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Evaluation of Porcine Gastric Mucin Magnetic Bead Enrichment for the Detection of Norovirus in Bivalve Mollusks

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Abstract **– Norovirus (NoV) represents one of the leading causes of foodborne illness worldwide. Standard methods for detection or quantification of NoV in bivalve mollusks are now available, but cannot discriminate between infectious and non-infectious virus particles. Based on the ability of intact NoV particle to bind to porcine gastric mucins conjugated to magnetic beads (PGM-MB), this study aimed to develop an enrichment method allowing to assess the infectivity of NoV particles in bivalve mollusks. To achieve this aim, we have investigated the effects of pH of binding buffer, amounts of PGM-MB and the use of additives on the recovery of NoV. The optimal parameters for the capture of NoV in modified Britton–Robinson buffer were pH 6.0; presence of 5% glycerol and 100 µl of PGM-MB. To avoid the negative effect of oyster matrix on the capture of NoV, oyster homogenate needs to be diluted three times in binding buffer. This optimal procedure allowed the detection of 2 × 10^2 NoV genome copies/ml in clean system and 5 × 10^3 NoV genome copies/g of oyster digestive tissues. The advantages of this new method are its simplicity and capability of assessing the infectivity of NoV particles.**

Keywords **– Bivalve Mollusks, Food Safety, Norovirus, Porcine Gastric Mucins.**

I. INTRODUCTION

Belonging to the family *Caliciviridae*, Norovirus (NoV) represents one of the leading causes of foodborne illness worldwide. According to a recent meta-analysis, it accounts for 18% of all cases of acute gastroenteritis worldwide [1]. In United States, it was estimated that 58% of foodborne illnesses are caused by NoV [2]. Among the foods at high risk of NoV contamination, bivalve mollusks and fruits are most commonly associated with foodborne outbreaks [3].

Standard methods for detection or quantification of NoV in bivalve mollusks are now available (ISO/TS 15216-1:2013 and ISO/TS 15216-2:2013). Nevertheless, these methods, based on extraction of viral RNA by guanidine thiocyanate and Real-time RT-PCR, cannot discriminate between infectious and non-infectious virus particles. Consequently, we could not rely on the Real-time RT-PCR results to confirm the presence of infectious NoV [4]-[5]. To assess the risk of infectious viruses in foods, several approaches have been developed including the use of Proteinase K and RNase pre-treatment, intercalators, and virus binding ligands [4]-[5]. The first step of NoV infection consists of the attachment of viral particles to cell surface through interaction between the viral capsid and human histo-blood group antigens (HBGAs) [6]. Therefore, HBGAs have been suggested to be a marker for NoV infectivity. However, due to ethical issues, it is difficult to use human HBGAs for the detection of infectious NoV in foods. Fortunately, HBGAs from porcine gastric mucins (PGM) were found to be able to bind to various genotypes of human NoV [7]-[8]. In addition, using PGM-conjugated magnetic beads (MB) allows concentrating NoV particles directly from food extracts, thus simplifying the detection procedure and increasing the sensitivity of the assays [7]-[8].

In this study, we evaluated the efficacy of PGM-MB for the detection of NoV in bivalve mollusks.

II. MATERIALS AND METHODS

A. NoV strain

NoV GII.4 strain was kindly provided by Dr. Elisabetta Suffredini (Department of Veterinary Public Health and Food Safety, Istituto Superiore di Sanità, Italy).

B. Preparation of Porcine Gastric Mucins-Conjugated Magnetic Beads (PGM-MB)

Porcine Gastric Mucins (Sigma-Aldrich) at a concentration of 20 mg/ml were desalted in Phosphate buffer (1.2 mM Na_2HPO_4, 15 mM NaH_2PO_4; pH = 7.4) by Zeba™ Spin Desalting Columns, 7K MWCO, 2 ml (Thermo Scientific). Carboxyl-Adembeads 300nm (Ademtech) was conjugated to desalted PGM following the manufacturer's protocol.

C. Enrichment of NoV

Enrichment of NoV in "Clean" System

To investigate the effects of pH, additives and quantity of PGM-MB on the recovery of NoV, we have used a "clean" system consisting of NoV GII.4 diluted in binding buffer which is a modified Britton–Robinson buffer with ionic strength of 0.15 M [9]. Firstly, PGM-MB were washed three times in 1.5 ml microcentrifuge tubes with one ml of binding buffer using a MagneSphere Magnetic Separation Stand (Promega). NoV GII.4, diluted in binding buffer, was then added to PGM-MB. The total volume of the mixtures was adjusted to 10 ml. After incubation at 4°C for one hour with agitation, samples were washed three times with one ml of binding buffer. The beads were then resuspended in 50 µl of RNA secure™ RNase Inactivation Reagent 1X (Ambion) and heated at 95°C for 5 minutes to release viral RNA. Subsequently, the supernatant containing viral RNA was transferred to a new 1.5 ml microcentrifuge tube and stored at -80°C for further analysis by Real-time RT-PCR.

To optimize the procedure, pH of binding buffer were varied from 4.0 to 9.0; two additives (5% glycerol and 10% Polyethylene glycol) have been used during the binding stage; and the quantity of PGM-MB were varied from 30 to 300 µl/reaction.

Enrichment of NoV from Pacific Oyster

Pacific oysters (uncontaminated with NoV), purchased from supermarkets in Hanoi, were dissected to collect digestive tissues. One gram of digestive tissues, spiked with a known amount of NoV, was then homogenized in 10 ml of homogenization buffer (100 mM Tris-HCl, 50 mM glycine, pH 9.5) by sonication. Homogenized samples were centrifuged at 10,000 × g for 30 minutes at 4°C and the supernatant was transferred to a new 50 ml centrifuge tube and its pH was adjusted to 6.0 by 1M HCl. To investigate the effect of oyster matrix on the recovery of NoV, the supernatant was diluted in binding buffer pH 6.0 at various levels ranging from non-diluted to tenfold. Subsequently, diluted samples were used to bind to PGM-MB as described above.

D. Quantification of Norovirus RNA by Real-time RT-PCR

Real-time RT-PCR assays were performed on a Master Cycler Realplex[4] system (Eppendorf) according to ISO/TS 15216-1:2013. Briefly, the RT-PCR master mix contained 5 µl of extracted RNA, 12.5 µl of 2X Reaction Mix (Superscript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase); 1 µl of Super Script® III RT/Platinum® Taq Mix (Superscript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase); 12.5 µM of QNIF2 primer (ATGTTCAGRTGGATGA-GRTTCTCWGA), 22.5 µM COG2R primer (TCGACG-CCATCTTCATTCACA), 6.25 µM of QNIFS probe (FAM-AGCACGTGGGAGGGCGATCG-TAMRA). Thermal cycling was then performed as follow: 1 cycle at 55°C for 60 min, 1 cycle at 95°C for 5 min, and 45 cycles at 95°C for 15 s, 60°C for 1 min, 65°C for 1 min.

III. Results and Discussion

A. Optimization of NoV Binding to PGM-MB in Clean System

In the pioneer work, Tian *et al*. found that NoV binding to PGM-MB was enhanced significantly at acidic pH conditions comparing to neutral and basic pH [10]. However, in this study, the authors investigated the effect of pH on NoV recovery only at three pH values of 3.6, 7.2, and 9.0. Therefore, in order to determine the optimal pH for NoV binding to PGM-MB, we have carried out the enrichment at more pH values ranging from 4.0 to 9.0. Real-time RT-PCR quantification of viral RNA extracted from PGM-MB-captured NoV at these pH conditions (Table I) showed that pH 6.0 is optimal for NoV recovery; therefore this pH was used for all subsequent experiments. Surprisingly, NoV recovery at acidic pH (0.25% at pH 4.0) was lower than that at neutral pH (1.25% at pH 7.0). The discrepancy in the pH effect on NoV recovery between the study by Tian *et al*. and our study might have been caused by the NoV strains or the types of the buffer used (citrate buffer and phosphate buffer in the study by Tian *et al*. and universal buffer in our study). Of note, NoV recoveries were relatively low because current analytical methods cannot quantify infectious NoV, therefore NoV recoveries were calculated based on the total amount of NoV in the samples, including both infectious and non-infectious virus particles.

Table I. Effect of pH on NoV recovery

pH	Average recovery ± SD (%)	Number positive/ Total number test
4.0	0.25[a]	½
5.0	2.12[a]	½
6.0	2.41 ± 0.23	2/2
7.0	1.25 ± 1.00	2/2
8.0	0.53 ± 0.48	2/2
9.0	NA	0/2

[a] One positive result out of two replicates
NA: None available

In order to improve the capture efficiency of NoV, we have investigated the effect of glycerol (5%) and PEG 8000 (10%) on the recovery of NoV. These compounds were found to enhance the recovery of *Campylobacter jejuni* by immunomagnetic separation (IMS) [11]. Furthermore, the addition of glycerol to samples before enrichment procedure decreases the non-specific interactions in IMS [12]. Used as a crowding agent, PEG was found to improve the performance of antibody-antigen reaction [13]. Results in Table II showed that addition of 5% glycerol improved significantly NoV recovery, whereas PEG 8000 affected negatively the capture of NoV. Consequently, in the subsequent experiments, glycerol (final concentration: 5%) was added to the samples before enrichment by PGM-MB.

Table II. Effect of PEG 8000 and Glycerol on NoV recovery

Additive	Average recovery ± SD (%)	Number positive/ Total number test
Control	1.57 ± 0.31	2/2
10% PEG 8000	NA	0/2
5% Glycerol	2.55 ± 0.22	2/2

NA: None available.

To determine the optimal amount of PGM-MB used in one reaction, capture of NoV was performed using various quantities of conjugated beads ranging from 30 to 300 µl. Results in Table III showed that the highest recovery was obtained when 100 µl of PGM-MB were used.

Table III. Effect of amount of PGM-MB on NoV recovery

Volume of PGM-MB	Average recovery ± SD (%)	Number positive/ Total number test
30 µl	2.48 ± 0.33	2/2
100 µl	3.15 ± 0.78	2/2
300 µl	2.19 ± 0.26	2/2

B. Sensitivity of the Optimized PGM-MB Enrichment Procedure in Clean System

To evaluate the sensitivity of the optimized enrichment procedure for the detection of NoV, different amounts of NoV were added to 10 ml of binding buffer at pH 6.0 containing 5% glycerol and 100 µl of PGM-MB. The

enrichment procedure was then conducted for these samples and NoV RNA was quantified by Real-time RT-PCR. Results in Table IV showed that the method still gave positive results even at the lowest amount of NoV (2×10^2 genome copies/ml). However, only one replicate was positive at the level of 5×10^2 genome copies/ml, suggesting that the limit of detection of the method is close to these concentrations.

Table IV. Sensitivity of the optimized PGM-MB enrichment procedure in clean system

Genome copies/ml	Average recovery ± SD (%)	Number positive/ Total number test
2×10^3	6.45 ± 0.07	2/2
10^3	2.20 ± 1.13	2/2
5×10^2	7.3[a]	½
2×10^2	15.53 ± 6.68	2/2

[a] One positive result out of two replicates

C. Effect of Oyster Matrix on NoV Recovery

The effect of oyster matrix has been assessed by performing enrichment from different dilutions of oyster extract in binding buffer (dilution range: non-diluted to tenfold). Results in Table V showed that the highest recovery of NoV was achieved when the dilution level was threefold. When oyster extracts were not diluted, NoV RNA was undetectable. It is likely that components in the oyster homogenate inhibit the binding of NoV particles to PGM-MB. In the subsequent experiments, oyster homogenates were diluted three times in binding buffer before enrichment by PGM-MB.

Table V. Effect of dilution levels of oyster homogenate on NoV recovery

Dilution level	Average recovery ± SD (%)	Number positive/ Total number test
Non-diluted	NA	0/2
1:3	1.75 ± 1.06	2/2
1:10	0.83 ± 0.30	2/2

NA: None available

D. Sensitivity of the Developed Procedure for the Detection of NoV in Pacific Oyster

To evaluate the sensitivity of the developed procedure for the detection of NoV in Pacific oyster, different amounts of NoV were spiked to one g of oyster digestive tissues, which were checked before for non-contamination of NoV by standard method. The extraction and enrichment were then conducted as described above and NoV RNA obtained was quantified by Real-time RT-PCR. Results in Table VI showed that this method could detect the presence of NoV even at the lowest amount tested (5×10^3 genome copies/g). However, only one replicate was positive at this level, meaning that the limit of detection of the developed method is likely near to this value.

Table VI. Sensitivity of the developed procedure for the detection of NoV in Pacific oyster

Genome copies/g	Average recovery ± SD (%)	Number positive/ Total number test
5×10^4	2.67 ± 0.23	2/2
2×10^4	2.05 ± 0.83	2/2
10^4	1.40[a]	1/2
5×10^3	2.40[a]	1/2

[a] One positive result out of two replicates

IV. Conclusion

In conclusion, based on the ability of PGM to bind to NoV, we developed a simple method for NoV enrichment and extraction of viral RNA from oyster samples. This method allows to assess the infectivity of NoV present in bivalve mollusks, requires only basic laboratory equipment and the whole procedure takes about two hours to complete.

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