farm (Fig. 2). The highest enumeration of coliphages was in market 3 whereas it was the lowest in market 1. Also, a statistically significant difference was found between market 3 and another sample collection site, whereas no statistically significant difference was observed between samples of different sites from farm (Fig. 3).

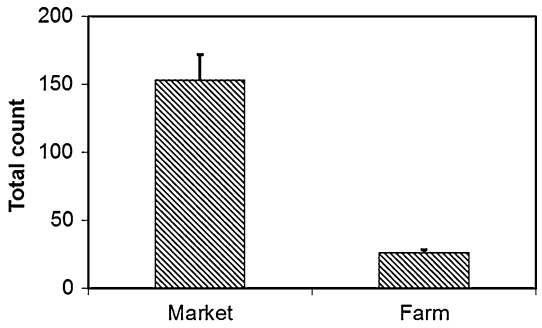

Fig. 2 Comparison of MS2 coliphage recovery efficiency from spiked lettuce with the PEG precipitation and ultracentrifugation method

Table 2 Detection of MS2 coliphage from lettuce samples spiked with three levels of MS2 using two virus recovery methods

Spiking level (pfu/ml)	No. of MS2-positive results/No. of samples tested	
	PEG precipitation	Ultracentrifugation
10^2	2/3	2/3
10^4	3/3	3/3
10^6	3/3	3/3

Table 3 Comparison of MS2 coliphage recovery efficiency from spiked lettuce with the PEG precipitation and ultracentrifugation method

Method	Spiking level (pfu/ml)	Recovery (pfu/g)	Recovery efficiency (%)
PEG precipitation	10^2	0.83	0.83^b
	10^4	1.52×10^3	15.02^a
	10^6	1.663×10^5	16.63^a
Ultracentrifugation	10^2	0.5	0.5^b
	10^4	3.34×10^2	3.34^c
	10^6	4.3×10^4	4.3^c

Numbers with the same letters have not significant difference statistically by Duncan's test at ($P < 5$ %)

Table 4 MS2 coliphage recovery (pfu/10gr) from naturally contaminated lettuce in the farm and market

	Location					
	Farm			Market		
	1	2	3	1	2	3
Enumeration of coliphage (pfu/10gr)	35	67	49	0	137	301
	50	0	51	99	87	179
	0	9	31	107	0	341
	0	0	39	101	236	205
	56	0	0	0	258	225

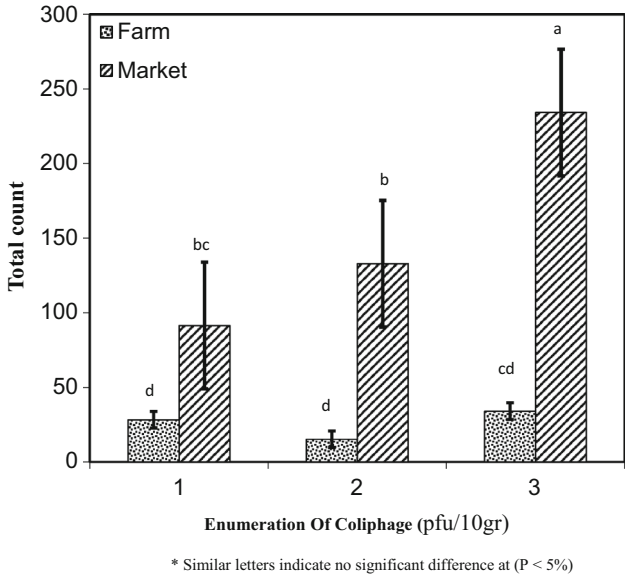

Fig. 3 Frequency distribution of presence of F$^+$RNA coliphage genotype I in the farm and market lettuce samples

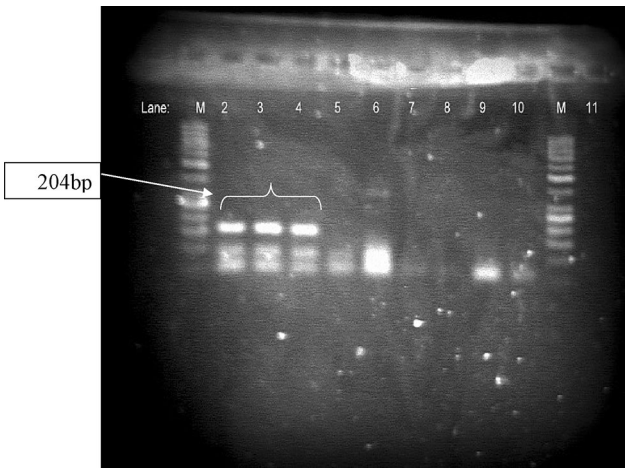

Fig. 4 RT-PCR products of F$^+$RNA coliphages (lettuce samples). *Lane 1* 50 bp DNA ladder, *lane 2–4* F$^+$RNA coliphage genotype I, *lane 5–6* F$^+$RNA coliphage genotype II, *lane 7–9* F$^+$RNA coliphage genotype IV, *lane 10* cDNA negative control, *lane 11* primer negative control

Prevalence of Different Genotypes of F$^+$RNA Coliphages in Farm and Market Samples

Using molecular method (RT-PCR) F$^+$RNA coliphages were detected at 9 and 13 out of 15 lettuce samples collected from farm and market, respectively (Table 5). Among market and farm samples, based on the primer sets used in this study, only group I was detected and the other three groups of II, III, and IV were not identified (Fig. 4). This finding revealed that animal feces probably are the biggest source of pollution in these areas. The sequencing results and comparison of RT-PCR amplicons with NCBI database demonstrated the presence of group I of F$^+$RNA coliphage (Table 6).

Discussion

Raw fruits and vegetables have been known to carry human diseases for at least a century. These products can be contaminated with food-borne pathogens in the farm or orchards, or during harvesting, transporting, processing, distribution, marketing, or at home (Cliver 1997).

Several strategies have been proposed for extraction, concentration, and detection of viruses in foods (Dubois et al. 2002; Sair et al. 2002; Dubois et al. 2002; Butot et al. 2007; Croci et al. 2008; Stals et al. 2012). Virus concentration methods have to be in accordance with the used eluent and the type of food stuff which is analyzed (Albertsson 1960). In the current study, the optimized method of PEG precipitation was used for washing the surface of naturally contaminated lettuce samples (without inoculation). However, the most

Table 5 Number of F$^+$RNA coliphages positive samples (DAL and RT-PCR method)

Sampling sites	No. of samples tested	No. of positive samples				
		DAL assay	RT-PCR			
			Genotype I	Genotype II	Genotype III	Genotype IV
Farm						
1	5	3 (60 %)	4 (80 %)	0	0	0
2	5	2 (40 %)	1 (25 %)	0	0	0
3	5	4 (80 %)	3 (60 %)	0	0	0
Market						
1	5	3 (60 %)	5 (100 %)	0	0	0
2	5	4 (80 %)	3 (60 %)	0	0	0
3	5	5 (100 %)	5 (100 %)	0	0	0

Table 6 F^+RNA coliphage genotype I characteristics identified in the lettuce samples

Characteristics	Identification percentage	Accession number
MS2 enterobacteriophage	100	GQ153927.1
Mutant Enterobacteriophage MS2	96	GQ153925.1
Enterobacteriophage MS2 clone pSMART_3528 A protein	96	GQ456167.1
Enterobacteriophage MS2 clone L3.G70.11 assembly protein (MS2g1)	96	FJ799708.1
Bacteriophage FR RNA genome	100	X15031.1
Bacteriophage for maturation and coat protein genes	100	M31635.1

available in vitro studies have been done based on seeding experiments, only few methods have been applied successfully to detect viruses in some naturally contaminated foods such as shellfish and sediment from open harvesting (Le Guyader et al. 2005; Williams 2005).

One of the major advantages of microbial source tracking is recognizing the origin of contamination.

Recently, in most studies carried out on viruses and fecal pollutions, F^+RNA coliphages have been proposed as a useful indicator and tracer (Sobsey et al. 2006; Yavarmanesh et al. 2010). These coliphages are not pathogenic for human, but they contaminate natural enteric bacteria in the mammalian's tracts and they are excreted in feces (Debartolomeis and Cabelli 1991).

In this study, we also focused on F^+RNA coliphages as a tracer to preliminarily identify the type of fecal contamination in lettuce. Firstly, in order to select an optimal method for virus detection in lettuce, two current virus extraction and concentration methods of ultracentrifugation and PEG precipitation, which are proposed recently, (Baert et al. 2008; Butot et al. 2007; Kim et al. 2008; Le Guyader et al. 2004b) were used and compared. Some studies have reported that the mean number of coliphage in the plants that were treated by sewage is 10^5 pfu/100 ml (Debartolomeis and Cabelli 1991). Thus, in this study, coliphage preparations for contamination were done in the range of 10^5, containing 10^6, 10^4, and 10^2 pfu/ml. A significant difference was observed between ultracentrifugation and PEG precipitation methods in two spiked solutions (10^4 and 10^6 pfu/ml) in which PEG precipitation method had higher efficiency than ultracentrifugation.

Recovery efficiency obtained by ultracentrifugation method in this study was similar to that of previous studies (Rzezutka et al. 2005, Rutjes et al. 2006). Rutjes et al. (2006) and Rzezutka et al. (2005) which reported that recovery efficiency of spiked norovirus on lettuce and soft fruits of raspberries and strawberries were 10 and 2.5–25 %, respectively.

Rzezutka et al. (2005) used washing buffer containing soy protein with sodium bicarbonate (alkaline solution) in order to facilitate desorption of virus particles from the surface of vegetables at the ultracentrifugation method. In this method, the pellet of viruses was formed in 100,000×*g* for 20 min (Rzezutka et al. 2005). Since this method requires expensive equipment, in this study, low speed with a longer time was used (50,000×*g* 3 h) similar to Summa et al. (2012) and the results obtained were similar to high speed. Some coliphages are discarded during decanting of supernatant in ultracentrifugation method, because of the forming of an often invisible pellet of viruses, therefore the difference at the recovery efficiencies between the replicates may occur at the final step (Summa et al. 2012).

In this study, PEG precipitation method did not show a significant difference in recovery efficiency of two spiking levels (10^6, 10^4 pfu/ml) (Table 3). In general, higher recovery efficiency was obtained at the spiking level of 10^6 pfu/ml (16.6 % 10 g) by PEG precipitation method. The recovery efficiency obtained by PEG precipitation method in this study was in line with the results of previous investigations (Dubois et al. 2002; Scherer et al. 2010; Sánchez et al. 2012; Summa et al. 2012). For example, in other studies Scherer et al. (2010) and Shahrampour (2014) reported that recovery efficiency of MS2 coliphage from spiked lettuce and leek and parsley samples was 6–10 and 12 %, respectively. Also, according to standard deviations in replicates and the frequency of negative results, the best repeatability and applicability method for all food matrices (lettuce) was obtained with PEG precipitation (Summa et al. 2012). Therefore, PEG precipitation method was applied for recovery of coliphages from the surface of naturally contaminated lettuce samples.

The higher MS2 coliphage recovery was obtained by the elution of the fruit or vegetable surface with a basic buffer TGBE (pH 9.5–7.4) supplemented with a salt (Tris–HCL), glycine amino acid, and 3 % beef extract protein, that is appropriate for a wide range of vegetables. Elution buffer in PEG precipitation method breaks electrostatic and hydrophobic interactions between the surfaces of vegetables and viruses (Dubois et al. 2002). Tris in this elution buffer prevents pH reduction, whereas glycine and beef extract were used to facilitate removal of coliphage particles from lettuce surfaces.

Similar buffers such as Tris–Glycine (pH 9.5) and beef extract have been noted for washing solution in lettuce and

herbs in earlier researches, respectively (Dubois et al. 2002; Bahreyni 2011). Moreover, the reduction of pH to the neutral condition causes the settling of the viral particles to reduce due to the nature of phage capsid proteins and their isoelectric point (Sobsey and Meschke 2003). Therefore, reducing of pH helps precipitate coliphage and, adding NaCl to the PEG solution causes the amount of coliphage recovery to increase (Bidawid et al. 2000).

In this study, some modifications were applied such as the elimination of pectinase for preventing gel formation at fruits because of samples having hard surface (lettuce). Moreover, in some other studies, chloroform–butanol was used for increasing transparency after adding PEG, decreasing the obtained sedimentation viscosity, preventing microtubes column saturation during RNA extraction, and eliminating PCR inhibitors. But, using chloroform–butanol, because of destruction of viral particles may cause some problems in enumerating spiked coliphage for calculating recovery efficiency and also enumerating of coliphage in naturally contaminated samples. Therefore, chloroform–butanol treatment is removed from the protocol.

The time of incubation after addition of PEG ranges from 4 h to overnight. There is no significant difference between the overnight and 4 h according to Kruskal–Wallis non-parametric comparison test (Kim et al. 2008). Also, there is a relation between the shape of viruses and the PEG concentration in which higher concentration of PEG between 7.5 and 30 % is needed to precipitate small spherical viruses (e.g., FRNA coliphages) in comparison with the rod-shaped ones (Yamamoto et al. 1970; Vajda 1978).

Factors that may contribute in increasing diseases associated with fruits and vegetables include the use of wastewater and incompatible composted manures to soils, changes in packaging technology, extended time between harvesting and consumption, and modifying food consumption patterns. Viruses may survive for weeks or even months on vegetables, crops, or in soil that have been irrigated or fertilized with sewage wastes (Larkin et al. 1978). Minimum dose to create enteric viruses infection in human are 1–100 units (WHO 2008). Therefore, due to low-dose enteric virus pathogenesis as well as the presence of enteric viruses on food surfaces, the role of food products to carry enteric viruses is very important (Mbithi et al. 1992; D'Souza et al. 2006). The use of F^+RNA coliphages for evaluation of the hygiene of fresh food was reported (Doré et al. 2000; Endley et al. 2003).

Negative results to enumerate coliphages can be related to inadequate accuracy for recovery and culture methods in this study. On the other hand, there is no infective risk in 33.3 % of samples which contained coliphage number $<$10 pfu/10 g (infective dose of viruses is 10–100 virus particles) Also, coliphage enumeration among farm samples is similar. It seems that sampling locations should be the same. Since vegetables' shelf life is short, after harvesting, vegetables should be sold quickly and not kept for a long time in the stores. Thus, it seems that the farm can be the main source of viral contamination in vegetables like lettuce.

Vegetables usually are kept cool to maintain their freshness. This low temperature may play a role in virus survival and transmission to humans. It was explained that MS2 reduction in fresh produce (such as parsley, cabbage, lettuce, etc.) was less than 1 log after 50 days storage at 4 and 8 °C (Dawson et al. 2005). Similar results were reported for virus survival in vegetables (celery, spinach, lettuce, and tomato) at 4 °C after irrigation with wastewater (Ward and Irving 1987). On the other hand, the highest numbers of F^+RNA coliphages are detectable during cold weather (Dryden et al. 2006). Therefore, the use of raw vegetables like lettuce after even 1 week of treatment with wastewater in farm may pose a high risk for consumers as it is kept in cool conditions at homes. Also, the average of environmental temperature in the city of Mashhad from April to July was usually lower than 28 °C which is relatively appropriate for human virus survival.

Detection and tracking of microbial fecal contamination in fresh products has created major challenges in environmental microbiology. Also, detection of different groups of F^+RNA coliphages would be appropriate for the control of virus contamination in food samples (Feachem 1975; Kasper et al. 1990). For this purpose, F^+RNA coliphage subgroups in lettuce samples were identified in this study. The presence of genotype I of F^+RNA coliphages was proved among detected positive farm and market samples. Irrigation with polluted water or using improper fertilizers (hens and cows) and sewage as well as the presence of animals like dogs and cats can be the main source of animal fecal contamination in farm and market lettuce samples. Also numerous viruses of human or animal origin can spread in the environment and infect the people via water and food, mostly through ingestion and occasionally through skin contact; therefore, there is a significant risk of contamination for workers in the markets. Viruses are transmitted via the fecal–oral route; so workers with poor personal hygiene can transfer enteric viruses or zoonotic viruses to foods or to work surfaces from fingers contaminated with animal or human feces. As a result, there is a possibility for the presence of group I of F^+RNA coliphage in the lettuce samples from the market.

The absence of subgroup II and III in lettuce samples probably indicated no pollution with human feces. Therefore, there was no chance of pathogenicity with human viruses in consumer.

Some of the previous studies have mainly reported that genotypes II, III, and I, IV have been isolated from human feces and animal feces, respectively. While this specificity can be changed, because genotype I of F^+RNA coliphages have also been isolated from human sewage (Griffin 2000; Furuse et al. 1981). Also, the applicability of MST method at different geographic locations such as Asia is not clearly understood yet (Long et al. 2002). On the other hand, analyzing based on nucleic acid sequence from specific genes of F^+RNA coliphages has not been done or too little has been carried out so far (Yee et al. 2006). Also, a comprehensive analysis based on nucleic acid sequencing has not been applied for bacteriophages.

Conclusion

In the present study, only genotype I of F^+RNA coliphage by RT-PCR was detected from the surface of lettuce. The nucleic acid sequencing managed to confirm lettuce contamination with animal feces. The genotyping of F^+RNA coliphages by RT-PCR used in this study allowed characterization and identification of different types of fecal pollution in lettuce samples and introduced these coliphages as a valid biomarker in microbial source tracking studies in food samples.

Acknowledgments We are grateful to Dr. Bassami from the Faculty of Veterinary for technical helps and Dr. Nasiri, Department of Agricultural Biotechnology, Faculty of Agriculture for invaluable advice.

References

Albertsson, P. A. (1960). *Partition of cell particles and macromolecules* (p. 231 S). Stockholm: International Publishers Printers & Booksellers. **Preis Sw.kr. 35**.

Baert, L., Uyttendaele, M., & Debevere, J. (2008). Evaluation of viral extraction methods on a broad range of Ready-To-Eat foods with conventional and real-time RT-PCR for Norovirus GII detection. *International Journal of Food Microbiology, 123*, 101–108.

Bahreyni, M. (2011). *Screening, isolation and identification of indicator bacteria and viruses of fecal contamination in ready to use vegetables in Mashhad*. Phd thesis (Ferdowsi university of Mashhad).

Berger, S. B., Oshiro, R. K. (2002). Source water protection. *Journal of Microbiology of source water*, pp. 2967–2978, In G. Bitton (editor-in-chief) Encyclopedia of environmental microbiology. New York: Wiley.

Bidawid, S., Farber, J. M., & Sattar, S. A. (2000). Rapid concentration and detection of hepatitis A virus from lettuce and strawberries. *Journal of Virological Methods, 88*, 175–185.

Butot, S., Putallaz, T., & Sánchez, G. (2007). Procedure for rapid concentration and detection of enteric viruses from berries and vegetables. *Journal of Applied and Environmental Microbiology, 73*, 186–192.

Cliver, D. O. (1997). Foodborne viruses. *Journal of Food Microbiology: Fundamentals and Frontiers,* 437–446.

Croci, L., Dubois, E., Cook, N., Medici, D., Rutjes, S. A., Hoorfar, J., et al. (2008). Current methods for extraction and concentration of enteric viruses from fresh fruit and vegetables: Towards International Standards. *Journal of Food Analytical Methods, 1*, 73–84.

D'Souza, D. H., Sair, A., Williams, K., Papafragkou, E., Jean, J., Moore, C., et al. (2006). Persistence of caliciviruses on environmental surfaces and their transfer to food. *International Journal of Food Microbiology, 108*(1), 84–91.

Dawson, D., Paish, A., Staffell, L., Seymour, I., & Appleton, H. (2005). Survival of viruses on fresh produce, using MS2 as a surrogate for norovirus. *Journal of Applied Microbiology, 98*(1), 203–209.

Debartolomeis, J., & Cabelli, V. J. (1991). Evaluation of an Escherichia coli host strain for enumeration of F male-specific bacteriophages. *Applied and Environmental Microbiology, 57*(5), 1301–1305.

Doré, W. J., Henshilwood, K., & Lees, D. N. (2000). Evaluation of F-specific RNA bacteriophage as a candidate human enteric virus indicator for bivalve molluscan shellfish. *Applied and Environmental Microbiology, 66*(4), 1280–1285.

Dryden, S. K., Ramaswami, B., Yuan, Z., Giammar, D. E., & Angenent, L. T. (2006). A rapid reverse transcription-PCR assay for F^+RNA coliphages to trace fecal pollution in Table Rock Lake on the Arkansas-Missouri border. *Journal of Water Research, 40*, 3719–3724.

Dubois, E., Agier, C., Traoré, O., Hennechart, C., Merle, G., Crucière, C., et al. (2002). Modified concentration method for the detection of enteric viruses on fruits and vegetables by reverse transcriptase-polymerase chain reaction or cell culture. *Journal of Food Protection, 12*, 1962–1969.

Endley, S., Johnson, E., & Pillai, S. D. (2003). A simple method to screen cilantro and parsley for fecal indicator viruses. *Journal of Food Protection®, 66*(8), 1506–1509.

Feachem, R. (1975). An improved role for faecal coliform to faecal streptococci ratios in the differentiation between human and nonhuman pollution sources. *Journal of Water Research, 9*, 689–690.

Furuse, K., Ando, A., Osawa, S., & Watanabe, I. (1981). Distribution of ribonucleic acid coliphages in raw sewage from treatment plants in Japan. *Journal of Applied and Environmental Microbiology, 41*, 1139–1143.

Grabow, W. (1998). *Evaluation of the application of bacteriophages as indicators of water quality*. Pretoria: Water Research Commission.

Grabow, W. (2004). Bacteriophages: Update on application as models for viruses in water. *Water Sa, 27*(2), 251–268.

Griffin, D. W., Stokes, R., Rose, J. B., & Paul III, J. H. (2000). Bacterial indicator occurrence and the use of an F^+ specific RNA coliphage assay to identify fecal sources in Homosassa Springs, Florida. *Microbial Ecology*, *39*(1), 56–64.

Havelaar, A., & Hogeboom, W. (1984). A method for the enumeration of male-specific bacteriophages in sewage. *Journal of Applied Bacteriology, 56*(3), 439–447.

Hedberg, C. W., MacDonald, K. L., & Osterholm, M. T. (1994). Changing epidemiology of food-borne disease: a Minnesota perspective. *Journal of Clinical Infectious Diseases, 18*, 671–682.

Hsu, F.-C., Shieh, Y.-S. C., & Sobsey, M. (2002). Enteric bacteriophages as potential fecal indicators in ground beef and poultry meat. *Journal of Food Protection*, *65*(1), 93–99.

Hsu, F. C., Shieh, Y. S., van Duin, J., Beekwilder, M. J., & Sobsey, M. D. (1995). Genotyping male-specific RNA coliphages by

hybridization with oligonucleotide probes. *Journal of Applied and Environmental Microbiology, 61*, 3960–3966.

Jofre, J., Stewart, J. R., & Grabow, W. (2011). *Phage methods microbial source tracking: Methods, applications, and case studies* (pp. 137–156). Berlin: Springer.

Kasper, C. W., Burgess, J. L., Knight, I. T., & Colwell, R. R. (1990). Antibiotic resistance indexing of *Escherichia coli* to identify sources of fecal contamination in water. *Canadian Journal of Microbiology, 36*, 891–894.

Kim, H. Y., Kwak, I. S., Hwang, I. G., & Ko, G. (2008). Optimization of methods for detecting norovirus on various fruit. *Journal of Virological Methods, 153*, 104–110.

Kirs, M., & Smith, D. C. (2007). Multiplex quantitative real-time reverse transcriptase PCR for F?-specific RNA coliphages: A method for use in microbial source tracking. *Journal of Applied and Environmental Microbiology, 73*(3), 808–814.

Koopmans, M. (2004). Round-robin comparison of methods for the detection of human enteric viruses in lettuce. *Journal of Food Protection, 67*, 2315–2319.

Larkin, E. P., Tierney, J. T., Lovett, J., Van Donsel, D., Francis, D. W. (1978). Land application of sewage wastes: potential for contamination of foodstuffs and agricultural soils by viruses, bacterial pathogens and parasites. In: *State of Knowledge in Land Treatment of Wastewater,* International Symposium. Vol. 2. Hanover, NH, USA. Army Corps of Engineers.

Le Guyader, F. S., Mittelholzer, C., Haugarreau, L., Hedlund, K. O., Alsterlund, R., Pommepuy, M., et al. (2004a). Detection of noroviruses in raspberries associated with a gastroenteritis outbreak. *International Journal of Food Microbiology, 97*, 179–186.

Le Guyader, F. S., Schultz, A. C., Haugarreau, L., Croci, L., Maunula, L., Duizer, E., et al. (2004b). Round-robin comparison of methods for the detection of human enteric viruses in lettuce. *Journal of Food Protection, 10*, 2315–2319.

Le Guyader, F. S., Schultz, A. C., Haugarreau, L., Croci, L., Maunula, L., Duizer, E., et al. (2005). Assessment of sources and diversity of male-specific coliphages for source tracking. *Journal of environmental-engineering and science, 22*, 367–377.

Leclerc, H., Edberg, S., Pierzo, V., & Delattre, J. M. (2000). Bacteriophages as indicators of enteric viruses and public health risk in groundwaters. *Journal of Applied and Environmental Microbiology, 88*, 5–21.

Long, S., Adak, G., O'Brien, S., & Gillespie, I. (2002). General outbreaks of infectious intestinal disease linked with salad vegetables and fruit, England and Wales, 1992–2000. *Communicable Disease and Public Health/PHLS*, *5*(2), 101–105.

Mbithi, J., Springthorpe, V., Boulet, J., & Sattar, S. (1992). Survival of hepatitis A virus on human hands and its transfer on contact with animate and inanimate surfaces. *Journal of Clinical Microbiology, 30*(4), 757–763.

Mesquita, M. M., & Emelko, M. B. (2012). *Bacteriophages as surrogates for the fate and transport of pathogens in source water and in drinking water treatment processes*. London: INTECH Open Access Publisher.

Rutjes, S. A., Lodder-Verschoor, F., van der Poel, W. H., van Duijnhoven, Y. T., de Roda, H., & Maria, A. (2006). Detection of noroviruses in foods: a study on virus extraction procedures in foods implicated in outbreaks of human gastroenteritis. *Journal of Food Protection, 69*, 1949–1956.

Rzezutka, A., Alotaibi, M., D'Agostino, M., & Cook, N. (2005). A centrifugation-based method for extraction of norovirus from raspberries. *Journal of Food Protection, 68*, 1923–1925.

Sair, A. I., D'Souza, D. H., Moe, C. L., & Jaykus, L.-A. (2002). Improved detection of human enteric viruses in foods by RT-PCR. *Journal of Virological Methods, 100*(1), 57–69.

Sánchez, G., Elizaquível, P., & Aznar, R. (2012). A single method for recovery and concentration of enteric viruses and bacteria from fresh-cut vegetables. *International Journal of Food Microbiology, 152*(1), 9–13.

Scherer, K., Johne, R., Schrader, C., Ellerbroek, L., Schulenburg, J., & Klein, G. (2010). Comparison of two extraction methods for viruses in food and application in a norovirus gastroenteritis outbreak. *Journal of Virological Methods, 169*, 22–27.

Schwab, K. J., Estes, M. K., Neill, F. H., & Atmar, R. L. (1997). Use of heat release and an internal RNA standard control in reverse transcription-PCR detection of Norwalk virus from stool samples. *Journal of Clinical Microbiology, 35*, 511–514.

Seymour, I. J., & Appleton, H. (2001). Foodborne viruses and fresh produce. *Journal of Applied Microbiology, 91*, 759–773.

Shahrampour, D. (2014). *Application of F^+RNA Coliphages as Source Tracking Enteric Viruses on Parsley and Leek using RT-PCR*. Ms thesis (Ferdowsi university of Mashhad). Iran: 'Unpublished results' or 'Personal communication'.

Sobsey, M. D., Love, D. C., & Lovelace, G. L. (2006). *F^+RNA coliphages as source tracking viral indicators of fecal contamination*. Final report to NOAA and CICEET.

Sobsey, M. D., & Meschke, J. S. (2003). *Virus survival in the environment with special attention to survival in sewage droplets and other environmental media of fecal or respiratory origin* (p. 70). Geneva, Switzerland: Report for the World Health Organization.

Stals, A., Baert, L., Van Coillie, E., & Uyttendaele, M. (2012). Extraction of food-borne viruses from food samples: a review. *International Journal of Food Microbiology, 153*(1), 1–9.

Summa, M., Bonsdorff, C. V., & Maunula, L. (2012). Evaluation of four virus recovery methods for detecting noroviruses on fresh lettuce, sliced ham, and frozen raspberries. *Journal of Virological Methods, 183*(2), 154–160.

US EPA. (2001). *Method 1602: male-specific (F^+) and somatic coliphage in water by single agar layer (SAL) procedure* (vol. EPA-821-R-01-029). Washington, DC: U.S. Environmental Protection Agency.

Vajda, B. (1978). Concentration and purification of viruses and bacteriophages with polyethylene glycol. *Folia Microbiologica, 23*(1), 88–96.

Vinjé, J., Oudejans, S. J. G., Stewart, J. R., Sobsey, M. D., & Long, S. C. (2004). Molecular detection and genotyping of male-specific coliphages by reverse-transcription- PCR and reverse line blot hybridization. *Journal of Applied and Environmental Microbiology, 70*, 5996–6004.

Ward, B. K., & Irving, L. G. (1987). Virus survival on vegetables spray-irrigated with wastewater. *Water Research, 21*(1), 57–63.

Williams, K. E. (2005). Detection of norovirus and indicator organisms on fresh produce. (*Doctoral Dissertation*)

Wolf, S., Hewitt, J., Rivera-Aban, M., & Greening, G. E. (2008). Detection and characterization of F^+RNA bacteriophages in waterband shellfish: Application of a multiplex real-time reverse transcription PCR. *Journal of Virological Methods, 149*(1), 123–128.

Yamamoto, K. R., Alberts, B. M., Benzinger, R., Lawhorne, L., & Treiber, G. (1970). Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. *Virology, 40*(3), 734–744.

Yan, T., Hamilton, M., & Sadowsky, M. J. (2007). High throughput and quantitative procedure for determining sources of Escherichia coli in waterways using host-specific DNA marker genes. *Journal of Applied and Environmental Microbiology, 73*, 890–896.

Yavarmanesh, M., Abbaszadegan, M., Mortazavi, A., Habibi Najafi, M. B., Bassami, M., & Nassiri, M. R. (2010). Impact of milk components in recovery of the MS2 bacteriophage as an indicator of enteric viruses. *Journal of Virological Methods, 168*, 103–107.

Yee, S. Y. F., Fong, N. Y., Fong, G. T., Tak, O. J., Hui, G. T., & Ming, Y. S. (2006). Male-specific RNA coliphages detected by plaque assay and RT-PCR in tropical river waters and animal fecal matter. *Journal of International Journal of Environmental Health Research, 16*, 59–68.