Norovirus-specific mucosal antibodies correlate to systemic antibodies and block norovirus virus-like particles binding to histo-blood group antigens

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Abstract

The best acknowledged correlate of protection from norovirus (NoV) infection is the ability of serum antibodies to block binding of NoV virus-like particles (VLPs) to histo-blood group antigens (HBGAs). We investigated mucosal NoV-specific antibody levels in adult volunteers and used saliva from a single donor to determine whether purified saliva antibodies confer blocking. NoV-specific IgG and IgA levels in saliva and plasma samples were measured against four NoV genotype VLPs. NoV-specific IgG and IgA titers in saliva and plasma samples correlated significantly. Antibodies were detected against all VLPs with the highest level of antibodies directed against ancestral GII.4 99 genotype. Affinity chromatography purified salivary IgA and IgG blocked binding of GII.4 99 VLPs to HBGAs. Saliva sampling is a non-invasive alternative to blood drawing and an excellent biological fluid to study NoV-specific immune responses. Mucosal anti-NoV antibodies block binding of NoV VLPs to HBGAs, and may therefore be protective.

Keywords: norovirus; mucosal immunity; saliva; blocking assay; secretory IgA

1. Introduction

Human noroviruses (NoVs) are major causative agents of acute gastroenteritis (AGE) globally [1]. NoV outbreaks and wintertime sporadic infections cause morbidity and mortality particularly in vulnerable populations [2-4] and put a huge burden on healthcare [5], as no preventive vaccine is available [6]. Genetically and antigenically divergent NoVs are divided into seven genogroups (GI- GVII) of which GI and GII comprise at least 28 different human infecting genotypes [7]. Over two decades, GII.4 has been recognized as the most prevalent genotype, and its variants escape herd immunity in 3-5 year intervals causing worldwide pandemics [8,9]. Last pandemic GII.4 variant, GII.4 Sydney, emerged in 2012 [10] and its recombination subvariants still persist as the most prevalent NoV genotype [11].

Humans from an early childhood have a very high prevalence of NoV antibodies and repetitive NoV exposures increase the magnitude of pre-existing NoV antibodies with age [12-14]. Serum NoV antibodies are broadly cross-reactive [15-17] and cross-reactivity is highest among closely related NoV variants and decreases gradually in relation to amino acid sequence divergence [18-21]. NoV seropositivity itself is not associated with protection in adults [15,22,23]. The protection against a certain NoV genotype is short-lived and natural cross-protection between different genotypes is controversial [24]. The best-recognized correlate of protection is the ability of serum antibodies to block NoV virus-like particles (VLPs) binding to histo-blood group antigens (HBGAs) in a surrogate neutralization assay [16,23,25]. HBGAs are expressed i.e. in bodily secretions and on mucosal surfaces where they are thought to facilitate NoV entry and infection [26,27]. The biosynthesis of complex HBGAs on mucosa and secretions is dependent on fucosyltransferase 2 (FUT2) enzyme activity and individuals with dysfunctional *FUT2*-gene (non-secretors) have reduced risk of NoV-infection [28,29].

Saliva is as an intriguing non-invasive alternative to blood to be used in serological assays [30]. It is readily and abundantly available, the collection is painless and the drawing does not require an authorized technician. Salivary samples can be used as a representative mucosal fluid in enteric infections [31-33], however, there are some drawbacks in using saliva that should be taken into the consideration; i.e. the viscosity, proteolytic degradation of antibodies and temporal variability of individual-specific antibody levels [34,35]. Also in the case of severe dehydration, saliva sample could be hard to obtain. Despite these disadvantages, there are several validated diagnostic applications using saliva as biological fluid [35,36]. Saliva is widely used as a biomarker to certain diseases such as the coeliac disease [37-39] and saliva-based serological assays have been utilized in the research of many viruses including NoV [40-46]. New immunological tools based on luminescence were recently published for the detection of NoV infection from saliva samples [41].

In the present study we used salivary samples from adult volunteers in qualification of an ELISA-based method for the detection of mucosal NoV antibodies. The results demonstrate, that affinity chromatography purified saliva anti-NoV antibodies block binding of NoV VLPs to HBGAs in a surrogate neutralization assay.

2. Materials and Methods

2.1 Volunteers

Saliva samples were collected in 2016 from 23 healthy adults (age-range 26-56 years) and a blood sample was obtained from ten of these volunteers (laboratory personnel) originally used to study NoV-specific T-cell and humoral immune responses [47]. Additional saliva samples were collected from two donors within 9 months follow-up period to study saliva antibody level fluctuations. An informed consent was obtained from each donor prior sample collection and all procedures were conducted in accordance of the ethical guidelines of the Declaration of Helsinki.

2.2 Sample collection and storage

Volunteers were asked to exude saliva in 15 ml tubes and saliva samples (5 – 10 ml/donor) were then centrifuged for clarification at 800 × g for 10 minutes. The clarified saliva was aliquoted and stored at -80 °C until use. All saliva samples were processed within 2 hours after collection to avoid proteolytic degradation of antibodies. Venous blood samples were drawn from ten donors and plasma was collected and stored as previously described [47].

2.3 Affinity column purifications

2.3.1 Jacalin column

Jacalin agarose column (Thermo Scientific, Rockford, USA) was used to purify saliva IgA. The column purification was conducted under gravity flow following the instructions provided by the manufacturer. Briefly, two milliliters of immobilized jacalin was packed in a disposable polypropylene column (Thermo Scientific) and the column was equilibrated with phosphate buffered saline (PBS) pH 7.5. Saliva sample (1 ml) was thawed and diluted 5 × with PBS. Diluted saliva was centrifuged 16 000 × g for 5 min at +4°C. The supernatant was filtrated through 0.2 µm sterile filter (VWR, Radnor, USA) and the filtrate was added on top of jacalin gel bed and let to flow through. After washing the column with PBS, the bound antibody was recovered with elution buffer (0.1 M melibiose in PBS) and 2 ml fractions were collected in separate test tubes. The resin was regenerated with 20 column volume of PBS. The fractions were stored at -20° C until use.

2.3.2 Protein G column

Protein G agarose column (Invivogen, San Diego, USA) was used to purify saliva IgG according to the instructions provided by the manufacturer. One milliliter of Protein G-agarose was packed in a disposable polypropylene column (Thermo Scientific). The column was equilibrated with PBS pH 7.2 and diluted saliva sample prepared as described in section 2.3.1 was added. After washing, the bound antibody was eluted with 0.1 M glycine pH 2.2. Elution fractions (1 ml/fraction) were collected and pH of the eluates was immediately neutralized with 1 M TRIS-buffer (pH 9.00). The column was regenerated with 20 % ethanol in PBS. The fractions were stored at -20° C until use.

2.4 Antibody assays

2.4.1 Saliva and plasma ELISA

NoV VLPs of genotypes GII.4 1999 (GII.4 99, original patient sequence, reference strain GII.4 1995/95-US GenBank ID: AF080551), GII.4 Sydney 2012 (GII.4 Syd, GenBank ID: AFV08795.1,), GII.12 1998 (GenBank ID: AJ277618) and GI.1-2001 (GenBank ID: AY502016.1) were used as antigens in enzyme-linked immunosorbent assays (ELISAs). The baculovirus-insect cell production, sucrose-gradient purification and characterization of the VLPs were described elsewhere [48,49]. An ELISA, originally developed to measure serum anti-NoV antibodies [12], was utilized to measure mucosal (saliva) and systemic (plasma) antibodies with few modifications. Polystyrene half-area 96-well plates (Corning Inc., New York, USA) were coated with NoV VLPs (25-50 ng/well) in PBS) overnight at +4°C in a closed chamber with wet tissues. After washing with 0.05% Tween 20 in PBS, the plates were blocked with 5% skimmed milk in PBS. Saliva samples were diluted from 1:4 and plasma from 1:100 two-fold and added to plates (50 µl/well) for 1 h incubation at +37°C. Elution fractions from jacalin and protein G purification columns were added to GII.4 99 coated plates without dilution. After throughout washing, secondary antibodies to detect anti-human IgG (Thermo Scientific) or IgA (Pierce by Thermo Fisher Scientific) were added at dilutions 1:6000 and 1:4000, respectively. After one hour incubation, 0.4 mg/ml o-phenylenediamine dihydrochloride (OPD)-substrate (Sigma Aldrich, Saint Louis, USA) was added and the plates were developed for 30 minutes in dark. After stopping the reaction with sulphuric acid, optical density (OD) at 490 nm was measured in a microplate reader (Victor2, PerkinElmer, Waltham, USA). Blank wells (dilution buffer only) and previously identified positive and negative serum samples were added to each plate as assay controls. All the assay volumes were 50 µl/well and the dilution buffer 1% milk and 0.05% Tween 20 in PBS was used for diluting all samples and antibody-reagents.

2.4.2 Blocking assays (surrogate neutralization assay)

Two different blocking assays based on boiled human saliva HBGAs [50,51] or pig gastric mucin (type III, Sigma Aldrich) [52,53] were used to measure blocking activity of purified saliva IgA and IgG antibodies. Briefly, boiled human type O saliva (1:3000) or pig gastric mucin (2.5 µg/ml) was coated on 96-well plate (Corning, New York, USA) and incubated o/n at +4°C. GII.4 99 VLPs at final concentration of 0.1 µg/ml were mixed with undiluted column elution fractions and controls (known blocking and non-blocking plasma samples) and the mixtures were incubated in low-binding tubes (Eppendorf, Fisher Scientific, Hampton, USA) 1.5 hours at +37°C. After 5% milk blocking step, the VLP-antibody mixtures were added to plates and incubated for 1.5 h. GII.4 positive detection serum (polyclonal mouse antiserum for saliva coated plates and polyclonal rabbit antiserum for PGM coated plates) were used to detect HGBA-bound GII.4 VLPs, following the corresponding HRP-conjugated secondary antibody incubation. The plates were developed with OPD substrate as described in section 2.4.1. Maximum binding control (VLPs lacking sample) and blank wells (lacking VLPs and sample) were added to each plate. PGM coated plates were at each incubation step kept at RT and saliva coated plates at +37°C for 1 h unless otherwise stated. The blocking index (%) was calculated as 100% - (OD wells with serum/OD wells without serum, maximum binding) × 100%.

2.4.3 Secretory component ELISA

An ELISA was used to test NoV-specific secretory IgA (SIgA) antibodies from saliva, plasma and column purification samples. The assay was conducted as described in section 2.4.1 with the exception that sample incubation step was followed by monoclonal anti-human secretory component (SC) antibody (1:5000, Sigma-Aldrich) incubation for 1 hour at +37°C. After washing, HRP conjugated anti-mouse IgG (Sigma-Aldrich) secondary antibody diluted 1:2000 was added and the plate was developed with OPD-substrate and measured as described in section 2.4.1. Boiled saliva was used as a negative control.

2.5 Data analysis

Data from ELISA plates were analyzed after subtracting background signal (mean of blank wells OD) from all OD readings of a plate. End-point titers were defined as the reciprocal of the final serum dilution giving an OD-value above the set cut-off value. For plasma ELISA, cut-off value was calculated as the mean OD + 3 × SD of negative control wells and at least 0.100 OD. For saliva ELISA the cut-off value was determined as the mean OD + 3 × SD of three different boiled (10 min at 96 C) saliva samples. In case the starting dilution of a given sample resulted in an OD value below the set cut-off value (detection limit of the assay) a reciprocal titer half of the starting dilution was used for statistical analyses. The cut-off values were determined for each NoV VLP antigen individually. Reciprocals of end-point titers were used to calculate the geometric mean titers with ± 95% confidence intervals.

2.6 Saliva ELISA qualification

Three different saliva samples (low, medium-high and high NoV-specific antibody content) were diluted two-fold and ran in three separate IgG and IgA ELISAs and the results were used to determine the intra- and inter-assay variation. Intra-assay coefficient of variation (CV) was calculated from triplicate wells within an assay by dividing SD of triplicate wells with the mean OD. Inter-assay CV was calculated by dividing the SD of the three individual assays with the mean OD at each sample dilution. In addition, the end-point titers of the three assays were compared and the precision (%) to obtain the same end-point titer between adjacent runs was evaluated.

2.7 Statistical analyses

Wilcoxon's signed rank test was used to determine the statistical differences between paired non-parametric datasets; the NoV genotype-specific IgG and IgA antibody levels in plasma or saliva. Friedman's two-way analysis of variance by ranks was used to compare the end-point titers of consecutive saliva samples in longevity study of NoV salivary antibody levels. Spearman rank correlation coefficient was used to assess the correlation between antibody titers of combined datasets including all antigen-specific observations. Statistical analyses were conducted using IBM SPSS statistics (SPSS, Chicago, USA) version 25. Statistical significance was defined at $p<0.05$.

3. Results

3.1 Qualification of NoV-specific saliva ELISA

Intra-assay precision was assessed by calculating CVs between positive OD values of triplicate wells of three serially diluted saliva samples. Intra-assay CVs were on average 4.1 ± 3.6% (range 0.07% to 12.7%) for IgG assay, and 2.9 ± 2.6% (range 0.27% to 9.24%) for IgA assay. Inter-assay precision was determined by calculating CV of three serially diluted saliva samples positive OD values from three consecutive assays. Inter-assay CVs were on average 11.8 ± 5.5% (range 5.5% to 21.3%) for IgG assay and 13.2 ± 5.0% (range 3.6% to 21.6%) for IgA assay. Saliva ELISA precision to repeatedly detect the same end-point titer between consecutive runs was on average 77.8 % and 88.9 % for IgG and IgA assays, respectively, and a maximum of two-fold titer increase or decrease between consecutive runs was detected. The qualification specifications obtained here corroborate findings on previously published immunoassays utilizing salivary samples [35,42].

3.2 The magnitude and correlation of NoV-specific mucosal and systemic IgG and IgA

The quantity of pre-existing anti-NoV antibodies in plasma (Fig. 1A) and saliva (Fig.1B) were determined in ELISA by using four different NoV VLPs (GII.4 99, GII.4 SYD, GII.12 and GI.1) as antigens. All 23 volunteers were seropositive to NoV, indicating that all donors had NoV exposure in the past. In plasma, NoV-specific IgG titers trended higher than IgA, although the differences in antigen-specific datasets were not statistically significant (Fig. 1A). In saliva, the level of saliva IgA was significantly higher ($p=0.001$-0.031, Fig. 1B) than the level of IgG. On the individual level, plasma IgG end-point titer was on average 3-fold higher than IgA; whereas saliva IgA end-point titer was on average 3-fold higher than IgG titer.

The correlation of NoV-specific IgG and IgA end-point titers within and between plasma and saliva samples was evaluated using Spearman rank correlation test (Fig. 2). A strong positive correlation was detected between NoV-specific IgG and IgA levels in both plasma ($r= 0.812$, $p<0.0001$; Fig. 2A) and saliva ($r=0.772$, $r<0.0001$; Fig. 2B). Furthermore, IgG and IgA levels in plasma significantly correlated with the corresponding isotype levels in saliva ($r=0.657$, $p<0.0001$ and $r=0.575$, $p=0.0001$, respectively; Fig. 2C-D).

3.3 NoV genotype-specific mucosal and systemic antibody levels

Figure 3 shows NoV genotype-specific reactivity of IgG and IgA in plasma (Fig. 3A-B) and saliva (Fig. 3C-D) to four different NoV VLPs used as antigens in an ELISA. Although there was considerable variation in the genotype-specific antibody levels among donors, the overall magnitude of systemic and mucosal antibody levels were the highest against GII.4 99 genotype. In plasma, the level of GII.4 99 -specific IgG (GMT 10400,

95% CI=2500-43100) was 1.5-5.6 fold higher than the GMTs of other genotypes although the difference compared to GII.12 IgG level was not statistically significant (p=0.05). Similar pattern was seen for genotype-specific plasma IgA with GII.4 99 -specific level (GMT 3940, 95% CI=840-18500) being the highest. In saliva, the quantity of GII.4 99 -specific IgG and IgA levels was significantly higher ($p<0.05$) compared to all other genotype-specific antibody levels. GII.4 99 -specific saliva IgG GMT (50, 95% CI=27-95) was 1.7-2.2-fold higher and IgA GMT (144, 95% CI=78-268) was 4.9-6.3 fold higher than the GMTs of other genotypes.

In addition, genotype-specific antibody titers were compared by using Spearman rank correlation test (data not shown). Only GII.4 99 IgG and IgA levels in plasma and saliva were significantly associated with all other genotype-specific antibody levels ($r=0.586-0.972$, $p<0.0001-0.044$). In addition, the strongest correlations in both plasma and saliva were detected between GII.4 99 and GII.4 SYD -specific antibody titers ($r=0.756-0.972$, $p<0.0001-0.01$), whereas the weakest associations were detected between GI.1 and GII.12 antibody levels ($r=0.409-0.586$, $p=0.005-0.240$).

3.4 Longevity of NoV-specific saliva antibody levels

Saliva NoV-specific IgG and IgA antibody levels of two donors were followed for 9 months to detect any temporal fluctuations in saliva antibody titers. Table 1 shows the results of all saliva end-point titers against different NoV genotypes during the 9-month follow-up time. No significant change ($p=0.135-0.670$) in NoV-specific antibody titers was observed during the collection period. There was a maximum of two-fold titer increase or decrease between the consecutive samples, indicating that pre-existing NoV-antibody levels in a certain saliva sample remain stable over time. In addition, the results confirm validity of the saliva ELISA to be used for detection of NoV-specific antibodies.

3.5 Blocking HBGA binding with purified saliva IgA and IgG antibodies

Affinity chromatography columns were used to purify IgA (Jacalin column) and IgG (Protein G column) antibodies from saliva. A saliva sample with considerable high GII.4 99-specific IgA and IgG levels (end-point titers 1:4096 and 1:512, respectively) was used for purification. Chromatography elution fractions were first screened for GII.4 99 -specific IgG and IgA content by ELISA, after which the fractions containing different levels of IgA and IgG antibody were further used for blocking analyses (Fig. 4A). The elution fractions containing purified saliva IgA (fractions a-e; OD 1.021-2.530) blocked very efficiently GII.4 99 VLPs binding to HBGAs present in human saliva and PGM. Jacalin column elution fractions contained also residual amount of IgG (OD 0.176-0.362), but this level of IgG is not sufficient to have any blocking activity [21]. Next, we tested the blocking activity of Protein G column purified saliva IgG. We managed to obtain only one elution fraction with high salivary IgG content (IgG OD 2.144 and IgA OD 0.211) and this sample conferred

69 % blocking in PGM blocking assay (data not shown). An elution fraction containing low level of IgG (OD 0.508) did not have any blocking activity (blocking index 0%,Fig. 4A horizontal line).

Furthermore, the saliva sample used in jacalin column purification and the resulting elution fractions were tested for the presence of SC. Figure 4B illustrates that the quantity of SIgA in the saliva sample was high and resulted in an end-point titer of 1:2048. Small amount of SIgA was also detected in plasma of the same donor with end-point titer of 1:400. The elution fractions containing GII.4 99 specific IgA used in blocking analyses (Fig. 4A) were all strongly positive for SC (Fig. 4C), indicating that considerable level of SIgA was present in the fractions.. The elution fractions negative for GII.4 99 -specific IgA were negative for SC.

4. Discussion

NoV-specific serum antibody responses, namely blocking antibodies, are the first established correlate of protection [12,25,54]. In addition to serum blocking activity, pre-challenge salivary IgA [46] and an early rise in NoV-specific salivary IgA post infection [28] have been identified as mucosal correlates of protection. Fecal NoV-specific IgA have also been shown to reduce the viral load [46]. The goal of this study was to employ a simple ELISA-based method for measuring mucosal antibodies and to further investigate previously unknown aspects of mucosal NoV-immunity in humans, particularly mucosal blocking antibodies.

We showed that salivary anti-NoV IgG and IgA levels correlate significantly with serum IgG and IgA levels, which is consistent with the results shown by others [42,46]. Therefore, saliva is a noteworthy non-invasive alternative for blood sampling and collecting salivary samples could be useful in settings where obtaining blood sample can be challenging such as in pediatric studies, outbreak settings and epidemiological studies in developing countries. Furthermore, determining the pre-existing antibody profile and secretor-status from saliva could be used as a first-line test to screen the suitable individuals for challenge studies [54,55]. Our findings were concordant with the previously published results [42] demonstrating that anti-NoV IgG was the predominating antibody in plasma and IgA in mucosal secretion. The levels of IgG and IgA in saliva significantly correlated with each other indicating that measuring either isotype gives sufficient information on pre-existing immunity. However, there are subtle differences in IgG and IgA induction and duration after acute infection that should be taken into account; i.e. NoV-specific IgG peaks after IgA and is more long-lasting [42,56-58]. A limitation of our current study was the lack of infection-related samples and therefore we were unable to test the antibody levels in response to infection. However, others have shown that 4-fold rise in antibody titer, a standard marker of NoV-infection [56,59], can also be detected from salivary samples [22,41,42].

SIgA is the first line immune effector against enteric pathogens on mucosal surfaces [60]. It is secreted in basolateral side of mucosa and exported through epithelial cells by polymeric Ig receptor (pIgR) which is cleaved on apical side of membrane releasing dimeric secretory IgA into lumen with a bound secretory component. A key finding of this study was that purified saliva IgA could block binding of NoV VLPs to HBGAs in a surrogate neutralization assay. A study by Ramani et al. [46] demonstrated that prechallenge salivary IgA is associated with protection and have a significant inverse correlation with the severity of the disease. The authors speculated that mucosal IgA might be serum-derived [46]. However, the results of the current study showed that salivary NoV-specific IgA is likely not serum derived as it is highly positive for secretory component. These results indicate that mucosal IgA has blocking activity and possibly plays a role in virus neutralization although the general limitation of this study was that a saliva sample used for purification and blocking assay was from a single donor. However, the objective was not to develop a saliva blocking assay to be used for routine mucosal sample testing as the chromatographical purification is very laborious and need

high concentration of the antibodies in the starting material, but rather to demonstrate that purified saliva IgA and IgG antibodies have blocking potential likewise to serum antibodies. It remains to be investigated, whether blocking mucosal IgA antibodies are associated with protection from NoV infection and/or disease.

Mucosal IgG is mostly derived from blood but some is produced by mucosal IgG plasma cells [34,61]. Similarly to saliva IgA, purified saliva IgG blocked the binding of NoV VLPs to HBGAs. Hypothetically, mucosal SIgA has more neutralizing potential than IgG, as the valency of antigen binding sites in dimeric IgA is more than is in monomeric IgG. An interesting recent finding was that *in vitro* produced monoclonal IgA, even in its monomeric form, appeared to be more potent in blocking VLP binding to HBGAs than IgG [62]. We also detected small amount of SIgA in serum, which happens when mucosal SIgA leaks back into systemic circulation [63]. What role does the SIgA play in systemic immunity is not clear, but others have shown that purified serum anti-NoV IgA confers cross-strain blocking activity [58].

The highest antibody levels in both serum and saliva were detected against GII.4 99 VLPs. Although it is not surprising that the antibody levels were skewed to GII.4 genotype, as it has been the predominating NoV strain since 1990s, the difference in the antibody response magnitude to the most recent circulating GII.4 SYD variant was surprising ($p=0.0001$-0.012, Fig 3). We have previously shown that GII.4 99 possesses the greatest cross-reactivity when compared to any other GII.4 variants or other NoV VLPs [12,21,47]. Here we reported that only anti-GII.4 99 antibody levels significantly correlated with all other genotype antibody levels in both saliva and serum. The reason for the superiority of this ancestral GII.4 genotype is not fully clear but it might hold the widely conserved key epitopes in its sequence. In addition, the structure might be more accessible to cross-reactive antibodies buried in conserved P1 or S-domains of NoV capsid VP1 [18,20,64]. A clinical study, investigating cross-reactive immune responses induced by NoV VLP, showed that the strongest blocking antibody response was developed against ancestral GII.4 97 VLP, not included into a vaccine composition [65]. The authors discussed that antibody response was characterized by a memory response to the cross-reactive epitopes present in GII.4 97 rather than immature response to vaccine component GII.4 VLP, a term named antigen seniority [66]. Our GII.4 99 VLP sequence is an original patient sequence belonging to the first pandemic GII.4 95/96-US subset of variants [67]. It is possible that our study subjects have initially been exposed to ancestral GII.4 and later GII.4 variant exposures have strengthen the affinity of antibodies generated to this strain. In outbreaks or prospective epidemiological study settings, where the infecting genotype-specific VLP might not (yet) be available for serological assays, the antigen that is able to capture the most cross-reactive antibodies, such as ancestral NoV genotype, should be preferably used. The current study showed the superiority of ancestral GII.4 99 VLP over other genotypes in capturing NoV-specific antibodies in adult population. However, for the younger age groups, who have had more recent GII.4 variant priming infection, different GII.4 antigen might be needed to detect the back-boost response.

There are acknowledged drawbacks of using saliva samples in diagnostics [34,35]. The viscosity of saliva samples has been pointed out as being one factor causing pipetting errors and hindering the accuracy of the

assays. However, when clarified saliva samples were diluted at least 4-fold prior to ELISA, the viscosity was drastically reduced. Another downfall reported with saliva is the fluctuations in the saliva antibody levels over time and flow rate [34,68]. In addition, due to the inadequate amount of saliva samples, we did not analyze total antibody levels and therefore did not report % antigen-specific antibody levels. However, we tested saliva samples of two donors at 3 - 4 different time points and the end-point titers remained stable over nine months test period. Variation in the end-point titers were at maximum two-fold up or down between adjacent time-points, which is within the range of precision of salivary ELISA determined in the qualification experiments (section 3.1).

Saliva is a convenient and adequate biological fluid to study NoV-specific antibody responses. Although saliva-based assays cannot fully replace serum-based assays, the advantage in using salivary samples is that they can be used as a reflection of systemic immune response and at the same time important knowledge on mucosal immunity can be gained. For the first time, to our knowledge, we showed that saliva purified IgA and IgG blocked VLP binding to HBGAs. We hypothesize that secretory blocking IgA might have a role in the first line defense against NoV prior passing the mucosal layer, but once the virus penetrates this mucosal barrier, the high level of systemic blocking IgG is most likely imperative in controlling the infection. Our previous studies in mice [69,70] have shown, that mucosal immunization route is needed to induce mucosal anti-NoV IgA, which raises the possibility, that naïve individuals such as children might require mucosal administration of NoV VLP vaccine in order to generate neutralizing IgA on mucosal surfaces. The results of this study encourage further investigating NoV-specific mucosal immune responses, especially in vaccine design.

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Conflict of interest

None of the authors have any conflicts of interest.

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Table 1. Longevity and reproducibility of the salivary NoV-specific IgG and IgA responses.

Time point		*0 weeks*		*10 weeks*		*12 weeks*		*36 weeks*		Friedman's two-way analysis of variance by ranks *p-value*	
Donor	**Antigen**	**IgG**	**IgA**	**IgG**	**IgA**	**IgG**	**IgA**	**IgG**	**IgA**	**IgG**	**IgA**
Donor 1	GII.4 99	64*	512	64	256	64	256	64	256	*0.392*	*0.573*
	GII.4 SYD	16	64	32	64	32	64	32	128		
	GII.12	32	64	16	64	32	64	64	64		
Donor 2	GII.4 99	128	64	64	64	ND**	ND**	64	128	*0.670*	*0.135*
	GII.4 SYD	16	32	32	32			64	64		
	GII.12	16	64	32	64			32	64		

**end-point titer*

***ND, not determined*

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FIGURE CAPTIONS

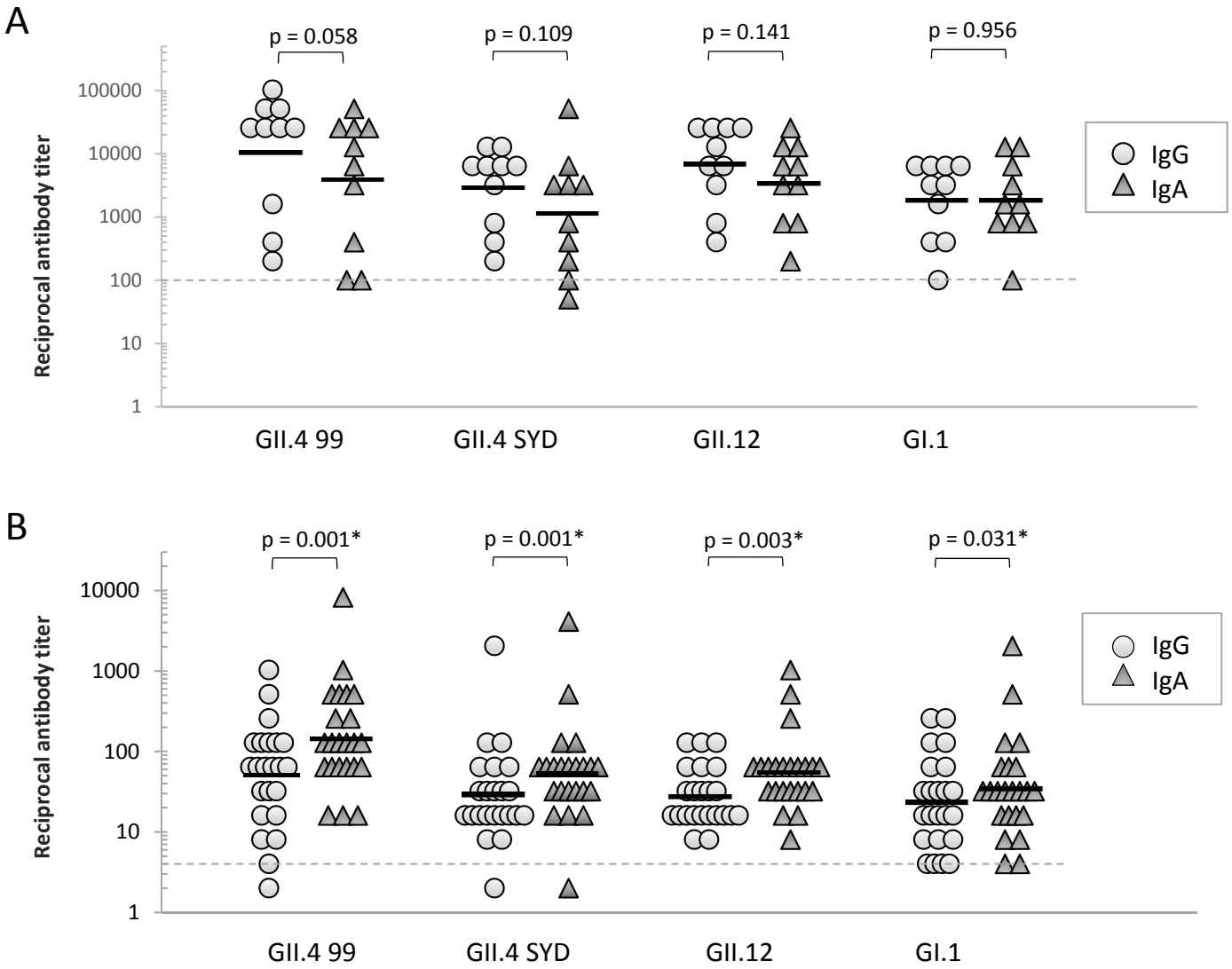
FIGURE 1. The level of norovirus (NoV)-specific immunoglobulin G (IgG) and IgA responses in plasma (A) and saliva (B) of human donors. The plasma of 10 donors and saliva samples of 23 donors were analyzed by enzyme-linked immunosorbent assay (ELISA) against GII.4 99, GII.4 Sydney, GII.12 and GI.1 virus-like particles (VLPs). Shown are the reciprocals of antigen-specific end-point titers and the geometric mean titers (horizontal full lines). Dashed line illustrates the limit of detection as described in the Materials and Methods. Statistical significance was determined by Wilcoxon's signed rank test and a p-value ≤ 0.05 was considered to be statistically significant (*).

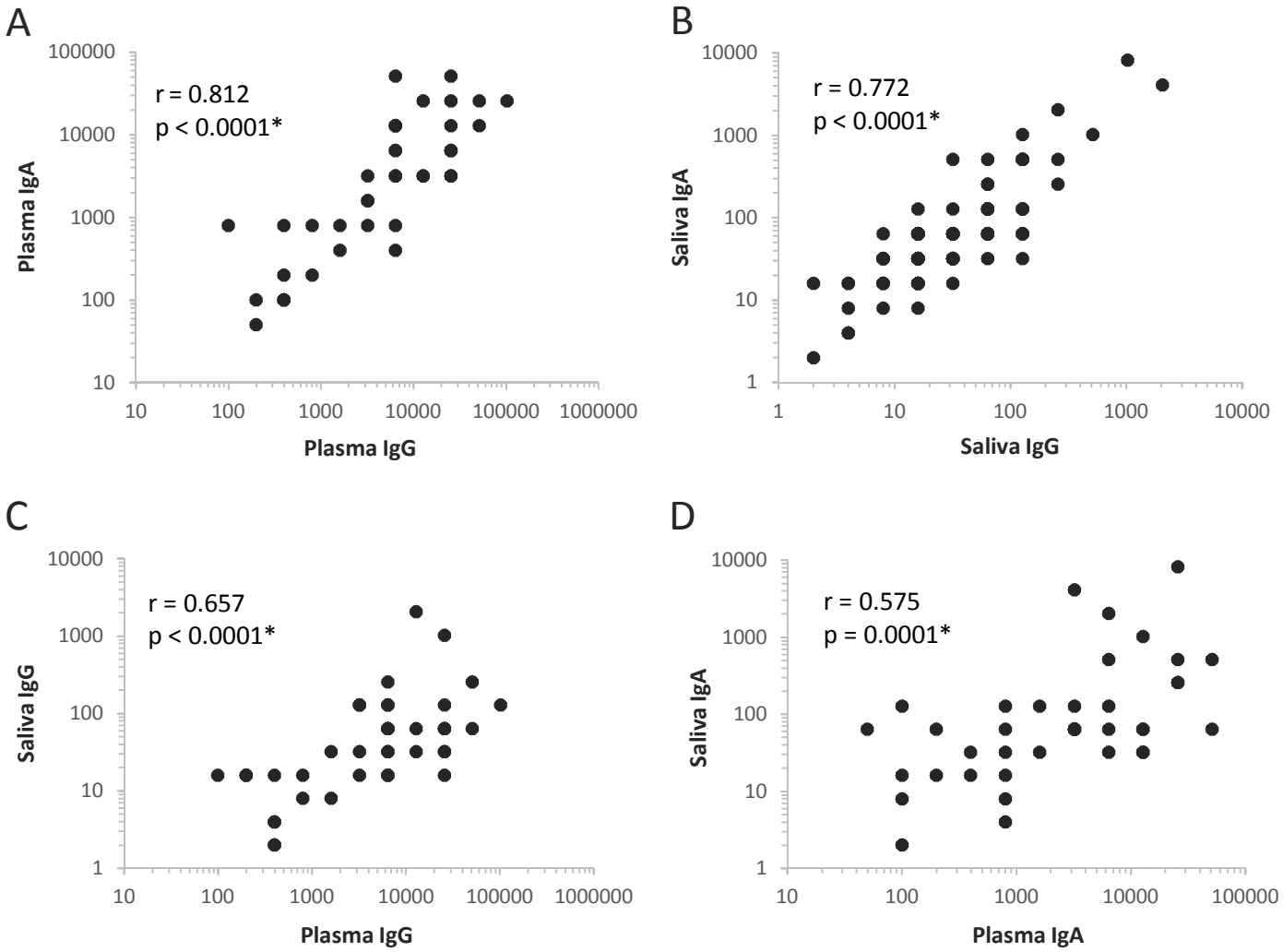
FIGURE 2. Correlation of norovirus (NoV) -specific immunoglobulin G (IgG) and IgA antibodies. Spearman's rank correlation (r) was determined between NoV-specific IgG and IgA levels in plasma (A) and saliva (B) samples and between the plasma and saliva levels of IgG (C) and IgA (D). Shown are the reciprocal end-point titers to four different NoV-genotype VLPs (GII.4 99, GII.4 Sydney, GII.12 and GI.1) as combined datasets based on sample type. A p-value ≤ 0.05 was considered to be statistically significant (*).

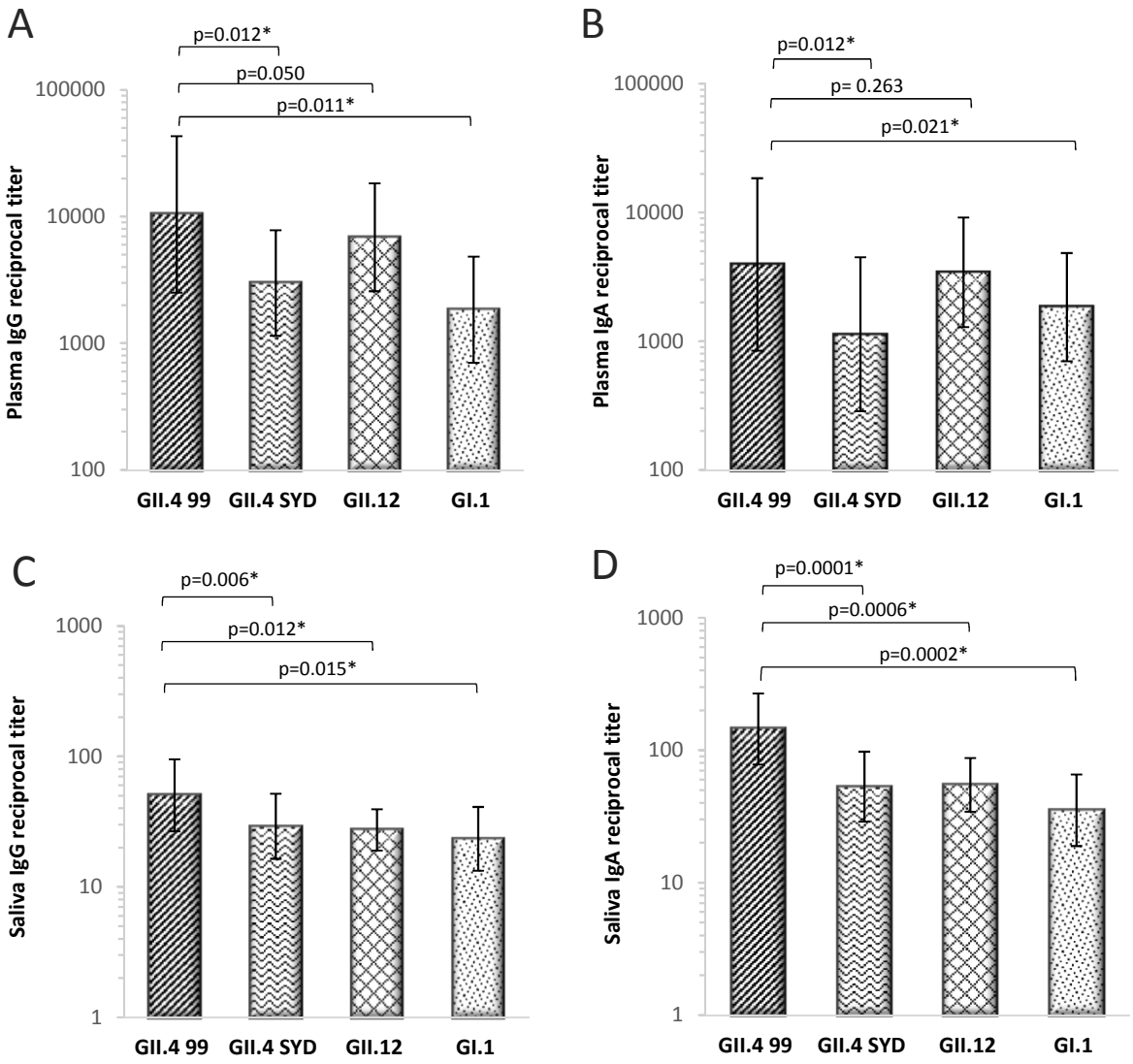
FIGURE 3. Norovirus genotype-specific systemic and mucosal antibody levels. The plasma and saliva samples of ten donors were analyzed by enzyme-linked immunosorbent assay (ELISA) against GII.4 99, GII.4 Sydney, GII.12 and GI.1 virus-like particles (VLPs) and the genotype-specific plasma IgG (A) and IgA (B) and saliva IgG (C) and IgA (D) were determined. The bars represent the geometric mean titers (GMTs) and the error bars illustrate 95% confidence intervals. The antigen-specific antibody levels were compared by Wilcoxon's signed rank test and a p-value ≤ 0.05 was considered to be statistically significant (*).

FIGURE 4. Blocking activity of affinity column purified salivary immunoglobulin A (IgA). (A) Saliva sample was purified by jacalin affinity chromatography and the elution fractions were used for blocking of GII-4 99 virus-like particle (VLP) binding (0.1µg/ml) to human saliva type O and pig gastric mucin (PGM). Enzyme-linked immunosorbent assay (ELISA) was used to determine the levels (optical density, OD) of anti-GII.4 99 -specific IgA and IgG in each elution fraction. The error bars represent the standard deviation of OD values of two independent assays. The horizontal dashed line represents the IgG level (OD-value) in a protein G column purified saliva sample that is negative for blocking activity (blocking index 0 %). Blocking index was calculated as 100% - (OD wells with serum/OD wells without serum, maximum blocking) × 100%. (B) The level of secretory component (SC) positive GII.4 99-specific antibodies in untreated saliva (1:8 – 1:4096 dilution), plasma (1:100 – 1:25600 dilution) and heated saliva (negative control) determined by ELISA as described in the Materials and Methods. (C) Jacalin chromatography purified elution fractions (a-g) tested for

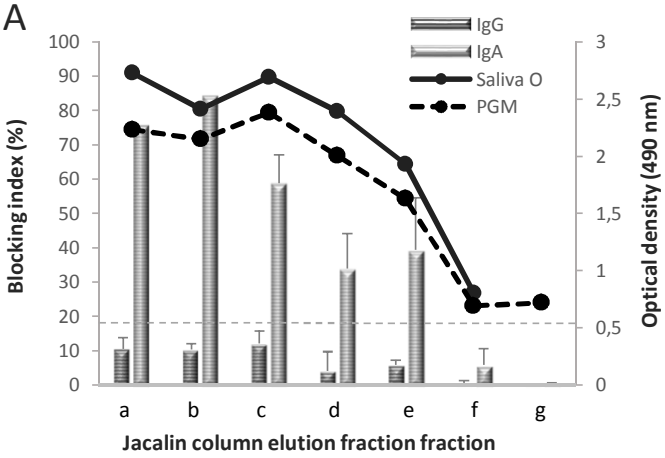
the SC presence. Optical density at 490nm represents the level of SC positive NoV GII.4 99-specific antibody in a sample.


A
p = 0.058
p = 0.109
p = 0.141
p = 0.956
Reciprocal antibody titer
100000
10000
1000
100
10
1
IgG
IgA
GII.4 99
GII.4 SYD
GII.12
GI.1
B
p = 0.001*
p = 0.001*
p = 0.003*
p = 0.031*
Reciprocal antibody titer
10000
1000
100
10
1
IgG
IgA
GII.4 99
GII.4 SYD
GII.12
GI.1

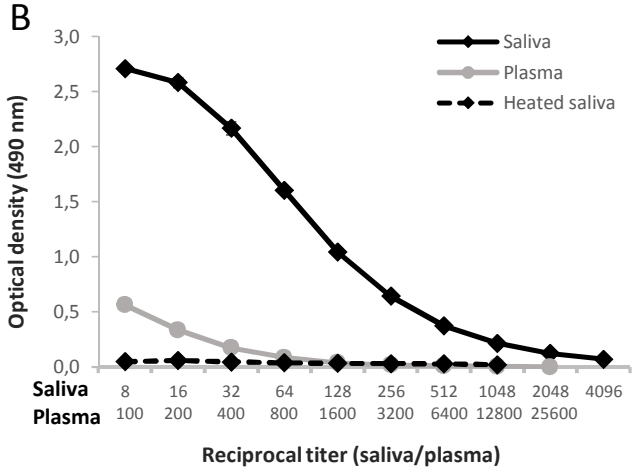

A
r = 0.812
p < 0.0001*
Plasma IgA
Plasma IgG
100000
10000
1000
100
10
10
100
1000
10000
100000
1000000
B
r = 0.772
p < 0.0001*
Saliva IgA
Saliva IgG
10000
1000
100
10
1
1
10
100
1000
10000
C
r = 0.657
p < 0.0001*
Saliva IgG
Plasma IgG
10000
1000
100
10
1
10
100
1000
10000
100000
1000000
D
r = 0.575
p = 0.0001*
Saliva IgA
Plasma IgA
10000
1000
100
10
1
10
100
1000
10000
100000


A
p=0.012*
p=0.050
p=0.011*
100000
10000
1000
100
Plasma IgG reciprocal titer
GII.4 99
GII.4 SYD
GII.12
GI.1
B
p=0.012*
p= 0.263
p=0.021*
100000
10000
1000
100
Plasma IgA reciprocal titer
GII.4 99
GII.4 SYD
GII.12
GI.1
C
p=0.006*
p=0.012*
p=0.015*
1000
100
10
1
Saliva IgG reciprocal titer
GII.4 99
GII.4 SYD
GII.12
GI.1
D
p=0.0001*
p=0.0006*
p=0.0002*
1000
100
10
1
Saliva IgA reciprocal titer
GII.4 99
GII.4 SYD
GII.12
GI.1

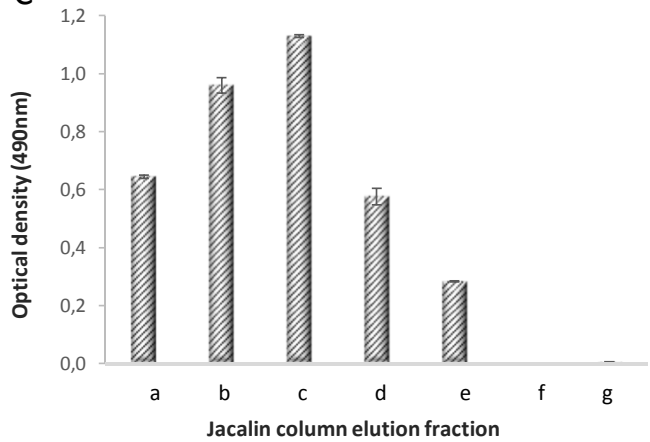
A


IgG
IgA
Saliva O
PGM
Blocking index (%)
Optical density (490 nm)
100
90
80
70
60
50
40
30
20
10
0
3
2,5
2
1,5
1
0,5
0
a
b
c
d
e
f
g
Jacalin column elution fraction fraction

B


Saliva
Plasma
Heated saliva
Optical density (490 nm)
3,0
2,5
2,0
1,5
1,0
0,5
0,0
Saliva 8 16 32 64 128 256 512 1048 2048 4096
Plasma 100 200 400 800 1600 3200 6400 12800 25600
Reciprocal titer (saliva/plasma)

C


Optical density (490nm)
1,2
1,0
0,8
0,6
0,4
0,2
0,0
a
b
c
d
e
f
g
Jacalin column elution fraction