Development of a Simple, Fast, and Cost-Effective Nanobased Immunoassay Method for Detecting Norovirus in Food Samples

Hani A. Alhadrami, Saleh Al-Amer, Yumna Aloraij, Fatimah Alhamlan, Raja Chinnappan, Khalid M. Abu-Salah, Shaihana Almatrrouk, and Mohammed M. Zouroob*

ABSTRACT: This study presents a quick, low-cost, and easy technique for the detection of norovirus in several food samples, including cucumber, lettuce, and chicken. The developed sandwich immunoassay method depends on employing nanotechnology for the detection step. Lactoferrin immobilized on activated Q-tips cotton swabs was used as a general capturing reagent to bind viruses from the sample surface. The cotton swabs were then submerged in a gold nanoparticle solution, which had previously decorated with a specific antibody for noroviruses. Positive samples retained the red color of the gold nanoparticles on the surface of Q-tips, even after washing, while the negative control samples easily lost their color through washing. The results confirmed that the developed assay has superior sensitivity and selectivity with a LOD between 10 and 53 pfu/mL for all tested samples. In light of the difficulty, complexity, and high cost of the methods recently used for detecting viruses in food samples, this method presents a promising reliable technique that can be employed for the rapid detection of norovirus in food samples with an acceptable accuracy.

1. INTRODUCTION

Recently, viruses have been reported as one of the major causes of foodborne illnesses. In the United States, viruses are responsible for 66.6% of food-related diseases compared with 9.7% caused by Salmonella and 14.2% caused by Campylobacter.1,2 Norovirus is one of the most well-known viruses causing foodborne diseases, being responsible for 45% recreational waterborne outbreaks followed by adenovirus, which is responsible for 24%.3,4 Norovirus was first recognized in 1968 in Ohio, United States, during the outbreak of a winter vomiting disease.5 Norovirus belongs to a genetically diverse group of single-stranded RNA nonenveloped viruses of the family called Caliciviridae. This group is subdivided into five subgroups: Norovirus, Vesivirus, Lagovirus, Sapovirus, and Nebovirus, with one or more species recognized in each genus. Noroviruses are responsible for acute gastroenteritis outbreaks around the world. In the United States, for instance, there are 5.5 million people who suffer from foodborne norovirus diseases annually,6 and in the United Kingdom, there are 600,000 cases of norovirus infections.7 Noroviruses are known to be resistant to many disinfectants, so they remain infectious for about 2 weeks on surfaces and for more than 2 months in water.8,9 Norovirus can be spread in different ways, including direct transmission from one person to another via feces or ingestion. Further, it can be transmitted indirectly through contaminated surfaces, water, or food.8 Norovirus is very dangerous and highly contagious because a very small dose, less than 10^2 particles/mL, is able to cause humans infection, and it is highly resistant to heat of up to 60 °C, acids, and antiseptics such as chlorine in tap water (up to 6.25 mg/L).10

Although there are many detection methods available for viruses in food, including norovirus, such as ELISA, electrophoresis, and chromatography, the molecular method remains the most commonly applied technique due to its high sensitivity and selectivity. The molecular detection of norovirus in food matrices by RT-PCR is a widely used method in research laboratories comprising three main steps: virus extraction, purification of RNA, and the molecular detection of RNA. Extraction methods used to isolate virus molecules from food matrix vary according to the biological composition of each matrix.11 There are three main categories of food matrices; the first one is water-based foods, including carbohydrates like fruits and vegetables, the second is protein-based, including fats (mainly ready-to-eat products and chicken meat), and the third category is shellfish, which contains viruses in their digestive systems. Virus extraction includes the elution of viral particles, the direct extraction of viral RNA from the food matrix, and the extraction of virus by proteinase K treatment. However, the sensitivity and the selectivity of this procedure is somehow complex, time...
consuming, and costly. In this study, we developed a new approach for detecting norovirus in vegetables and chicken meat by an immunoassay method, which is simple, fast, and cost-effective. This sandwich immunoassay technique uses Q-tips cotton swabs immobilized with lactoferrin as a general binding agent and gold nanoparticles decorated with a secondary antibody as a specific binding agent.

2. MATERIALS AND METHODS

Lactoferrin was purchased from Monojo (Amman, Jordan), colloidal gold nanoparticles (30 nm) and anti-norovirus specific antibodies GI Mab (clone NG28) and anti-norovirus GII Mab (clone NP8) were purchased from Atlas Medical Company (Amman, Jordan). Sodium periodate (NaIO4), sulfuric acid, phosphate buffer saline (PBS) buffer, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Cotton buds were purchased from the local market.

2.1. Activation of Cotton Swab. Polyhydroxyle groups of cellulose cotton were transformed to active aldehyde groups by sodium periodate oxidation (NaIO4). Sulfuric acid (H2SO4, 1 mL) and 2.4 g of NaIO4 were dissolved in 100 mL of water, mixed for 10 min, and the cotton swabs were submerged in the solution overnight. Cold distilled water was used to remove the excess oxidizing agent of the oxidized cotton. The formation of active aldehyde groups was confirmed from the shape of a characteristic peak at 1730 cm⁻¹ in the FTIR spectra.

2.2. Lactoferrin Immobilization on Cotton Swab. Lactoferrin (40 μL) was mixed with 2 mL of PBS buffer, and the activated cotton was immersed in the solution overnight at 4 °C. PBS buffer was used to extensively wash the lactoferrin-linked cotton to remove the unbound lactoferrin molecules. Active aldehyde groups were blocked with bovine serum albumin (BSA) and then washed with PBS. The lactoferrin-immobilized cotton swabs were stored in PBS until further use. Control samples were prepared using the same protocol; however, BSA (1 mg/mL) was used instead of lactoferrin.

2.3. Screening Assay Procedure. The norovirus screening procedure consists of two steps: the first is the virus capturing and collection and the second is the sandwich assay formation with the secondary antibody and color development. In this study, lactoferrin (LF) was employed as a universal recognition receptor as it can bind any virus in the sample. Lactoferrin was conjugated to the cotton swab, and the secondary detection antibodies (D-mAb) was attached physically to the gold nanoparticles to be used for the color development. In the first step, the cotton-immobilized lactoferrin was swabbed over the contaminated sample surfaces to capture the norovirus. The cotton-immobilized lactoferrin-virus complexes were then washed twice with PBS buffer to remove unbound viral particles.

In the second step (the detection step), the virus was sandwiched between the cotton-immobilized lactoferrin and secondary detection antibody conjugated with colored gold nanoparticles. As shown in Scheme 1, the cotton swab lactoferrin-virus (cotton-LF-virus) complexes were immersed for 2 min in a PBS solution of gold nanoparticles linked with the secondary antibody. The LF-virus secondary antibody (LF-virus-mAb-D) sandwich complex was washed with PBS buffer to remove the unbound particles. The color of the cotton swab indicates the specific viral strain exist on the contaminated surfaces.

3. RESULTS AND DISCUSSION

In this study, a simple sandwich immuno-based colorimetric method was evaluated for the detection of norovirus in chicken and two types of vegetable samples: lettuce and cucumber as common potential foodstuffs. They are more likely to be contaminated with the virus during harvesting, transportation, and storage processes.

3.1. Principle and Efficiency of the Assay. Samples (chicken, lettuce, and cucumber) artificially spiked with norovirus were swabbed using lactoferrin-immobilized cotton buds to allow the capturing of the virus and formation of LF-cotton virus particle complexes. Then, the cotton Q-tips were incubated with red-colored gold nanoparticles, which were previously decorated with a secondary antibody specific to norovirus. The combination of the LF-virus complex and gold nanoparticles immobilized with secondary antibodies leads to change in the color of the cotton swap to red. The linearity of the assay was evaluated in the range of 10⁻¹⁰⁵ pfu/mL, which was the same concentration series used to spike the samples.

The results showed a very high sensitivity and good linear regression between the virus concentration and color intensity in all samples, which can be explained by the high number of nanoparticles collected in turn. The detection limit of norovirus in this assay in both lettuce and cucumber samples was reported around 10 pfu/mL, as illustrated in Figures 1 and 2. The linearity and the sensitivity of the assay in chicken samples was not significantly different from that in vegetable samples, i.e., the detection limit of 53 pfu/mL, as presented in Figure 3. The limit of detection was as low as 10 pfu/mL for the cucumber and lettuce samples, but the color variation between different concentrations obtained from the lettuce samples was not as clear, as shown in Figure 2. This is because of the low intensity of the color in general and may be related to the physical characteristics of the cucumber surface, which is smoother than lettuce, so less virus particles may adhere to its surface than to that of lettuce samples. The correlation of the color intensity of a cotton swab with the concentration of virus particles captured in the three sample matrices was confirmed by establishing a relationship between integrated intensity values calculated by Photoshop software and concentration, as presented in Figure 4.

3.2. Comparison between the Developed Assay and Other Techniques. The newly developed technique resolves some of the many challenges of known techniques for the detection of norovirus. For instance, electron microscopy (EM) was the first tool used for detecting norovirus but requires a high viral load in the sample for analysis. However, the sensitivity can be improved by using immuno-EM as well as other techniques like traditional immunoassay, ELISA, and RT-PCR, which are nowadays the most widely used molecular techniques for detecting norovirus. The major disadvantage of ELISA and immunoassays is the low sensitivity, which falls
between $10^3$ and $10^6$ virus particles/g of the sample. Furthermore, the main limitation of using EM and RT-PCR is the fact that noroviruses are genetically highly diverse, and this complicates the design of protocols to detect multiple strain variants. In addition, inhibitors (humic acid, complex polysaccharides, microorganism debris, metal ions, and nucleases) in samples may inhibit the amplification process during RT-PCR. Table 1 highlights the main differences between the most widely used techniques for the detection of

**Table 1. Comparing the New Assay with some Other Techniques in Terms of Diversity, Simplicity, Complexity, and Cost**

| technique            | sensitivity | process simplicity/complexity | limitation                                                                 | cost   |
|----------------------|-------------|-------------------------------|---------------------------------------------------------------------------|--------|
| microscopy (EM)      | low         | simple                        | need high viral load in the sample                                        | high   |
| traditional immunosassay (ELISA) | low | complex | high diversity complicates the protocol, and need high viral load in the sample | high   |
| molecular (RT-PCR)  | good        | complex                       | high diversity complicates the protocol                                   | high   |
| nanobased immunosassay | good        | simple                        | cannot be used with colored liquid samples                                | low    |
norovirus in different samples and the advantages of the developed assay over the other techniques. The comparison of the study results with other studies shows that the developed assay has a superior sensitivity in terms of the detection limit. The limit of detection in a previous study using phage nanoparticle reporters in a lateral flow assay was $10^7$ virus particles/mL. In another study, the limit of detection using a three-dimensional paper-based slip device for one-step point-of-care testing was $9.5 \times 10^4$ copies/mL for human norovirus.

4. CONCLUSIONS
This study developed a simple, fast, easy, and cost-effective method for detecting norovirus in food samples. The developed technique relies on a sandwich immunoassay principle using lactoferrin immobilized on Q-tips cotton swabs as a general binding agent, which can bind many biological molecules, including bacteria, viruses, and DNA. The detection of the virus was achieved using gold nanoparticles, which had been previously immobilized with a specific antibody; the color change of the cotton Q-tips after immersion in the nanoparticle solution indicates positive results. The results indicate that the developed method is highly reliable for the detection of norovirus in various food samples, particularly, lettuce, cucumber, and chicken. The correlation between color intensity and concentration of the analyte in solution indicates that the assay can be used for quantification. Furthermore, the limit of detection was between 10 and $5 \times 10^4$ particles/mL in the samples tested, so this assay could be suitable for many applications in the food industry, food safety, and biodefense.

AUTHOR INFORMATION

Corresponding Author

Mohammed M. Zourob — Department of Chemistry, Alfaisal University, Riyadh 11533, Saudi Arabia; King Faisal Specialist Hospital and Research Center, Riyadh 12713, Saudi Arabia; orcid.org/0000-0003-2187-1430; Email: mzourob@alfaisal.edu

Authors

Hani A. Alhadrami — Faculty of Applied Medical Sciences, Department of Medical Laboratory Technology and Special Infectious Agent Unit, King Fahd Medical Research Centre, King Abdullah University, Jeddah 21589, Saudi Arabia

Saleh Al-Amer — Department of Chemistry, Alfaisal University, Riyadh 11533, Saudi Arabia

Yumna Aloraj — Department of Chemistry, Alfaisal University, Riyadh 11533, Saudi Arabia

Fatimah Alhamlan — King Faisal Specialist Hospital and Research Center, Riyadh 12713, Saudi Arabia; College of Medicine, Alfaisal University, Riyadh 11533, Saudi Arabia

Raja Chinnappan — Department of Chemistry, Alfaisal University, Riyadh 11533, Saudi Arabia

Khalid M. Abu-Salah — Department of Nanomedicine, King Abdullah Medical City, King Abdullah International Medical Research Center/King Saud bin Abdulaziz University for Health Sciences, Riyadh 11481, Saudi Arabia

Shaihana Almatrouk — National Health Laboratory, Ministry of Health, Riyadh 13354, Saudi Arabia

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c00502

Author Contributions

H.A.A. and S.A.-A. have equal contribution.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding

This project was funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, under grant no. G-1267-142-1440. The authors, therefore, acknowledge with thanks, to the DSR for technical and financial support.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Mead, P. S.; Slutsker, L.; Dietz, V.; McCaig, L. F.; Bresee, J. S.; Shapiro, C.; Griffin, P. M.; Tauxe, R. V. Food-related illness and death in the United States. Emerging Infect. Dis. 1999, 5, 607–625.

(2) Vasicova, P.; Dvorska, L.; Lorencova, A.; Pavlik, I. Viruses as a cause of foodborne diseases: a review of the literature. Vet. Med. 2005, 50, 89–104.

(3) Sinclair, R. G.; Jones, E. L.; Gerba, C. P. Viruses in recreational water-borne disease outbreaks: a review. J. Appl. Microbiol. 2009, 107, 1769–1780.

(4) La Rosa, G.; Fratini, M.; Spuri Vennarucci, V.; Guercio, A.; Purpari, G.; Muscillo, M. GIV noroviruses and other enteric viruses in bifalves: a preliminary study. New Microbiol. 2012, 35, 27–34.

(5) Adler, J. L.; Zickl, R. Winter vomiting disease. J. Infect. Dis. 1969, 119, 668–673.

(6) Hall, A. J. Noroviruses: The perfect human pathogens? J. Infect. Dis. 2012, 205, 1622–1624.

(7) Wheeler, C.; Vogt, T. M.; Armstrong, G. L.; Vaughan, G.; Weltman, A.; Nainan, O. V.; Dato, V.; Xia, G.; Waller, K.; Amon, J.; Lee, T. M.; Highbaugh-Battle, A.; Hembree, C.; Evenson, S.; Ruta, M. A.; Williams, I. T.; Fiore, A. E.; Bell, B. P. An outbreak of hepatitis A associated with green onions. N. Engl. J. Med. 2005, 353, 890–897.

(8) Seitz, S. R.; Leon, J. S.; Schwab, K. J.; Lyon, G. M.; Dowd, M.; McDaniels, M.; Abdulhafid, G.; Fernandez, M. L.; Lindsmith, L. C.; Banic, R. S.; Moe, C. L. Norovirus infectivity in humans and persistence in water. Appl. Environ. Microbiol. 2011, 77, 6884–6888.

(9) Pathak, G. W.; Sobsey, M. D. Simultaneous comparison of murine norovirus, feline calicivirus, coliphage MS2, and GI-4 norovirus to evaluate the efficacy of sodium hypochlorite against human norovirus on a fecally soiled stainless steel surface. Foodborne Pathog. Dis. 2011, 8, 1005–1010.

(10) Neethirajan, S.; Tuteja, S. K.; Huang, S.-T.; Kelton, D. Recent advancement in biosensors technology for animal and livestock health management. Biosens. Bioelectron. 2017, 98, 398–407.

(11) Baert, L.; Wobus, C. E.; Van Coillie, E.; Thackray, L. B.; Debevere, J.; Uyttendaele, M. Detection of murine norovirus 1 by using plaque assay, transfection assay, and real-time reverse transcription-PCR before and after heat exposure. Appl. Environ. Microbiol. 2008, 74, 543–546.

(12) Alamer, S.; Eissa, S.; Chinnappan, R.; Zourob, M. A Rapid colorimetric immunoassay for the detection of pathogenic bacteria on poultry processing plants using cotton swabs and nanobeads. Microchim. Acta 2018, 185, 164.

(13) Leval, P. F.; Viljoen, M. Lactoferrin: A General Review. Haematologica 1995, 80, 252–267.

(14) Alamer, S.; Eissa, S.; Chinnappan, R.; Herron, P.; Zourob, M. Rapid colorimetric lactoferrin-based sandwich immunoassay on cotton swabs for the detection of foodborne pathogenic bacteria. Talanta 2018, 185, 275–280.

(15) Zheng, D.-P.; Widdowson, M.-A.; Glass, R. I.; Vinjé, J. Molecular epidemiology of genogroup II–genotype 4 noroviruses in the United States between 1994 and 2006. J. Clin. Microbiol. 2010, 48, 168–177.