



# Concentration of hepatitis A virus in milk using protamine-coated iron oxide (Fe<sub>3</sub>O<sub>4</sub>) magnetic nanoparticles

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## ABSTRACT

Hepatitis A virus (HAV) continues to be the leading cause of viral hepatitis. HAV outbreaks have been linked to the consumption of milk, but methods for HAV detection in milk are very limited. We developed a method to concentrate HAV in milk using protamine-coated iron oxide (Fe<sub>3</sub>O<sub>4</sub>) magnetic nanoparticles (PMNPs). In this study, protamine was covalently coated on the surface of the MNPs (20–30 nm) by a three-step chemical reaction. The successful linkage of protamine to the MNPs was confirmed by Fourier transform infrared spectroscopy (FTIR), zeta potential, and transmission electron microscopy (TEM). When used for concentrating HAV from 40 mL of milk, 50 μL of PMNPs were added to the sample and mixed for 20 min by gentle rotation, followed by a magnet capture for 30 min. The captured PMNPs were washed with glycine buffer (0.05 M glycine, 0.14 M NaCl, 0.2% (v/v) Tween 20, pH 9.0) and HAV RNA was extracted using the QIAamp MinElute Virus Spin Kit and quantified by real-time RT-PCR. The method showed a detection limit of  $8.3 \times 10^0$  PFU of HAV in milk. The whole concentration procedure could be completed in approximately 50 min. The developed method was simple, inexpensive, and easy-to-perform.

## 1. Introduction

An increase in the globalization of food trade in recent years has presented new challenges for food safety and resulted in an increased number of foodborne outbreaks (Stals et al., 2012). It was estimated by the World Health Organization (WHO) that 31 foodborne hazards caused approximately 600 million cases of foodborne illness and 420,000 deaths worldwide in 2010 (Havelaar et al., 2015). The growing burden of foodborne outbreaks due to viral contamination of food has become a significant public health concern. Hepatitis A viruses (HAV), belonging to the *Hepatovirus* genus of the *Picornaviridae* family, plays a very important role in foodborne infections (Carneiro et al., 2018). In 2010, HAV ranked ninth among the 31 identified major hazards causing foodborne diseases, accounting for approximately 13 million foodborne illnesses and around 27,731 deaths worldwide (Havelaar et al., 2015). Although hepatitis A vaccines were introduced in the mid-1990s (Wasley et al., 2006), HAV continues to be the leading cause of viral hepatitis and foodborne outbreaks have caused considerable economic losses (Minor et al., 2015; Scharff, 2012).

A wide variety of foods have been implicated in outbreaks of HAV, including shellfish (clam, oyster, mussel, and scallops) (CDC, 2016;

Coelho et al., 2003; Croci et al., 2003; Goswami et al., 2002), fresh produce (green onion, lettuce, tomato, fig, date, berry fruits, and pomegranate) (Boxman et al., 2012; CDC, 2015; Scavia et al., 2017; Sun et al., 2012; Wheeler et al., 2005), ready-to-eat foods (sandwich, coleslaw, French fries, sushi, and deli meat) (Sun et al., 2012), and orange juice (Frank et al., 2007). Milk and milk products (ice cream, yogurt, and milk shake) are able to serve as vehicles for transmitting HAV (Sun et al., 2012; Zaher et al., 2008). It was reported that an HAV outbreak of 10 cases in Forsyth, Georgia, was due to the consumption of contaminated milk (Murphy et al., 1946). In another report, around 424 people were sick because of the consumption of milk or milk products contaminated with HAV in Czechoslovakia (Raska et al., 1966). Actually, the number of foodborne HAV outbreaks in milk is underestimated, because the average incubation period of HAV is 28 days and the implicated foods usually have been consumed or discarded before the appearance of symptoms (Castrodale et al., 2002; Nainan et al., 2006). In up to 50% of cases of HAV infections, the source is not identified. Fewer than 5% of the infections are classified as foodborne, but a greater percentage may be transmitted by this route (Le Guyader and Atmar, 2008).

It is challenging to develop methods for the detection of HAV in

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foods due to low contamination levels. And tissue culture cultivation of HAV is unrealistic for routine detection in foods (Boxman et al., 2012; Felix-Valenzuela et al., 2012). Typically, prior to viral genome detection by polymerase chain reaction (PCR), viral particles in contaminated food need to be separated and concentrated in order to enhance the efficiency of virus detection. Currently, polyethylene glycol (PEG) precipitation, ultracentrifugation, ultrafiltration, positively or negatively charged membrane filtration, and immunomagnetic separation are commonly used to concentrate HAV from foodstuffs (Stals et al., 2012). However, each of these methods has inherent disadvantages, which limits their application. For example, the ultrafiltration method requires viral eluates free of food components; the ultracentrifugation method needs an expensive ultracentrifuge and specialized personnel; the immunomagnetic separation method is only suitable for processing samples with small volume due to the high price of the antibodies, and the rapid occurrence of novel antigenic variants of HAV weakens the binding between the virus and the antibody, causing risk of false negative detection (Costa-Mattioli et al., 2002; Gharbi-Khelifi et al., 2007; Pérez-Sautu et al., 2011; Sáñchez et al., 2002); and the PEG precipitation method is time-consuming (Stals et al., 2012). The concerns in regards to the charged membrane filtration method include the risk of a considerable number of viral particles eluding the electrostatic force and passing through the pores of the charged membrane (Di Pasquale et al., 2010). To overcome these limitations, it is desirable to develop a new method to concentrate HAV from food samples.

In the past decade, there has been increasing focus on iron oxide ( $\text{Fe}_3\text{O}_4$ ) magnetic nanoparticles (MNPs) for the separation and concentration of foodborne pathogens, due to their unique physiochemical properties such as high surface-to-volume ratio, rapid diffusion, good dispersability, and many unique size-dependent qualities (Yang et al., 2008). MNPs have been used to concentrate pathogenic bacteria from food samples, such as *Escherichia coli* O157:H7 from ground beef, milk, and spinach (Cheng et al., 2009; Ravindranath et al., 2009; Varshney et al., 2005; Yang et al., 2013), *Salmonella* Typhimurium from ground beef, lettuce, tomato, and spinach (Ravindranath et al., 2009; Yang et al., 2013), *Vibrio parahaemolyticus* from oysters (Zeng et al., 2014), and *Listeria monocytogenes* from ground beef, lettuce, and milk (Amagliani et al., 2006; Kanayeva et al., 2012; Luo et al., 2017; Wang et al., 2017; Yang et al., 2007, 2013). MNPs have demonstrated higher capture efficiency than magnetic microbeads (MMBs) in isolating bacteria from foods (Varshney et al., 2005; Yang et al., 2007). However, to the best of our knowledge, there is no report on the application of MNPs in capturing viruses such as HAV in foods.

It has been previously shown that HAV could bind to positively charged polyethyleneimine (PEI)-coated MMBs (Uchida et al., 2007). Cationic MNPs might be an effective alternative for the concentration of HAV in foods. We therefore decided to coat the surface of  $\text{Fe}_3\text{O}_4$  MNPs with positively charged proteins for the concentration of HAV from food samples. Protamine is a unique food protein found in fish sperm. Because of the extremely high composition of arginine, a basic amino acid, this protein is highly positively charged (Hoffmann et al., 1990). The isoelectric point (pI) of protamine was reported to be 12.1–13.8 (Hoffmann et al., 1990; Tang et al., 1993). The pI of HAV was reported to be 2.8 (Michen and Graule, 2010). Thus, when the pH is between 2.8 and 12.1, HAV is negatively charged and protamine is positively charged. Based on this mechanism, we hypothesized that  $\text{Fe}_3\text{O}_4$  MNPs coated with protamine could be used to capture HAV in food due to strong electrostatic attractive force between them at pH 2.8–12.1. Protamine-coated MNPs (PMNPs) have demonstrated good performance in concentrating hepatitis C virus (HCV) from human plasma (Yassin et al., 2014). The aim of the current study was to investigate efficiency of PMNPs for concentrating HAV from milk.

## 2. Materials and methods

### 2.1. HAV propagation

HAV strain HM175/24A, a cytopathic clone of strain HM175 adapted to cell culture, was obtained from the culture collection of the Canadian Research Institute for Food Safety (CRIFS) and propagated in fetal rhesus monkey kidney cells (FRhk-4), as described by Morales-Rayas et al. (2010). Briefly, FRhk-4 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Cat. No. 12800017, Life Technologies) supplemented with 1% (v/v) streptomycin/penicillin (Cat. No. 15140-122, Life Technologies) and 10% (v/v) heat-inactivated fetal bovine serum (FBS, Cat. No. 16140-063, Life Technologies) in a 75 cm<sup>2</sup> tissue culture flask (Cat. No. 430720, Corning, Tewksbury, MA, USA). Cells were incubated at 37 °C and 5% CO<sub>2</sub> for 2 days until confluent growth was achieved. The confluent monolayer was then washed once with PBS (pH 7.4, Cat. No. 10010031, Life Technologies) warmed at 37 °C. Five mL of DMEM and 200 µL of viral stock were inoculated on the surface of the monolayer with a MOI (multiplicity of infection) of 0.1. The flask was incubated at 37 °C for 1.5 h, with gentle shaking every 10 min to distribute the inoculum. After 1.5 h, the inoculum was removed and 8 mL of DMEM supplemented with 2% (v/v) FBS and 1% (v/v) streptomycin/penicillin were added to the flask. Cells were incubated for 8 days until 80% of them showed a cytopathic effect. The viruses were released from the cells by three freeze-thaw cycles, and cell debris was removed by centrifugation at 1600 × g for 10 min using a Beckman Coulter Allegra 21 centrifuge with an S4180 rotor (Beckman Coulter Canada). The supernatant was then filtered through a EMD Millipore Millex 0.22 µm (GV) low protein binding filter (Cat. No. SLGV033RS, EMD Millipore, Etobicoke, ON, Canada) and dispensed into cryogenic vials (Cat. No. 430488, Corning, Tewksbury, MA, USA) in 1-mL aliquots and stored at –80 °C.

### 2.2. HAV plaque assay

Viral stock was quantified by plaque assay as described by Cromeans et al. (1987). Briefly, FRhk-4 cells were inoculated into 6-well tissue culture plates (Cat. No. C3506, Corning, Tewksbury, MA, USA) with  $5 \times 10^5$  cells dispensed in each well. The plates were incubated at 37 °C and 5% CO<sub>2</sub> for 24 h. After washing the monolayer with the PBS (pH 7.4), 500 µL of 10-fold serial dilutions of viral stock in DMEM were inoculated onto the plates, with each dilution tested in triplicate. The plate was incubated for 1.5 h with gentle shaking every 10 min. The inoculum was removed and 3 mL of overlay medium, containing 1.5 mL of 1.2% (w/v) SeaKem LE Agarose (Cat. No. 50002, Lonza, Rockland, ME, USA) added with 26 mM MgCl<sub>2</sub> and 1.5 mL of 2 × DMEM supplemented with 4% (v/v) FBS, 2% (v/v) streptomycin/penicillin, were added to each well. The plate was incubated for 10 days. After fixing with 10% formalin (Cat. No. 245684, Thermo Scientific) for 24 h, the overlay was removed, and the monolayer was stained with 0.1% (w/v) crystal violet (prepared in 0.85% saline, w/v) for 20 min. The plaques were counted after the removal of crystal violet. The viral titer was expressed as plaque forming units (PFU) per mL.

### 2.3. Extraction of viral RNA

Viral RNA was extracted from 200 µL of viral stock using the QIAamp MinElute Virus Spin Kit (Cat. No. 57704, Qiagen, Toronto, ON, Canada) according to the manufacturer's instructions. Briefly, 200 µL of virus were mixed with 25 µL of Qiagen protease and 200 µL of buffer AL provided in the kit. The mixture was incubated at 56 °C for 15 min, followed by the addition of 250 µL of ethanol (100%) and incubation at room temperature for 5 min. The sample was loaded onto the QIAamp MinElute column and centrifuged at 6000 × g for 1 min using a Beckman Coulter Allegra 21 centrifuge with an F2402H rotor (Beckman Coulter

**Table 1**  
Primers and probe for the detection of HAV using real-time RT-PCR<sup>§</sup>.

Primers	Sequence (5'-3')	Position	Polarity	Reference
Forward	ATAGGGTAACAGCGGGGATAT	448–469	+	Gardner et al. (2003)
Reverse	CTCAATGCATCCACTGGATGAG	516–537	–	Gardner et al. (2003)
Probe	FAM-CCATTCAACGCCGGAGG-MGB	492–508	+	This study

<sup>§</sup>The positions of primers are in accordance with the wild-type strain of HAV (GenBank accession number: M14707.1).

Canada). After washing the column sequentially with 500  $\mu$ L of buffer AW1, AW2, and ethanol (100%) by centrifugation at  $6000 \times g$  for 1 min, the column was dried by centrifugation at  $20,000 \times g$  for 3 min. Finally, 40  $\mu$ L of RNase-free water (Cat. No. AM9937, Applied Biosystems) were used to elute RNA from the column and the eluted RNA was stored at  $-80^\circ\text{C}$ .

#### 2.4. Real-time RT-PCR of HAV

Ten  $\mu$ L of the extracted RNA were reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Cat. No. 4374966, Applied Biosystems) according to the manufacturer's instructions. Briefly, the RNA was mixed with 2  $\mu$ L of  $10 \times$  RT buffer, 2  $\mu$ L of RT random primers, 0.8  $\mu$ L of  $25 \times$  dNTP mix, 1  $\mu$ L of multiscribe reverse transcriptase, 1  $\mu$ L of RNase inhibitor, and 3.2  $\mu$ L of RNase-free water in a 20  $\mu$ L reaction volume. The reagents were mixed on ice. The mixture was incubated using a GeneAmp PCR system 9700 (Applied Biosystems) at  $25^\circ\text{C}$  for 10 min,  $37^\circ\text{C}$  for 120 min,  $85^\circ\text{C}$  for 5 min, and then at  $4^\circ\text{C}$  until PCR analysis.

Real-time PCR was performed with a ViiA 7 system (Applied Biosystems) using the primers and Taqman probe as shown in Table 1. The real-time PCR was performed in a 20- $\mu$ L reaction mixture consisting of 10  $\mu$ L of  $2 \times$  Taqman Fast Advanced Master Mix (Cat. No. 4444557, Applied Biosystems), 700 nM of each primer, 250 nM of probe, and 4  $\mu$ L of cDNA. Thermal cycling conditions included  $50^\circ\text{C}$  for 2 min and  $95^\circ\text{C}$  for 20 s, followed by 40 cycles of  $95^\circ\text{C}$  for 1 s and  $60^\circ\text{C}$  for 20 s. Fluorescence was measured at the end of each real-time PCR cycle. A standard curve was constructed by testing 10-fold serial dilutions of cDNA. The slope of the standard curve was generated by plotting the log virus titer versus the cycle threshold (Ct) value. The amplification efficiency was calculated using equation  $E = 10^{-1/\text{slope}-1}$  (Klein et al., 1999).

#### 2.5. Production of protamine-coated $\text{Fe}_3\text{O}_4$ MNPs

Protamine was coated on the surface of MNPs through a three-step chemical reaction. The synthesis procedure for PMNPs is schematically illustrated in Fig. 1.

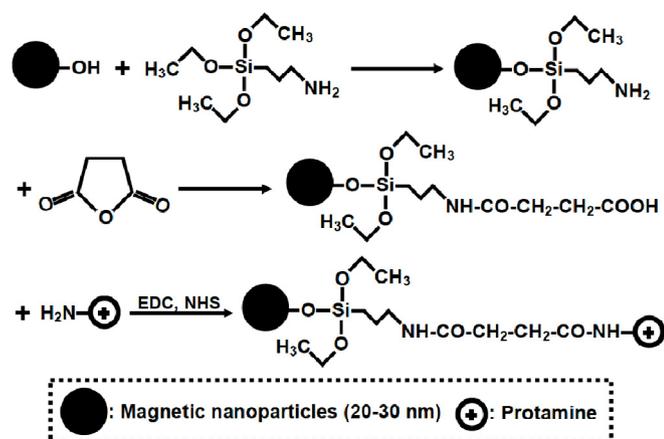


Fig. 1. Schematic illustration of the fabrication of PMNPs.

**Step 1,  $-\text{NH}_2$  coating:** Amino groups were coated on the surface of MNPs according to protocols described in the literature (Bruce and Sen, 2005; Chang and Adriaens, 2006; Kralj et al., 2011). One hundred mg of  $\text{Fe}_3\text{O}_4$  MNPs (Cat. No. 637106, Sigma-Aldrich, Oakville, ON, Canada) were added to 23.7 mL of Milli-Q water and mixed by vortex and sonication at 40 kHz in a FS-20 ultrasonic cleaner (Fisher Scientific, Ottawa, ON, Canada) until the suspension was completely homogenized. Then, 1.3 mL of 3-aminopropyltriethoxysilane (APTES, Cat. No. A3648, Sigma-Aldrich, Oakville, ON, Canada) were added to the homogenized suspension, which was then shaken at 250 rpm for 24 h at  $50^\circ\text{C}$ . After that, the MNPs were recovered using a permanent magnet with a surface magnetization of 13,000 G (Cat. No. 44207-20, Indigo Instruments, Waterloo, ON, Canada), followed by thoroughly washing the MNPs with Milli-Q water for five times to remove unbound APTES. Finally, the  $\text{NH}_2$ -coated MNPs (NMNPs) were re-suspended in 10 mL of Milli-Q water and stored at  $4^\circ\text{C}$ .

**Step 2,  $-\text{COOH}$  coating:** Carboxyl groups were then coated on the surface of the NMNPs (the product of Step 1) according to the method described by Zhao et al. (2004). After washing three times with *N,N*-dimethylformamide (DMF) (Cat. No. D4551, Sigma-Aldrich, Oakville, ON, Canada), NMNPs were suspended in 25 mL of DMF containing 10% (w/v) succinic anhydride (Cat. No. 239690, Sigma-Aldrich, Oakville, ON, Canada). The chemical reaction was performed at  $50^\circ\text{C}$  under nitrogen gas with shaking at 250 rpm for 6 h. The nanoparticles were harvested using the magnet and washed thoroughly with Milli-Q water for five times to remove unbound reactants. The  $\text{COOH}$ -coated MNPs (CMNPs) were re-suspended in 10 mL of Milli-Q water and stored at  $4^\circ\text{C}$ .

**Step 3, protamine coating:** Protamine sulfate from salmon (mol. wt.  $\sim 5.1$  kDa, Cat. No. P4020, Sigma-Aldrich, Oakville, ON, Canada) was coated on the surface of the CMNPs (the product of Step 2) by EDC/NHS coupling chemistry (Ravindranath et al., 2009; Satoh et al., 2003; Zhao et al., 2004). EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) (Cat. No. PI-22980) and NHS (N-hydroxysuccinimide) (Cat. No. 130672) were purchased from Fisher Scientific Inc. (Ottawa, ON, Canada) and Sigma-Aldrich Co. (Oakville, ON, Canada), respectively. One mL of CMNPs was washed with 0.1 M MES (4-morpholinethanesulfonic acid) (Cat. No. M8250, Sigma-Aldrich, Oakville, ON, Canada) buffer (pH 6.0) three times, and was then activated with 0.4 M EDC/0.1 M NHS prepared in 0.1 M MES buffer (pH 6.0) by rotation at 30 rpm for 40 min at room temperature using an Orbitron Rotator II (Block Scientific, NY, U.S.A.). The MNPs were magnetically recovered, washed three times with 0.1 M PBS (pH 7.5), and subsequently mixed with 2 mL of 10 mg/mL protamine in 0.1 M PBS (pH 7.5). The reaction mixture was rotated at 30 rpm at  $25^\circ\text{C}$  for 24 h, followed by washing of the MNPs three times with 0.1 M PBS (pH 7.5), twice with 3 M NaCl, once with 10 mM HCl, and three times with Milli-Q water. Finally, the protamine-coated MNPs (PMNPs) were suspended in 1 mL of Milli-Q water and stored at  $4^\circ\text{C}$ .

#### 2.6. Chemical characterization of surface modified $\text{Fe}_3\text{O}_4$ MNPs

##### 2.6.1. Fourier transform infrared spectroscopy (FTIR)

The  $\text{Fe}_3\text{O}_4$  MNPs obtained from each step of the aforementioned reactions were scanned using a Digilab FTS-7000 Fourier transform infrared spectrometer (Bio-Rad, Randolph, MA, USA) equipped with a

DTGS (deuterated triglycine sulfate) detector and a Golden Gate single reflection diamond ATR (attenuated total reflectance) accessory. The sample for FTIR spectroscopy was vacuum-dried at 40 °C. The spectra were collected from 800  $\text{cm}^{-1}$  to 4000  $\text{cm}^{-1}$  at 4  $\text{cm}^{-1}$  resolution with 64 co-added scans per spectrum.

### 2.6.2. Transmission electron microscopy (TEM)

The morphology of the protamine-coated  $\text{Fe}_3\text{O}_4$  MNPs was observed by a LEO 912 AB energy filtered transmission electron microscope (Carl Zeiss Inc. Oberkochen, Germany) operating at 100 kV. The sample preparation for TEM analysis included pipetting 5  $\mu\text{L}$  of MNP suspension onto a formvar and carbon foil covered copper grid (200 mesh). The excess liquid was removed by blotting with filter paper, and then the copper grid was floated on a drop of 2% (w/v) uranyl acetate for 30 s. Images were captured with an Olympus/SIS Cantega 2K digital camera using Olympus/SIS item software.

### 2.6.3. Determination of zeta potential at different pH

The zeta potentials of  $\text{Fe}_3\text{O}_4$  MNPs obtained from each step of the aforementioned reactions were measured using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd. Malvern, Worcestershire, United Kingdom). After equilibrating the samples for 24 h at room temperature, the zeta potentials of the MNPs were tested in aqueous suspension at pH of 3.0–11.0 adjusted by 0.1 M HCl or NaOH.

## 2.7. Optimization of RNA extraction from HAV captured by cationic $\text{Fe}_3\text{O}_4$ MNPs

Fifty  $\mu\text{L}$  of PMNPs or NMNPs were mixed with 50 mL of glycine buffer (0.05 M glycine, 0.14 M NaCl, 0.2% (v/v) Tween 20, pH 9.0) containing  $8.3 \times 10^4$  PFU of HAV particles. The mixture was rotated at 10 rpm at room temperature for 20 min using the Orbitron Rotator II, followed by magnetic capture for 30 min. Viral RNA was extracted from the captured particles using three different extraction methods described below. Each extraction method was performed in three independent replicates.

**Kit method:** The captured MNPs were re-suspended in 200  $\mu\text{L}$  of PBS (pH 7.4) and vortexed for 5 min to separate the MNPs. Viral RNA in the suspension was extracted using the QIAamp MinElute Virus Spin Kit according to the manufacturer's instructions, reverse transcribed to cDNA, and then quantified using real-time RT-PCR as described above.

**HCl and Kit method:** The captured MNPs were re-suspended in 200  $\mu\text{L}$  of 0.1 M HCl solution, followed by vortexing for 15 min to release viral particles from the MNPs. After magnetic capture for 5 min, the supernatant was transferred to a 1.5 mL Eppendorf centrifuge tube and neutralized with 1 M NaOH solution. Two hundred  $\mu\text{L}$  of the HAV suspension were then used for RNA extraction using the QIAamp MinElute Virus Spin Kit, followed by quantification using the real-time RT-PCR method described above.

**Heating method:** The captured MNPs were re-suspended in 40  $\mu\text{L}$  of RNase-free water, and the suspension was sonicated for 1 min at 40 kHz in the FS-20 ultrasonic cleaner to separate MNPs, and then heated at 95 °C for 5 min to lyse the virus capsid. After removing the MNPs with the magnet, viral RNA in 10  $\mu\text{L}$  of the suspension was reverse transcribed to cDNA and then tested by real-time RT-PCR as described above.

## 2.8. Concentration of HAV in milk using cationic $\text{Fe}_3\text{O}_4$ MNPs

Pasteurized partly skimmed milk (2%, Neilson, Canada) was purchased from a local supermarket and stored at 4 °C. Forty mL of the milk (in a 50 mL centrifuge tube) were inoculated with 100  $\mu\text{L}$  of HAV dilutions prepared in the PBS (pH 7.4). The concentration of HAV in the inoculum was  $8.3 \times 10^6$ ,  $8.3 \times 10^4$ ,  $8.3 \times 10^2$ , or  $8.3 \times 10^1$  PFU/mL. The artificially contaminated milk was mixed well and then left at room

temperature for 1 h. One tube of milk without HAV inoculation was used as a negative control. Before recovering HAV particles using the cationic MNPs, the milk samples were treated in four different ways shown as follows: (1) Forty mL of milk were centrifuged at  $4100 \times g$  for 20 min at 4 °C to be separated into three layers. The upper layer of cream and the lower layer were removed. The middle layer of skim milk was transferred to a new centrifuge tube (50 mL) for further analysis. (2) pH of the milk (40 mL) was adjusted to 4.6 by adding 2 mL of 1 M HCl, which resulted in the coagulation of milk protein. The protein was removed by centrifugation at  $4100 \times g$  for 20 min at 4 °C. The resulting supernatant was transferred to a new centrifuge tube (50 mL) for further analysis. (3) Six  $\mu\text{L}$  of chymosin (CHY-MAX<sup>®</sup> Extra, Cat. No. 73863, Chr. Hansen, Milwaukee, WI, USA) were added to 40 mL of milk and clotting was allowed to take place at 37 °C for 20 min, followed by centrifugation at  $4100 \times g$  for 20 min at 4 °C to precipitate protein. The resulting supernatant was transferred to a new centrifuge tube (50 mL) for further analysis. (4) No treatment. The milk (40 mL) was kept in the 50 mL tube for further analysis.

Fifty  $\mu\text{L}$  of the PMNPs or NMNPs were added to each of the aforementioned 50 mL centrifuge tubes containing different milk samples, followed by rotating at 10 rpm at room temperature for 20 min using the Orbitron Rotator II. Then, the MNPs were captured by holding the magnet against the bottom of the 50 mL tube for 30 min. The supernatant was discarded and the captured MNPs were washed with 1 mL of glycine buffer (0.05 M glycine, 0.14 M NaCl, 0.2% (v/v) Tween 20, pH 9.0) once to remove the residual milk components. After that, the viral RNA was extracted from the recovered HAV particles using the optimized extraction method described above. The extracted viral RNA was then reversely transcribed to cDNA and quantified using real-time RT-PCR as described previously. The obtained Ct values were used to estimate the HAV quantities in the concentrated samples based on the standard curve generated previously. Viral recovery rate was then calculated according to the equation: recovery rate (%) = (total viral particles captured / total viral particles input in milk before separation)  $\times$  100 (Morales-Rayas et al., 2010).

Both NMNPs and PMNPs were used to recover HAV in this test. The recovery efficiency of HAV using these two different MNPs was compared. Each experiment was carried out as three independent replicates.

## 2.9. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) and subsequent Tukey's test with IBM SPSS Statistics (version 21; IBM Corporation, New York, USA). In all cases, *P*-value < 0.05 indicated a significant difference.

## 3. Results

### 3.1. Quantification of HAV via plaque assay and real-time RT-PCR

The titer of the propagated HAV HM175/24A determined by plaque assay was  $8.3 \times 10^6$  PFU/mL. By analyzing the Ct values of 10-fold serial dilutions of HAV cDNA, a standard curve for real-time RT-PCR ( $y = -3.327x + 40.238$ ,  $R^2 = 0.998$ ) was obtained (Fig. 2). The slope of the curve indicated a PCR efficiency of 99.7%. The limit of detection of the real-time RT-PCR assay was found to be  $8.3 \times 10^0$  PFU/mL.

### 3.2. Chemical characterization of surface modified $\text{Fe}_3\text{O}_4$ MNPs

#### 3.2.1. FTIR analysis

The manufacture of PMNPs consisted of three steps and the product of each step was structurally examined using FTIR to verify the success of each coating. FTIR spectra for  $\text{Fe}_3\text{O}_4$  MNPs without and with chemical modifications were collected in the range from 800 to 4000  $\text{cm}^{-1}$  (Fig. 3). The FTIR spectrum of NMNPs demonstrated a strong band at 990  $\text{cm}^{-1}$ , which was due to the asymmetric stretching vibration of Si-

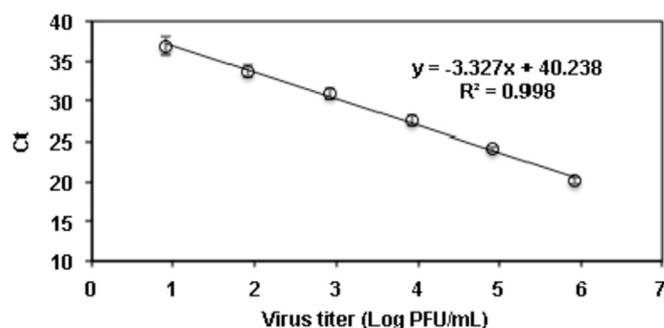


Fig. 2. Standard curve of real-time RT-PCR made by 10-fold serial dilutions of HAV cDNA using ViiA 7 system. Each value is the mean of three independent replicates. Bars show standard deviation.

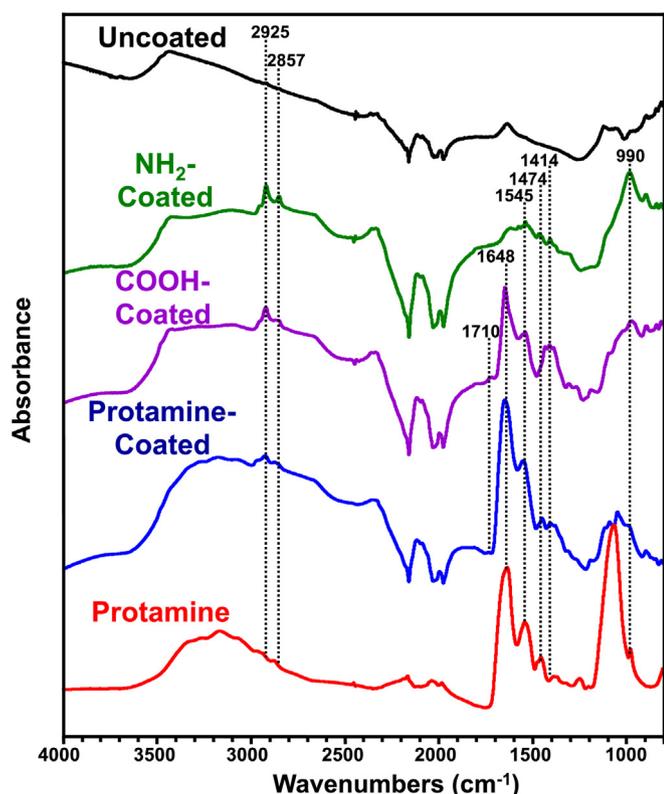


Fig. 3. FTIR spectra of  $\text{Fe}_3\text{O}_4$  magnetic nanoparticles with different surface coatings.

O-Si (Das et al., 2008; Lapin and Chabal, 2009; Pasternack et al., 2008). It was apparent that the peaks at  $2925$  and  $2857$   $\text{cm}^{-1}$  were caused by the asymmetric and symmetric stretching vibration, respectively, of  $-\text{CH}_2$  groups in the NMNPs (Das et al., 2008; Pasternack et al., 2008; Zhang et al., 2002). The success of  $-\text{NH}_2$  group coating was further confirmed by the low-intensity bands at  $1300$ – $1600$   $\text{cm}^{-1}$  generated from both associated and free  $-\text{NH}_2$  groups (Zhang et al., 2002). The intensity of the bands related to APTES was relatively weak, in accordance with the small proportion of the surface layer of APTES compared to the bulk of reagent, which was in good agreement with previous reports (Kouassi et al., 2005).

For the  $-\text{COOH}$  coating, the  $-\text{NH}_2$  groups on the surface of NMNPs were reacted with succinic anhydride by attacking the carbonyl C atoms of the succinic anhydride molecule to form amide bonds, resulting in free terminal  $-\text{COOH}$  groups from the elongated chains. The bands at  $1648$   $\text{cm}^{-1}$  and  $1545$   $\text{cm}^{-1}$  were generated by the  $\text{C}=\text{O}$  stretching vibration of Amide I and the N-H bending vibration of Amide II, respectively (Kim et al., 2010). The successful coating of  $-\text{COOH}$  groups

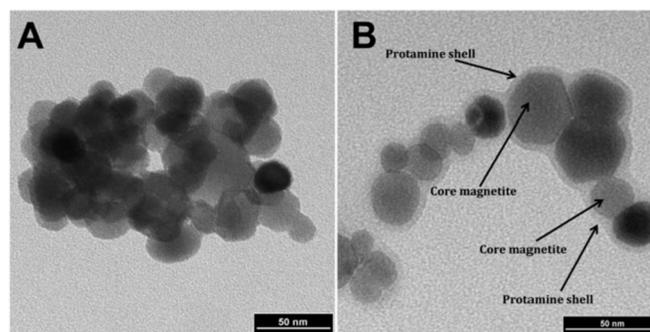


Fig. 4. TEM images of uncoated (A) and protamine-coated (B)  $\text{Fe}_3\text{O}_4$  MNPs.

was further confirmed by the existence of the stretching vibrational mode of  $-\text{COOH}$  at  $1710$   $\text{cm}^{-1}$  and symmetric stretching vibration mode of carboxylate ( $\text{COO}^-$ ) at  $1405$   $\text{cm}^{-1}$  (Frey and Corn, 1996).

For the coating of protamine, the surface  $-\text{COOH}$  groups of CMNPs were activated by EDC to form an amine-reactive *O*-acyl-isourea intermediate that could react with the  $-\text{NH}_2$  groups of protamine to form an amide bond (Grabarek and Gergely, 1990). The typical vibration mode of amide bonds appeared at  $1648$   $\text{cm}^{-1}$  and  $1545$   $\text{cm}^{-1}$  in the spectrum of PMNPs. The characteristic absorption peak from symmetric stretching vibration of  $\text{NH}_3^+$  in protamine was found at  $3200$   $\text{cm}^{-1}$  (Fukushima et al., 2011). These results indicated successful covalent coating of protamine onto the surface of MNPs.

### 3.2.2. TEM analysis

TEM analysis of protamine-coated and uncoated MNPs showed that both MNPs were approximately spherical in shape and were about  $20$ – $30$  nm in diameter (Fig. 4). Two regions with different electron densities were observed for the MNPs coated with protamine: a relatively dense magnetite core and a surrounding protamine layer with lower density. The TEM images suggested that the protamine coating was fairly homogeneous and the thickness of the protamine layer was approximately  $2$  nm (Fig. 4).

### 3.2.3. Zeta potential analysis

Zeta potentials of the uncoated MNPs and the MNPs coated with  $-\text{NH}_2$ ,  $-\text{COOH}$ , or protamine were analyzed at different pH ( $3.0$ – $11.0$ ) by testing the electrophoretic mobility of the nanoparticles in the aqueous suspension. Results showed that the zeta potential of each kind of MNPs decreased as pH increased (Fig. 5). The isoelectric point (pI) of a charged nanoparticle is the pH at which the particle has zero zeta potential. The original MNPs without any coating had a pI of around  $8.0$ . Coating  $-\text{NH}_2$  groups on the surface of the MNPs resulted in a net

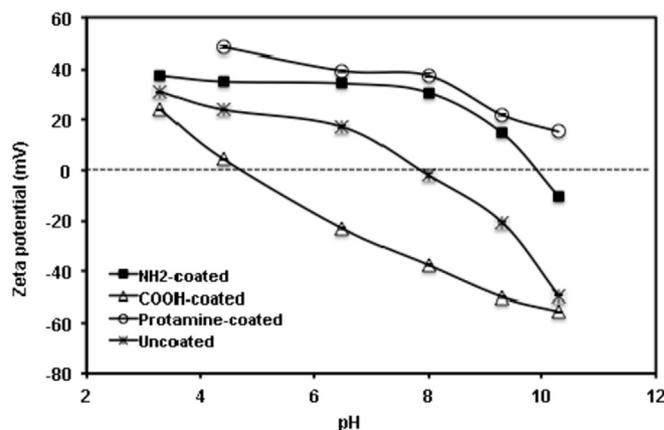


Fig. 5. Zeta potentials of  $\text{Fe}_3\text{O}_4$  MNPs with different coatings. Each value is the mean of five replicates. Bars show standard deviation.

**Table 2**  
Comparison of different RNA extraction methods<sup>§</sup>.

RNA release method	Ct value	
	NMNPs	PMNPs
HCl and Kit	20.8 ± 0.4	21.5 ± 0.3
Kit	29.3 ± 0.4	19.9 ± 0.8
Heating	ND	36.5 ± 1.3

<sup>§</sup>HAV concentration was  $8.3 \times 10^4$  PFU/50 mL and the volume of MNPs used was 50  $\mu$ L; Each value is the mean  $\pm$  standard deviation (n = 3); ND means not detectable; PMNPs: protamine-coated magnetic nanoparticles; NMNPs: NH<sub>2</sub>-coated magnetic nanoparticles; For each MNP, the differences of Ct values of RNA among three methods were significant ( $P < 0.01$ ).

positive surface charge and an increase of pI to around 10.0 for NMNPs. When the surface of the NMNPs was further modified with -COOH groups that are negatively charged under physiological conditions, the pI of CMNPs decreased to about 5.0. The PMNPs had a pI higher than 10.3 due to the coating of MNPs with protamine that is rich in L-arginine, a highly positively charged amino acid. When the pH was lower than 10.0, both the PMNPs and NMNPs were positively charged. The zeta potential of PMNPs was much higher than that of NMNPs at any given pH, indicating that the surface charge of the former was more intense than that of the latter.

### 3.3. Comparison of different RNA extraction methods

Three different methods (Kit method, HCl and Kit method, and Heating method) were compared for extracting RNA from the viral particles concentrated from 50 mL of glycine buffer by PMNPs and NMNPs (Table 2). The Ct values given by real-time RT-PCR reflected the RNA extraction efficiency of different methods. For PMNPs, the Kit method demonstrated higher efficiency than the HCl and Kit method and the Heating method ( $P < 0.01$ ). For NMNPs, the HCl and Kit method worked best ( $P < 0.01$ ). Therefore, the Kit method was used for studies involving PMNPs, and the HCl and Kit method was used for NMNPs in the following experiments.

### 3.4. Concentration of HAV in milk pre-treated with different methods

Artificially contaminated milk was centrifuged to remove fat and somatic cells, or was treated with either chymosin or HCl to precipitate and remove casein before HAV recovery using PMNPs or NMNPs. The results showed that the combination of chymosin precipitation and PMNP capture had greater repeatability than other methods (Table 3).

**Table 3**  
Detection of HAV from milk pre-treated with different methods<sup>§</sup>.

Treatment before virus concentration	Virus concentration method	No. of positive samples / No. of tested samples at inoculation level (PFU) of			
		10 <sup>5</sup>	10 <sup>3</sup>	10 <sup>1</sup>	10 <sup>0</sup>
No treatment	PMNPs	3/3	3/3	1/3	1/3
	NMNPs	3/3	3/3	1/3	0/3
Centrifugation	PMNPs	3/3	3/3	1/3	0/3
	NMNPs	3/3	3/3	0/3	0/3
HCl precipitation	PMNPs	3/3	3/3	1/3	0/3
	NMNPs	3/3	3/3	1/3	0/3
Chymosin precipitation	PMNPs	3/3	3/3	2/3	1/3
	NMNPs	3/3	3/3	1/3	0/3

<sup>§</sup> PMNPs: protamine-coated magnetic nanoparticles; NMNPs: NH<sub>2</sub>-coated magnetic nanoparticles; NA: not analyzed; The inoculum level of HAV was  $8.3 \times 10^5$ ,  $8.3 \times 10^3$ ,  $8.3 \times 10^1$ , or  $8.3 \times 10^0$  PFU/40 mL of milk; Virus was recovered on three distinct occasions and detection of viral RNA by real-time RT-PCR was carried out in duplicates. A sample was considered positive if both tests of viral RNA were positive.

However, the treatments did not improve the detection limit of HAV as the value for milk without any treatment was also  $8.3 \times 10^0$  PFU/40 mL (Table 3). So, PMNPs showed better performance than NMNPs for concentrating HAV from milk. Considering lower the cost of the method, milk did not need to be treated to remove any protein prior to HAV concentration by PMNPs.

## 4. Discussion

Milk has been widely consumed all over the world for thousands of years and acts as an excellent source of high-quality proteins. It also acts as an important food providing the essential nutrients such as fat, carbohydrates, calcium, potassium, phosphorus, iodine, vitamin A, vitamin B<sub>12</sub>, and riboflavin (Fayet-Moore, 2016). It is estimated that milk is consumed by around 6 billion people in the world, and its worldwide yearly production has reached 730 million tons (Visioli and Strata, 2014). Considering such high levels of consumption, it is very important that milk is free from hazards and poses no health risk to consumers. However, milk is an important potential reservoir of pathogenic microorganisms due to contamination from dairy farm environments, unhygienic milk production facilities, dirty udder surface, and infected dairy farmers (Griffiths, 2010). The pathogens can also be excreted from the udders of infected milk-producing animals (Oliver et al., 2005; Vissers and Driehuis, 2008). Among various milk-borne pathogens, HAV is a common contaminating organism causing outbreaks of infections and is an indicator of unhygienic condition during collection, processing, or storage of milk (Daudi et al., 2012; Murphy et al., 1946; Terzi et al., 2010; Zaher et al., 2008). HAV in milk can survive pasteurization because fat can protect HAV from inactivation by heat, and the higher the fat content, the stronger is the heat stability of HAV. Thus, routine pasteurization cannot guarantee the complete inactivation of HAV in milk (Bidawid et al., 2000). The source of milk contamination might also be from post-pasteurization contamination by infected food handlers. Reports on detection of HAV in milk are very limited. In the current study, we chose pasteurized milk as a food vehicle to test the concentration efficiency of cationic MNPs. The incubation period of HAV is very long and the implicated milk usually has been consumed or discarded before the appearance of symptoms (Nainan et al., 2006). Thus, it was extremely difficult to obtain naturally contaminated milk samples, and we could only use artificially contaminated milk in our research.

Because of rapid viral mutation, the appearance of new antigenic variants of HAV strains makes immunocapture unreliable (Atreya, 2004; Jothikumar et al., 2005; Sánchez et al., 2007). Also the high price of antibodies makes the detection of HAV using immunoassays expensive. It is necessary to find an ideal material to coat the surface of MNPs for capturing HAV in food. Since the pI of the HAV capsid is 2.8 (Michen and Graule, 2010) and the initial pH of milk is around 6.6 (Sinaga et al., 2017), HAV is net negatively charged in contaminated milk. Protamine, a low-molecular-weight protein (~4.3 kDa), is highly positively charged under physiological conditions due to its extremely high content of arginine (Hoffmann et al., 1990). It exists naturally in the sperm nuclei of salmon and plays an important role in binding to DNA and compacting the configuration of chromatin in the nucleus of sperm (Awasthi and Singh, 2017). Because of its advantageous charge property, protamine has been compacted with nucleic acid to form a complex and applied in gene delivery applications (Rahme et al., 2015) and used as a drug in pharmacotherapy for the neutralization of overdosed heparin, the most highly negatively charged biological molecule (Hoffmann et al., 1990). We hypothesized that the highly positively charged PMNPs would work effectively in separating and concentrating negatively charged HAV in milk.

Protamine was covalently coated onto the surface of MNPs by a three-step chemical reaction. The first step was to fabricate NMNPs. The protocol for making NMNPs was based on published literature (Bruce and Sen, 2005; Chang and Adriaens, 2006; Kralj et al., 2011) and has

been optimized in our research. Different types of solvent were used in coating APTES on the surface of MNPs. Toluene was used as solvent by [Chang and Adriaens \(2006\)](#) for the coating of MNPs with  $-NH_2$  groups. When toluene was used in the current experiment, strong bands at 2925 and  $2857\text{ cm}^{-1}$  were found in the FTIR spectrum of MNPs even when APTES was not present in the solvent. Additionally the band at  $1710\text{ cm}^{-1}$  in the spectrum of CMNPs was not found, indicating the efficiency of  $-COOH$  group and further  $-NH_2$  group coating was low. Additional concern is raised by the fact that toluene is a hazardous chemical, causing unconsciousness and even death ([Canadian Centre for Occupational Health and Safety, 2017](#)). These findings coupled with its hazardous nature resulted in toluene being not used for making NMNPs. Ethanol was also reported as solvent for linking APTES with MNPs ([Bruce and Sen, 2005](#); [Kralj et al., 2011](#)), but when used in our experiment, the ethanol led to less APTES on the surface of MNPs than Milli-Q water based on the FTIR and zeta potential analysis (data not shown). So, Milli-Q water was used subsequently used as the solvent. The reaction time used for protein cross-linking varied greatly in published protocols ([Cheng et al., 2009](#); [Varshney et al., 2005](#); [Yang et al., 2007](#); [Zhao et al., 2004](#)). We extended the reaction time from 4 h to 24 h and found that the zeta potentials of PMNPs were increased at given pH values, indicating higher coating efficiency (data not shown). In order to ensure the successful coating of  $-NH_2$  groups,  $-COOH$  groups, and protamine on the surface of MNPs, all the samples before and after surface modification were subjected to FTIR and zeta potential analysis. It was reported that when viewed under TEM, no difference was observed between the uncoated MNPs and NMNPs, indicating that molecules like APTES or succinic anhydride do not result in obvious changes to the morphology of MNPs ([Bruce and Sen, 2005](#); [Cheng et al., 2009](#)). Therefore, in the current study, only uncoated MNPs and PMNPs were tested by TEM. It was very evident that protamine was coated on the surface of PMNPs ([Fig. 4](#)). But only several nanometers of protamine were observed, which might be due to the relatively small size of protamine (approximately 5.1 kDa).

Two different cationic MNPs, NMNPs and PMNPs, were fabricated in our study for the concentration of HAV. PMNPs had an overall better performance than NMNPs with regards to sensitivity and repeatability ([Table 3](#)). In our experiment, both MNPs were stored at  $4^\circ\text{C}$  after production. We found that PMNPs could be suspended in water for longer time than NMNPs during storage, indicating that the former was more stable than the latter. The stability of MNPs might affect the capturing efficiency of virus. Another explanation for the better performance of PMNPs is the attraction force between PMNPs and HAV particles. As is shown in [Fig. 5](#), the pI of PMNPs is higher than NMNPs, making the electrostatic attraction force between PMNPs and HAV particles stronger than that between NMNPs and the virus. Aside from electrostatic attraction force, hydrophobic interactions and hydrogen bonding between PMNPs and HAV capsid might contribute to the better performance of PMNPs. Cationic MNPs with different surface modification exhibited varying concentration efficiencies for the same virus, which was in good agreement with the results obtained by ([Uchida et al., 2007](#)). In their research, three cationic magnetic beads, respectively coated with polyethyleneimine (PEI), polyarylamine (PAA), and poly-L-lysine (PLL), were used to concentrate different viruses including HAV, HBV (hepatitis B virus), HCV, SV-40 (simian virus 40), HSV-1 (herpes simplex 1 virus), PPV (porcine parvovirus), and adenovirus from viral suspension in cell culture medium. For each virus, the PEI-coated magnetic beads showed the highest virus recovery efficiency and the beads coated with PEIs of different molecular weights showed dramatically different efficiency in capturing viruses ([Uchida et al., 2007](#)). In our study, protamine was selected to coat MNPs instead of PEI, since the latter is cytotoxic and hazardous to human health ([Hunter, 2006](#); [Moghimi et al., 2005](#)). Our results also indicated that, compared with NMNPs, PMNPs showed higher efficiency to concentrate HAV in milk because the viral RNA extraction procedure for PMNPs was at least 20 min shorter than that for NMNPs. The RNA

extraction protocols for PMNPs and NMNPs were optimized in glycine buffer (0.05 M glycine, 0.14 M NaCl, 0.2% (v/v) Tween 20, pH 9.0) rather than milk. This is because the PMNPs and NMNPs were fabricated to concentrate HAV not only in milk in the current study but also in other foods that are associated with HAV outbreaks, such as green onion, berry fruits, and sea food. The glycine buffer works effectively in eluting HAV particles from these foods and has been used by Health Canada ([Mattison et al., 2010](#)).

For the virus inoculum level of  $8.3 \times 10^5$ ,  $8.3 \times 10^3$ ,  $8.3 \times 10^1$ , or  $8.3 \times 10^0$  PFU, the recovery rate of HAV from 40 mL of milk without pre-treatment was 0.1%, 0.2%, 0.7% and 4.9%, respectively. With the increase of virus inoculum level, the recovery rate of HAV decreased. This feature was also observed in other studies for the concentration of norovirus (NoV) ([Escudero-Abarca et al., 2014](#); [Fumian et al., 2009](#); [Moore et al., 2015](#)). [Fumian et al. \(2009\)](#) used a negatively charged membrane filtration protocol to concentrate norovirus (NoV) from lettuce and cheese. Their results showed that with the decrease of NoV inoculum levels from  $1.9 \times 10^7$  genome copies to  $9.5 \times 10^3$  genome copies in lettuce, the recovery rate of NoVs increased from 5.2% to 72.3%, and when the inoculum levels of NoV decreased from  $1.5 \times 10^7$  genome copies to  $8.9 \times 10^3$  genome copies in cheese, the recovery rate of the virus increased from 6% to 56.3%. [Escudero-Abarca et al. \(2014\)](#) used nucleic acid aptamers to capture NoV from artificially contaminated lettuce. The capture efficiency of the aptamers decreased from 2.5% to 36% when the inoculum levels of NoV increased from 1 Log<sub>10</sub> RNA copies to 5 Log<sub>10</sub> RNA copies per 3 g of lettuce. In another study conducted by the same research group ([Moore et al., 2015](#)), two nucleic acid aptamers were used to capture NoV in serial diluted human stool solutions. The capture efficiency of NoV by aptamers for the  $10^{-1}$  dilutions of stool was much lower than that for the  $10^{-2}$  and  $10^{-3}$  dilutions of human stool. The results in the current study implied that milk components might compete with viral particles for PMNPs and the concentration of PMNPs (50  $\mu\text{L}$ ) was not enough for capturing high contamination levels of virus in milk. However, because the natural contamination level of virus in food is usually low, foodborne virus detection should target the lower contamination levels. Thus, a volume of 50  $\mu\text{L}$  of PMNPs was chosen for performing the experiment.

The use of PMNPs allowed detection of  $8.3 \times 10^0$  PFU of HAV in 40 mL milk ([Table 3](#)). The limit of detection of this technique was believed to satisfy the need for diagnostic applications in a food microbiological laboratory, because HAV infectious dose was found to be between 10 and 100 viral particles ([Yezli and Otter, 2011](#)). [Hirreisen et al. \(2009\)](#) used cationic magnetic beads to concentrate HAV from 250 mL of milk and found the sensitivity of the method was only  $10^6$  TCID<sub>50</sub>/mL (equivalent to approximately  $10^6$  PFU/mL). In order to improve the efficiency of the PMNP capture method, we hypothesized that the milk components, especially casein, might interfere with the electrostatic attraction between HAV and PMNP, because casein comprises around 80% of milk protein and is negatively charged in milk because the pI of casein is 4.6 ([Ye and Harte, 2013](#)). In our study, casein in milk was precipitated by HCl or chymosin and removed by centrifugation before HAV recovery by cationic MNPs. Milk somatic cells and fat were also removed by centrifugation. However, none of the treatment methods greatly improved the detection efficiency. So, for applying PMNPs to concentrate HAV in milk, no treatment of the sample for protein removal was needed. There may be a possibility that the removal of casein did increase the concentration efficiency of PMNPs, but the reaction efficiency of real-time RT-PCR was inhibited by non-proteinaceous components of milk. In a future study, we can make efforts to make it clear.

The PMNP capture method developed in the current study took approximately 50 min to complete, which included  $\sim 20$  min for the complex formation between HAV and PMNP, and  $\sim 30$  min for magnetic capture of the complex. We used  $\sim 50$  min in our experiment to make sure as many viral particles as possible could be captured. But the total optimal operation time could be much shorter than 50 min

because nanoparticles have some unique physicochemical properties such as high surface-to-volume ratio, rapid diffusion, good dispersability, etc. (Yang et al., 2008). The capture time could be optimized in a future study. Currently, the whole procedure for the detection of HAV in milk took approximately 4.5 h to perform, including virus concentration (~50 min), RNA extraction (~40 min), and real-time RT-PCR amplification (~3 h). In order to reduce the time of the whole procedure, more efficient RNA detection methods are needed.

The protocol developed in the current study for surface modification of Fe<sub>3</sub>O<sub>4</sub> MNPs can be used to covalently attach other molecules to MNPs for the purpose of concentration of pathogens in food. For example, porcine gastric mucin (PGM) can be coated on the surface of MNPs for the concentration of norovirus in food, because PGM contains cell receptors for norovirus (Tian et al., 2008). HAV RNA has been found in breast milk of women who were infected with HAV (Daudi et al., 2012). PMNPs can also be used for the concentration of HAV in human breast milk. Apart from milk, PMNPs could potentially be used to concentrate HAV in other food matrices, such as green onion, strawberry, and mussel. PMNPs showed good performance in concentrating HAV from cow's milk without any pre-treatment, but different food matrices have different compositions, and also because PMNP capture method is non-specific to HAV, inhibitors to real-time RT-PCR in other foods might be concentrated by PMNPs. It is probably necessary to remove inhibitors in other foods prior to concentration of HAV using PMNPs.

In conclusion, PMNPs can effectively concentrate HAV in cow milk without the need to remove any milk component (e.g., casein and fat). Compared with the existing methods, the developed technique is rapid, simple, inexpensive, and easy-to-perform without the need of any special infrastructure and can be used in laboratories where resources are limited.

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