Broad-range and Effective Detection of Human Noroviruses by Colloidal Gold Immunochromatographic Assay Based on the Shell Domain of the Major Capsid Protein

Meng Xu
Shanghai Jiao Tong University
Feifeng Lu
Shanghai Jiao Tong University
Qingping Wu
Guangdong Institute of Microbiology
Jumei Zhang
Guangdong Institute of Microbiology
Peng Tian
US Department of Agriculture
Liang Xue
Guangdong Institute of Microbiology
Chenang Lyu
Shanghai Jiao Tong University
Ting Xu
Shanghai Jiao Tong University
Dapeng Wang (norovirus@163.com)
Shanghai Jiao Tong University

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Abstract

Background: Human noroviruses (HuNoVs) are a major cause of nonbacterial gastroenteritis in all ages worldwide. As the replication of HuNoVs *in vitro* is immature, the detection of HuNoVs is depended on molecular assays such as RT-PCR and RT-qPCR. However, these molecular-based techniques require special equipment, unique reagents, experienced operators to perform, and extended time to get results. In addition, the diversity of viral genotypes is high. Therefore, a method for rapidly, broad-range, and effective detectionf of HuNoVs was desiderated for screening the excrement or vomit from infected people when outbreaks occur.

Results: In this study, a colloidal-gold-based immunochromatographic assay (ICA) was developed for highly effective detection of HuNoVs in clinical samples. Monoclonal antibodies (MAbs) against the shell (S) domain in the major capsid protein of HuNoVs were used in the ICA. The limitations of detection for HuNoVs in clinical samples were 1.2×10^6 genomic copies per gram of stool sample (gc/g) and 4.4×10^5 gc/g for genogroup I and II (GI and GII) HuNoVs, respectively. A total of 122 clinical samples were tested for HuNoVs by ICA and compared against that by RT-qPCR. The relative sensitivity, specificity and agreement of the ICA was 84.2 % (95% CI: 83.6-84.8 %), 100.0 % (95 % CI: 98.5-100.0 %) and 87.7 % (95% CI: 85.6-89.8 %), respectively. No cross-reaction with other common enteric viruses or bacteria was observed. The ICA could detect a broad range of genotypes, including GI.1, GI.3, GI.4, GI.6, GI.14, GII.2, GII.3, GII.4, GII.6, GII.13, and GII.17 HuNoVs.

Conclusions: Our results demonstrated that ICA targeting the S domain of VP1 is a promising candidate for effectively improve identifying different genotypes of HuNoVs in clinical samples with high sensitivity and specificity.

Background

Human noroviruses (HuNoVs) are single-stranded RNA, non-enveloped viruses in the *Caliciviridae* family. The genome has three open reading frames (ORFs, 1 through 3), where ORF2 encodes a major capsid protein, VP1. VP1 consists of a shell (S) domain and a protruding (P) domain. The S domain, the most highly conserved region in VP1, forms a shell surrounding the RNA genome, while the P domain of VP1 contains the most variable sequence [1-3]. HuNoVs are divided into 5 genogroups based on the VP1, including genogroup I (GI), II (GII), IV (GIV), VIII (GVIII), and IX (GIX) [4]. GI and GII HuNoVs are the epidemic strains circulating worldwide [5].

HuNoVs are the leading cause of epidemic gastroenteritis in the world [6-8]. The viral diseases are attributed to about 700 million infections and 200 thousand deaths globally, annually [9, 10]. HuNoVs are highly-infectious, and infected patients can shed high titers of virus particles with strong resistance to the environmental factors [11, 12]. As cell culturing *in vitro* is still immature to replicate HuNoVs effectively [13], the detection of the virus mainly depends on the molecular approaches such as RT-PCR and RT-qPCR [14, 15]. These molecular approaches require specialized equipment, unique reagents, and

significant time for sample preparation and assay execution, all of which make them impractical for either clinical use or on-site assays of field samples requiring quick results. To efficiently control the spreading of HuNoVs in time, a highly effective, safe, and portable point-of-care assay is of great commercial and academic interest in monitoring the occurrence of outbreaks.

An immunochromatographic assay (ICA) is straightforward to screen samples for the presence of a target analyte, such as pathogens or biomarkers [16, 17]. A schematic diagram of ICA was shown in Fig. 1. In an ICA test, an antibody against the viral capsid is labeled with gold particles. In the process of chromatography, the viral capsid is captured by the labeled antibody and another antibody coated on the solid-phase carrier, so that virus-antibody-colloidal gold particles combinations are also aggregated and show positive red results. ICAs are designed to be used without any equipment and require minimal training to do. The results can be read visually within a couple of minutes. Currently, several ICAs used for the detection of bacterial pathogens and viral pathogens had been reported [18, 19]. However, many limitations of the ICA kits in HuNoVs detection were obvious, such as the detection of only specific genotypes of HuNoVs and the low sensitivity [20]. Therefore, a broad-range and highly effective ICA was needed for point-of-care assay of HuNoVs.

Li et al. reported a MAb against S domain of VP1 could cross-react with GI, GII, GIII, and GV of noroviruses (NoVs) [21]. Yoda et al. reported that two MAbs generated against the S domain of GII HuNoVs capsid protein also recognized the viral protein of GI HuNoVs [22]. In addition, Parra et al. identified a broadly cross-reactive epitope in the S domain of the NoVs capsid [2]. Therefore, the S domain of VP1, highly conserved, is a good candidate for detecting multiple genotypes of HuNoVs. However, few immunoassays were used to detect the HuNoVs targeting the S domain of VP1. In this study, three MAbs were generated against the S domain of VP1 from a GII.4 HuNoV and used to develop an ICA for broad and effective detection of both GI and GII HuNoVs.

Results

Selection of MAbs that recognize different epitopes on S domain of VP1 for ICA

Selection MAbs with different epitopes on S domain of VP1 could enhance the sensitivity and specificity of the assay. Three MAbs were selected and named H9E, B4H, and J5D, further abbreviated as H, B, and J, respectively. The values of displacement factor (*I*) of B-H, H-B, B-J, J-B, H-J and J-H combinations determined by an ELISA assay was 32.8 %, 28.9 %, 34.1 %, 29.6 %, 31.2 % and 22.7 %, respectively. *I* value greater than 10.0 % indicates that two MAbs recognizes different epitopes on the S domain of VP1. Among the *I* values of six pairs, B-J had the best matching effect. The results were further tested by the ICA assay to find the best combination of MAbs binding to gold particles (Table 1). Finally, H was selected as the antibody for the labeled colloidal gold, and J as the T-line capture antibody as double plus were shown in both test line and control line.

Table 1 Results obtained from different combinations of the three antibodies on the colloidal gold platform

	Colloidal-gold MAb	Test line MAb	Test line	Control line
BH:	B4H	H9E	+	+
HB:	H9E	B4H	++	+
BJ:	B4H	J5D	+	++
JB:	J5D	B4H	++	+
HJ:	**H9E**	**J5D**	**++**	**++**
JH:	J5D	H9E	+	+

"+" represented the color depth. The score was evaluated by multiple operators with hidden labels.

Pretreatment methods to expose S domain of VP1 in the clinical samples

Unlike the P domain of VP1, S domain of VP1 was hided inside of viral capsid. Two methods were tested for exposing the S domain of VP1 in clinical samples and to inactivate the virus. Under the temperature from 60 °C to 80 °C, the OD readings were gradually increased (roughly from 0.2 to 1.0). However, the OD readings were dramatically reduced when the temperature was greater than 90 °C (less than 0.2). A 3-minute at 80 °C treatment obtained the best result for heat-treatment (Fig. S1, see additional file 1). A progressive improvement of detection was displayed when pH was increased from 7.0 to 9.0 (optimal at pH=9.0, consistent with the best effect of heat treatment) (Fig. S2, see additional file 1). Better than heat-treatment, OD readings in alkali treatment samples were easy to control and stable over the time tested since there was no significant difference between the best 10-minute and the worst 5-minute treatment ($p = 0.131 > 0.05$). For simplicity and repeatability of the experimental operation, alkali treatment (samples were treated to pH=9.0 at room temperature for 10 min) was used to expose S domain of VP1 in all clinical samples before testing by ICA.

The sensitivity and specificity of ICA

The sensitivity of ICA was reflected by the limit of detection (LOD). LOD of ICA was determined by applying serially diluted HuNoVs on ICA strip. The results were judged by visualization of both test line and control line. The C_t data and the copies were recorded in Table S1 (Additional file 2). The visual LOD of the ICA for the determination of S domain of VP1 was 1.4 ng/ml. The LODs of viral genomic copies of

sample 57404 (GI) and 1717 (GII) were 1.2×10^6 gc/g and 4.4×10^5 gc/g, respectively. The sensitivity test was performed in triplicate, and representative results were shown in Fig. 2.

The specificity of ICA was determined in a set of clinical samples with HuNoVs and other pathogens; and compared with results from RT-qPCR. When clinical samples were detected by ICA, only samples containing HuNoVs showed positive results. Clinical samples containing other enteric viruses (4 Rotaviruses, 3 Sapoviruses, 2 Astroviruses, and 4 Adenoviruses) or bacteria causing gastroenteritis (3 *Salmonellas*) were all tested negative. Parts of the results were shown in Fig. 3. The comparison results of ICA and RT-qPCR were shown in Table 2. Eighty samples were positive by ICA from 95 RT-qPCR HuNoVs positive samples. The sensitivity was 84.2 % (95% CI: 83.6-84.8 %) (80/95). No false-positive results indicated a specificity of 100.0 % (95% CI: 98.5-100.0 %) (27/27). The overall agreement of ICA and RT-qPCR was 87.7 % (95% CI: 85.6-89.8 %) (107/122). In addition, the titers of HuNoVs in 15 negative samples (1 GI.6, 1 GII.3, 12 GII.4, and 1 GII.6) were shown in Table S2 (Additional file 2). The viral loads of 15 ICA false-negative samples were below the LOD of ICA.

Table 2 Comparison of HuNoVs detection in stool samples between the ICA and the RT-qPCR

		ICA		
		Positive	Negative	Total
RT-qPCR	Positive	80	15	95
	Negative	0	27	27
	Total	80	42	122

Sequencing results indicated that GII.4 was the dominant genotype (72/95) followed by GI.1 (4/95) in RT-qPCR HuNoV-positive samples. Other genotypes were GI.3 (2/95), GI.4 (3/95), GI.6 (2/95), GI.14 (1/95), GII.2 (2/95), GII.3 (3/95), GII.6 (3/95) GII.13 (1/95), and GII.17 (2/95) (Table 3). The ICA detected both GI and GII HuNoVs, including GI.1, GI.3, GI.4, GI.6, GI.14, GII.2, GII.3, GII.4, GII.6, GII.13, and GII.17 genotypes. All 11 genotypes could be detected by our ICA. In addition, the 15 false-negative samples were missed because of the low titer according to the above described.

Table 3 Detection results of genotypes by ICA

HuNoVs genotypes	Positive number in RT-qPCR	Number in ICA		Sensitivity (95 % CI)
		Positive	Negative	
GI.1	4	4	0	
GI.3	2	2	0	
GI.4	3	3	0	
GI.6	2	1	1	
GI.14	1	1	0	
(GI total)	**(12)**	**(11)**	**(1)**	**91.7 % (88.6-94.8)**
GII.2	2	2	0	
GII.3	3	2	1	
GII.4	72	60	12	
GII.6	3	2	1	
GII.13	1	1	0	
GII.17	2	2	0	
(GII total)	**(83)**	**(69)**	**(14)**	**83.1 % (80.0-86.3)**
Total	**95**	**80**	**15**	

Stability of ICA strips

Results demonstrated that acceptable activity (90.0 %) of the initial activity was retained for 21 days when the test strip was exposed at 60 °C. We expect that the shelf-life of ICA strips should be stable more than 21 days at room temperature.

Discussion

HuNoVs are one of the most influential foodborne pathogens in the world [23]. The burden of HuNoV outbreaks is high, reflected in the extensive infectivity and severe economic losses [24]. Acute gastroenteritis outbreaks associated with HuNoVs are particularly challenging to control because of their stability in the environment and efficient transmission in hospitals, hotels, schools, and domestic homes [25-27]. Therefore, a broad and highly effective assay is needed to identify the HuNoVs for issuing emergency treatment.

Fecal specimens from patients could contain up to 10^9 gc/g of HuNoVs [28]. It is highly risky for the operators. Therefore, the inactivation of the viral particles in clinical samples is crucial before detection.

To address this need, physical and chemical treatments were evaluated in this study. Our results indicated that the capsid was greatly disassembled after treating by alkaline (pH 9.0 for 10 min) and heating (70.0 °C for 3 min). The alkali treatment was much better than heat treatment in terms of the stability of the results and the simplicity of operation. More important, the alkali environment also kept the colloidal gold in a stable state to bind MAbs.

VP1 proteins (P plus S domains) could self-assemble to form virus-like particles (VLPs) which morphologically and antigenically resemble the viral particles [29, 30]. The protruding (P) domain of VP1 is highly immunogenic similar to VLP [31]. The antigenicity of VLP and P particles is often strain-specific and is not suitable for virus screening. On the other hand, the S domain of VP1 in HuNoVs is highly conserved [7]. It was reported that two MAbs (1B4 and 1F6) of S domain of VP1 against the capsid protein (NoV GII) were also found to react with NoV GI [22, 32]. In this study, MAbs against S domain of VP1 were prepared to develop an ICA kit, which recognized VP1 from either GI or GII HuNoVs. Moreover, the results demonstrated that our kit had good sensitivity and specificity for detection of both GI and GII HuNoVs (Table 3).

The epitopes on the S domain of VP1 were hidden in the inner layer of viruses. They could not be recognized by the antibodies against S domain of VP1 in the intact viral particles. It is necessary to expose S domain of VP1 for virus detection. In our ICA assay, the viral particles were disassembled by alkaline treatment and exposed S domain of VP1. It is an excellent way to minimize the risk of infection and much safer for testers than detection without inactivated samples. In addition, each viral particle contains 90 dimers of viral capsid [33]. After treatment, in theory, many more copies of S domain of VP1 were exposed to the MAbs, and the sensitivity of the immunoassay could be improved. Therefore, it was a one-stone-two-bird solution. As limited research was done on antigenicity of S domain of VP1, it will be interesting to identify the epitopes of S domain of these MAbs in later study.

The current molecular assays require special equipment, reagents, and experienced personnel. A simple approach with minimal hands-on time is more applicable and requisite as a screening assay. There were several commercial immunoassays for HuNoV detection available, such as Quick Ex-Norovirus, RIDA® QUICK (N1402), Immuno Search NV kit, NOROTOP+, and SD Bioline Norovirus [34-40]. However, these assays performed better for a single genotype of HuNoVs. To detect multiple strains of HuNoVs, a couple of MAbs were selected for cross-reactions with various genotypes. A simple approach is urgently needed for broad-range genotypes of HuNoVs.

It did report that IAC was used for the detection of NoVs. IP-NoV kit and the kit developed by Takanashi was mainly for GII.3 and GII.4 HuNoVs [41-43]. Immuno Search NV kit was developed for several genotypes (GI.1, GI.11, GII.2, GII.3, GII.4, GII.5, and GII.6 VLPs), but the limit of detection was not shown [29]. SD Bioline Norovirus could detect GI.3 for GI but except GII.2, GII.6, and GII.16 for GII HuNoVs [31, 32]. Limited genotypes of HuNoVs were detected by those kits. It is noteworthy that our ICA could detect a much broader range of genotypes (including GI.1, GI.3, GI.4, GI.6, GI.14, GII.2, GII.3, GII.4, GII.6, GII.13 and GII.17) (Table 3).

Compared to RT-qPCR, the sensitivity, specificity and agreement of our ICA were 84.2 % (95% CI: 83.6-84.8 %), 100.0 % (95% CI: 98.5-100.0 %) and 87.7 % (95% CI: 85.6-89.8 %), respectively (Table 2). The overall results were competitive with other commercial kits. The sensitivity (84.2%) of our ICAs was higher than IP-NoV kit (ranged from 72.7 % to 78.9 %), Quick Ex-Norovirus (54.5 %), RIDA® QUICK (from 68.8 % to 82.5 %), Immuno Search NV kit (75.4 %) and NOROTOP+ (51.4 %) (See Table S3, Additional file 3). In addition, Rotavirus, Sapovirus, Astrovirus, Adenovirus and *Salmonella* are the most common non-norovirus viral and bacterial pathogens that cause gastroenteritis in China [44]. None of the 16 specimens were tested positive in the evaluation of specificity in our study. The identification of the result from an ICA was depended on visualization of both test line and control line. We will improve the assay in the future study.

These performance characteristics of ICA kits were dependent on the tested individual and the titer of HuNoVs in the sample. There were several possibilities for false-negative results, including lower viral titer in the tested clinical samples and sensitivity of the MAbs selected in ICA tests. In our study, 15 false-negative samples had viral titers below the LOD. In the future, the LOD of our kit should be improved by quantum dot or signal amplification system. We believe the ICA targeting S domain of VP1 is a promising approach for screening HuNoVs from clinical samples, especially treating the outbreaks.

Conclusions

In this study, an ICA targeting the S-domain of the HuNoV VP1 for the broad-range detection of HuNoVs was developed. This assay was able to reduce detection times and achieve a sensitivity of 84.2 % (95% CI: 83.6-84.8 %) and a specificity of 100.0 % (95% CI: 98.5-100.0 %), which is competitive to other immunological kits without using special equipment and reagents. We believe this assay represents a promising approach for screening HuNoVs from the field and clinical samples.

Methods

Preparation of VP1s and S domain of VP1

HuNoV GI.1 and GII.4 ORF2 gene fragments (GenBank Nos. M87661 and KM114291, respectively) were subcloned separately into the pET-28a prokaryotic expression plasmid. HuNoV GI and GII VP1 proteins were expressed from respective recombinant plasmid vectors pET28a-ORF2 GI.1 and GII.4 in *Escherichia coli* (*E. coli*) BL21 cells [45].

HuNoV GII.4 ORF2 (GenBank No. KM114291) was used as a template to amplify the nucleic acid fragment of the S domain. The upstream primer was 5'-GAATTCATGAAGATGGCGTCGAGTG-3'; the downstream primer was 5'- CTCGAGCTCAACTGTGGGTGGCAC-3'. *Eco*R I and *Xho* I restriction sites were appended to the 5’ ends of forward and reverse primers, respectively. After amplification and digestion, the nucleic acid fragment of the S domain was inserted into a pSmart vector (Frdbio, China) to generate

recombinant pSmart-S. The recombinant S domain of VP1 was induced and expressed, as described in the previous report [46].

Preparation of anti-S domain of VP1 MAbs

The MAbs against S domain of VP1 were prepared as we previously described [47]. Three MAbs (H9E, B4H, and J5D, further abbreviated as H, B, and J, respectively) were selected for their ability to bind pSmart-S expression product, but not with *E. coli* residual proteins. These three MAbs also demonstrated specificity against both GI and GII HuNoV VP1 via Western Blot.

Selection of MAbs that recognize different epitopes of S domain of VP1 by ELISA assay

Each reaction of the ICA assay utilizes a labeled MAb and another immobilized ("capture") MAb. As the conditions for labeled MAb (40 μg/ml) and the immobilized Mab (2.0 mg/ml) were different, all six pair permutations of the three MAbs (B-H, H-B, B-J, J-B, H-J, and J-H) were tested respectively. First, the three MAbs (B, H, and J) were two-fold serially diluted and tested for their single epitope saturation concentrations using recombinant S domain of VP1 (100.0 μg/well). A concentration one step lower than the concentration with a significant decrease in OD_{450} value was defined as a single epitope saturation concentration and named as B_1 (or H_1, J_1). Then S domain of VP1 (100.0 μg/well) was coated overnight at 4 °C, and a 100.0 μl epitope-saturated MAb (e.g., B) was incubated at 37 °C for 1 h. After washing, 100.0 μl second saturated MAb (e.g., H) in the pair tested was incubated under the same conditions. OD_{450} was recorded as BH_2. Displacement factor (*I*) was defined as the ratio of the increased effect of cross-reactivity over the separate effect of the second added antibody. If $I > 10$ % [48], it indicates that the recognition sites of the two MAbs are distinct. *I* values were calculated according to the formula $I_{(e.g., BH)} = (BH_2 - B_1)/H_1 \times 100$ %.

Preparation of the colloidal gold labeled MAbs

Colloidal gold particles with a mean particle diameter of 25.0 nm were produced under the following procedures. One hundred ml 0.01% (w/v) chloroauric acid ($HAuCl_4$) (Aladdin, Shanghai, China) was boiled thoroughly for 3 min. Then 2.0 ml 1.0 % (w/v) sodium citrate (Aladdin, Shanghai, China) was added quickly into the solution on a magnetic stirring apparatus over 30 min. The color changed gradually from yellow to black–blue and finally brilliant red. After stirring for a few minutes at low speed, the colloidal gold suspension was rested to cool down and stored in the dark case at room temperature. Total volume was made up to the original volume (100.0 ml) by adding ultrapure water. To measure the size and size distribution of these gold nanoparticles, the colloidal gold solution was scanned under a transmission electron microscopy (Tecnai G2 spirit Biotwin, USA) at 120 KV. The OD value of the colloidal gold solution was measured at 400-680 nm using an ultraviolet spectrophotometer (Tecan Sunrise, Switzerland).

Both physical and chemical crosslinking methods have been used for conjugation of gold colloids and MAbs [49]. Chemical crosslinking is more stable but the functional groups in MAb might be impacted. All functional domains are maintained when MAbs conjugated by physical method [50]. In this study,

physical method was adapted to make the conjugation [49]. The optimal pH, dose and concentration of BSA for conjugation of gold colloids and MAbs were evaluated (Fig. S3 and Table S4-6, Additional file 4-5). Briefly, 200.0 μl of a MAb (i.e.H9E) was incubated with 0.5 ml of colloidal gold (pH 9.0) for 30 min at room temperature with stirring gently. Then, 50.0 μl bovine serum albumin (BSA, Amresco, United States) of different final concentration were dripped into the colloidal gold as the blocking buffer to stabilize the gold-labeled antibody. After incubated for 15 min, the colloidal gold-antibody complex was collected in pellet and unmarked antibodies were remained in supernatant by a centrifugation at 8000 ×g at 4 °C for 20 min. Centrifugal process should be avoided the presence of black massive deposits on the wall of the tube. Then, the conjugated colloidal gold-antibody was finally resuspended to 50.0 μl dissolution buffer pH 9.0 PBS containing 10.0 % w/v sucrose (Sangon Biotech, Shanghai, China), 0.2 % (w/v) PVA-205 (Aladdin, Shanghai, China), 0.2 % (v/v) Tween-20 (Aladdin, Shanghai, China) and BSA (3.0 %, 2.5 %, 2.0 %, 1.5 %, 1.0 % and 0.5 %, w/v), respectively. The conjugation was confirmed by UV-vis spectroscopy using the same method as unlabeled gold particles. Ultimately, 50.0 μl of colloidal gold-antibody mixture was dispensed evenly on the 0.5 cm×2.5 cm conjugated pad and was dried for 3 h at room temperature.

Selection of MAbs used on the colloidal gold platform

The performance of ICA depended critically upon the combination of the optimal antibody sandwich pair with colloidal gold. ELISA results needed to be considered in conjunction with ICA testing. The capture antibody on the Test line (T line) and the control antibody (goat-anti-mouse Ig G, Beyotime Biotech, Shanghai, China) on the Control line (C line) (Fig. 1) was both 2.0 mg/ml, and the labeled antibody was exceeded (40 μg/ml). The concentration of S domain of VP1 used in the test was 0.3 μg/ml. The final selection was based on the combination of the color effect of both C and T lines.

Source of HuNoVs clinical samples (including suspected HuNoVs)

A total of 122 fecal specimens were collected and tested. Five HuNoVs clinical samples [57404 (GI.1), 3010 (GI.1), 1704 (GII.4), 1717 (GII.4), 1028 (GII.4)] were kindly provided by Dr. Ningbo Liao (Zhejiang Provincial Center for Disease Control and Prevention (CDC)). Total 117 clinical diarrheal samples which kindly provided by the Affiliated Hospital of Guangzhou Medical University (3), the Affiliated Hospital of Sun Yat-sen University (7), Chinese CDC (39) and Anhui Provincial CDC (68) were also tested by both RT-qPCR and our ICA. Clinical samples provided by Zhejiang Provincial and Chinese CDC were from patients with acute gastroenteritis in 2018. The samples of Anhui provincial CDC and Affiliated Hspital of Guangzhou Medical University were all in 2017. The samples of the Affiliated Hospital of Sun Yat-sen University were from 2015 to 2017.

Detection of HuNoVs by RT-qPCR and calculation of viral genomic copies

Real-time RT-PCR was performed using a commercial one-step RT-qPCR kit (Sangon Biotech, China), consisting of 12.5 μl 2 × one-step RT-qPCR Master Mix (with SYBR Green), 0.65 μl RT enzyme Mix, 0.4 μl for each primer (0.16 μmol/l) (See Table S7, Additional file 6) [51]. RNA template (2.0 μl) and RNase free ddH_2O were added to a total volume of 25.0 μl. Amplification reactions were as follows: reverse

transcription at 50 °C for 30 min; heat-denatured at 95 °C for 3 min; 40 cycles with denaturation at 95 °C for 10 s, annealing, and extension at 60 °C for 30 s. Fluorescence signals were collected at the end of each extension step. The highest dilution of real-time RT-PCR to generate a positive cycle threshold (C_t) signal was one real-time RT-PCR unit (RT-qPCRU) [14]. C_t values versus $\log_{10}$ viral genomic copies linear standard curves were generated from a continuous 10-fold dilution (See Fig. S4, additional file 7). Amplified DNA fragments were sequenced on ABI 3730XL at Personalbio (Shanghai, China). Automated genotypes were analyzed with Norovirus Typing Tool Version 2.0 (www.rivm.nl/mpf/norovirus/typingtool).

Conditions for exposing S domain of VP1 from viral capsid in clinical samples

Pretreatment comprises of physical or chemical methods. Heat-denaturation was used for the physical treatment. Briefly, 10.0 % (w/v) fecal sample diluted with PBS was put in a water bath at a set temperature (60 °C, 70 °C, 80 °C, 90 °C, 100 °C) for 1 to 5 min with an interval of 1 min. The reducing agent (DTT) (Thermo Fisher, China) and alkaline were used for chemical treatment. The pH in PBS was adjusted to 6.0 to 10.0. Fecal samples were dissolved to 10.0 % with PBS buffers (pH 6.0, 7.0, 8.0, 9.0 or 10.0) at room temperature (25 °C) for 5, 10, 15, 20, 25 and 30 minutes. DTT was added to the homogenized solution of clinical sample at a final concentration of 1.0 %. A sandwich ELISA was undertaken to detect HuNoVs, as described previously [52].

Performing an ICA test

The procedure of preparing ICA strips was described in the Additional file 8. The sample preparation process was as follows: 10.0 % (w/v) homogenized solution of stool slurries was prepared with PBS (pH 9.0) (10,000×g, 10 min). DTT was added to a final concentration (1.0 %). After 10 minutes at 25 °C, 50 µl mixtures were added to the sample pad. If enough HuNoVs antigens were present, the binding of the gold-labeled antigen complex occurred at both the T and C lines. The presence of C line confirmed that the test was valid or not.

Limit of detection (LOD) of ICA for purified S domain of VP1 and clinical samples

Purified S domain of VP1 was used at a concentration of 22.4 ng/ml, 11.2 ng/ml, 5.6 ng/ml, 2.8 ng/ml, 1.4 ng/ml and 0.7 ng/ml. PBS buffer was used as blank control. Two HuNoVs positive samples (57404 GI.1 and 1717 GII.4) were two-fold diluted with a range of 1.6×10^5 to 5×10^6 gc/g (GI) and 1.1×10^5 to 3.5×10^6 gc/g (GII). All analyses were conducted in triplicate to determine the repeatability of the results.

Evaluation of the specificity of ICA

To further evaluate the specificity of antibodies in the colloidal gold test, 4 Rotavirus, 3 Sapovirus, 2 Astrovirus and 4 Adenovirus (stool sample provided by Zhejiang CDC, the affiliated Hospital of Guangzhou Medical University and the affiliated Hospital of Sun Yat-sen University) and 3 *Salmonella*

(ATCC 14028, CMCC 50115 and CICC 21482) were tested. The copies of Rotavirus, Sapovirus, Astrovirus, and Adenovirus RNA were more than 10^7 gc/g feces.

Stability test

To determine the stability of ICA strips, they were examined by thermal acceleration tests at 60 °C [53]. The activity of the antibodies on each assay was tested by using the lowest detectable S domain of VP1 concentration (1.4 ng/ml). The identical strips were tested at appropriate intervals (1, 2, 3, 7, 14, 21, and 28 days). The shelf-life could be estimated at room temperature.

Statistical analysis

One-way ANOVA was used for data analysis. SPSSAU, an online data analysis tool, was used for statistical analysis (www.spssau.com).

Abbreviations

CI: confidence interval; ATCC: American type culture collection; CMCC: National Center for Medical Culture collections; CICC: China Center of Industrial Culture Collection; ELISA: enzyme-linked immunosorbent assay; DTT: dithiothreitol

Declarations

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MX: methodology, formal analysis, and writing the original draft. FL: software, investigation, and validation. QW: methodology and supervision. JZ: project administration and data curation. PT: conceptualization, reviewing, editing, and validation. LX: resources and supervision. CL: resources and visualization. TX: resources and visualization. DW: conceptualization, supervision, and funding acquisition. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no conflict of interest.

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Figures

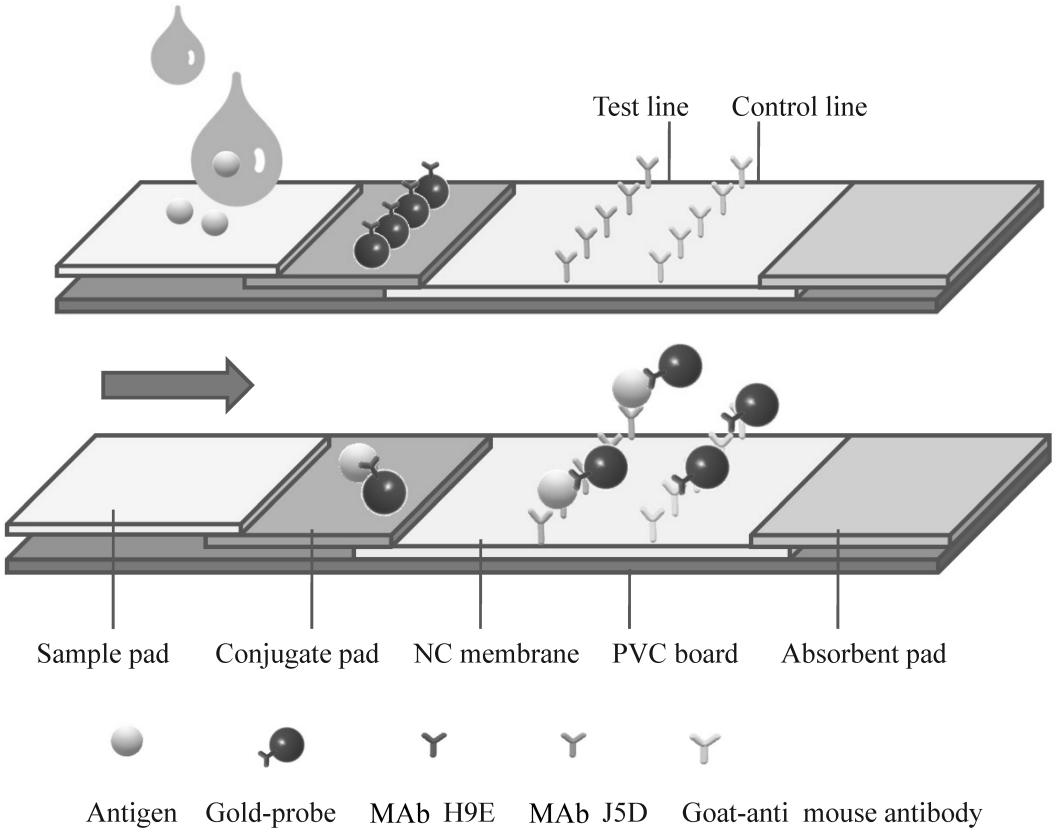

Figure 1

Schematic diagram of ICA test. MAb H9E was used as labeled antibody with colloidal gold particles (in red); MAb J5D was used as capture-antibody in the T line; goat-anti mouse MAb Ig G was used in the C line. Arrow indicates the direction of the movement of antigens (capsid protein of noroviruses).

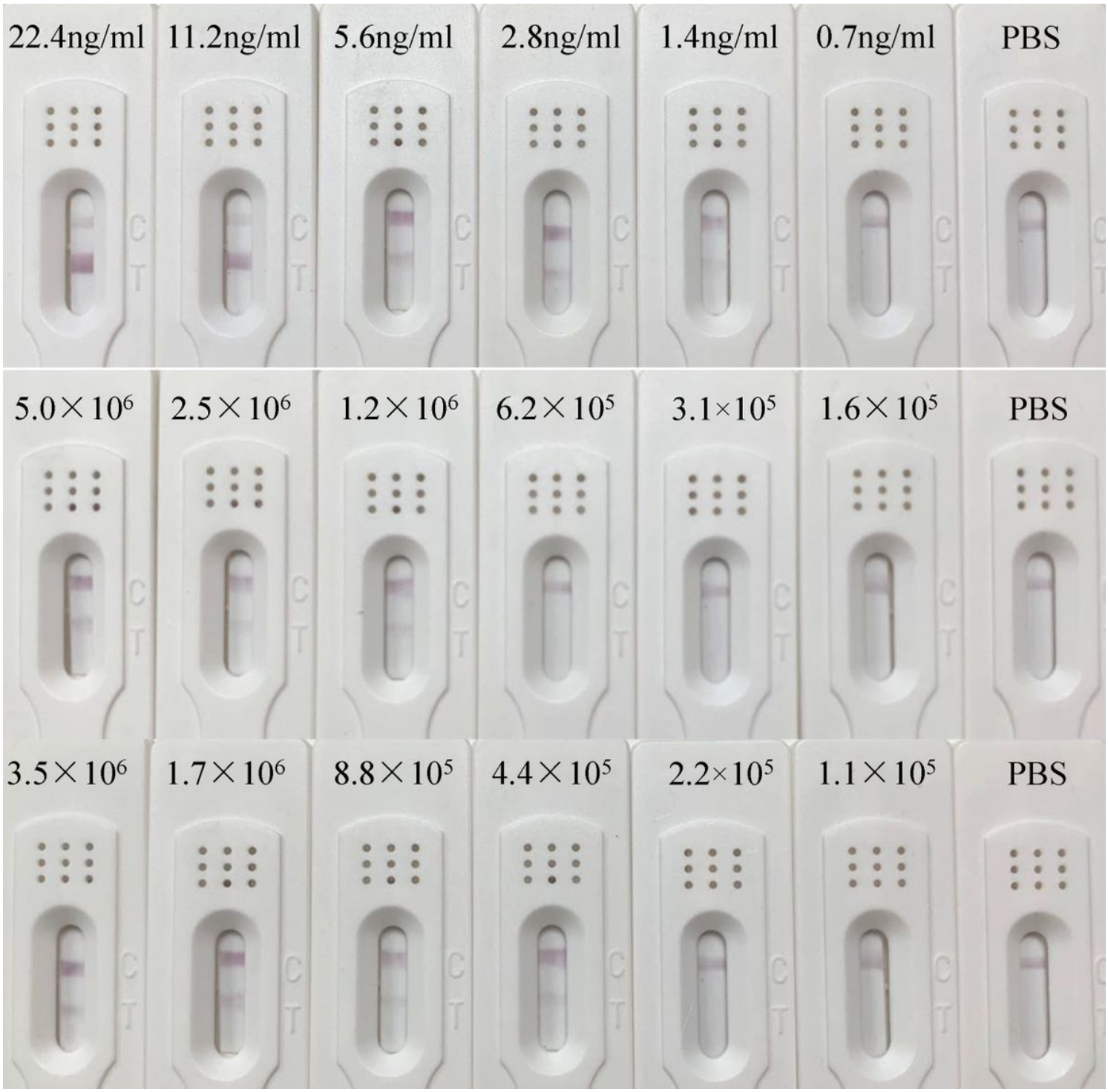

Figure 2

2 LOD of ICA for purified S domain of VP1 and clinical samples Top row: (A) Sensitivity for S domain of VP1 (two-fold dilutions from 22.4 ng/ml to 0.7 ng/ml), Middle row: (B) Sample 57404 (GI.1) (two-fold dilutions from 5.0×106 to 1.6×105 gc/g) and Bottom row:(C) Sample 1717 (GII.4) of different virus copies (two-fold dilutions from 3.5×106 to 1.1×105 gc/g) were detected with test strips. PBS buffer (pH 7.4) was used as blank control.

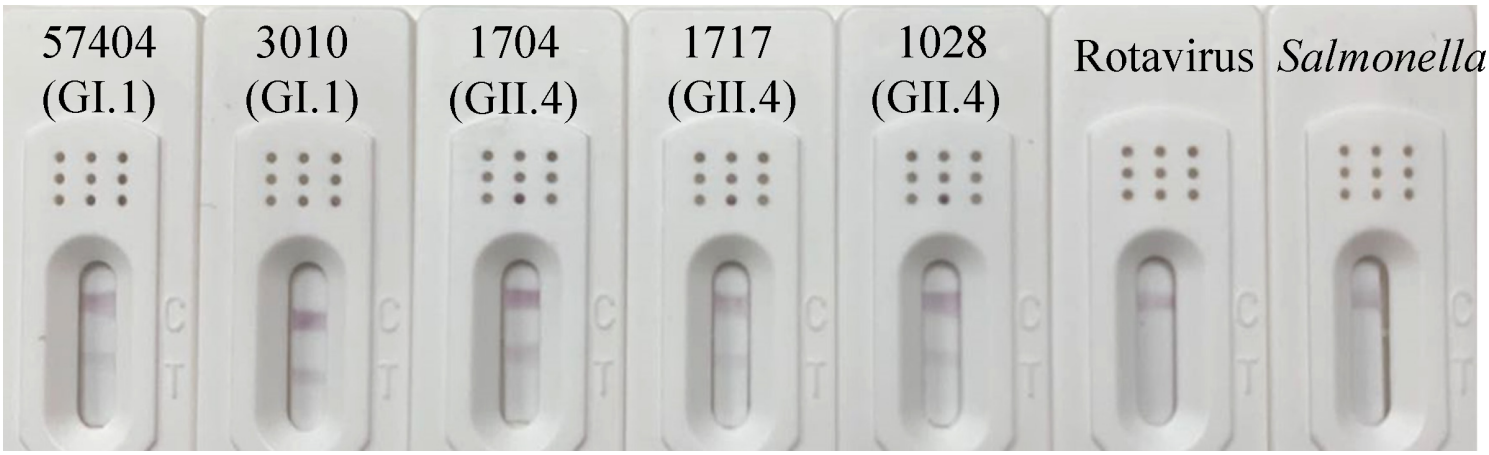

Figure 3

Specificity of ICA Five HuNoVs clinical samples, Rotavirus, and Salmonella cultured samples were tested with the strips.

Supplementary Files

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