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Full Paper

Identification of antigens recognized by salivary IgA using microbial protein microarrays

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Secretory IgA plays an important role in the mucosal immune system for protection against pathogens. However, the antigens recognized by these antibodies have only been partially studied. We comprehensively investigated the antigens bound by salivary IgA in healthy adults using microbial protein microarrays. This confirmed that saliva contained IgA antibodies that bind to a variety of pathogenic microorganisms, including spike proteins of severe acute respiratory syndrome coronavirus 2, severe acute respiratory syndrome coronavirus, Middle East respiratory syndrome coronavirus, and other human coronavirus species. Also, many subtypes and strains of influenza virus were bound, regardless of the seasonal or vaccine strains. Salivary IgA also bound many serogroups and serovars of *Escherichia coli* and *Salmonella*. Taken together, these findings suggest that salivary IgA, which exhibits broad reactivity, is likely an essential element of the mucosal immune system at the forefront of defense against infection.

Key words: IgA, saliva, antigen, virus bacteria

INTRODUCTION

Mucosal surfaces, unlike the skin, are fragile and thus serve as entry points for microorganisms and foreign entities, such as viruses, bacteria, and allergens. The immune system provides a two-tiered defense to maintain human health. The mucosal immune system acts as the first barrier to prevent invasion, and the systemic immune system defends against invaders that have succeeded in gaining access. Secretory immunoglobulin A (IgA) plays an important role in mucosal immunity, and because of the ease of its collection, it is often used as a mucosal immunity biomarker. Most of the immunoglobulin in mucosal secretions is IgA, which plays a role in preventing pathogen invasion [1], in particular by inhibiting adherence and colonization; neutralizing viruses, enzymes, and toxins; mucus trapping; and inhibiting antigen uptake. A protective role of secretory IgA against pathogens, such as influenza virus, respiratory syncytial virus, *Streptococcus pneumoniae*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium, has been reported [2]. Salivary IgA decreases with age, mental stress, and physical stress [3–9]. These situations are well known to increase susceptibility to infection, and a relationship between lower salivary IgA levels and increased risk of upper respiratory tract infections has been suggested [10–12]. Thus, enhancement or preservation of salivary IgA secretion is considered to contribute to better health. This has been reported to be influenced by lactic acid bacteria and food factors [13–17].

Although most of the IgA in human serum is monomeric, secretory IgA forms polymers, such as dimers, trimers, and tetramers, in external secretions [18]. Polymerization of IgA confers two unique features, an enhancement of neutralizing capacity and cross-reactivity against different antigens. These features were documented in influenza virus and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections [19–23]. The protective function of IgA has been studied for several individual pathogenic microorganisms [2], but cross-reactivity was investigated mainly using influenza viruses, not on a wider variety of pathogens together. Therefore, in the present study, we used microbial protein microarrays to comprehensively investigate the antigens to which salivary IgA binds in healthy adults.

MATERIALS AND METHODS

Study participants and saliva collection

Healthy men and women were recruited for this study with the inclusion/exclusion criteria shown in Table 1. The study specifics were explained to the participants, and written informed consent was obtained from each of them before the study was performed. Subsequently, the investigator interviewed and examined the

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Table 1. Inclusion/exclusion criteria

Criteria
Inclusion criteria
Males and females aged 20–79 years
Non-smokers for more than 1 year
BMI of 18.5–34.9 kg/m^2
Ability to comply with dietary and behavioral restrictions on the day before and on the day of the test
Exclusion criteria
Regular consumer of food for specified health uses (FOSHU), food with functional claims, supplements and/or health foods (for >3 days per week) which could affect the study results
Taking medication (e.g., antibiotics, steroids, antihistamines) which could affect the study results
Heavy drinker
Participant in another clinical study with medicine/food within the last 4 weeks before this study, or planning to join one after giving informed consent
Vaccinated with different vaccines during the month leading up to the test and during the test period (e.g., those who are waiting for an appointment or on standby for coronavirus vaccination)
With a previous or current medical history/anamnesis of severe cardiac, hepatic, renal or digestive disease
Pregnant, lactating, or intent to become pregnant during the study period
Allergic to medicines and food
Past blood donation as follows:
—males/females: 200 mL of blood components within a month
—males: 400 mL of whole blood within the last 3 months
—females: 400 mL of whole blood within the last 4 months
Blood donors whose blood collection would have reached the following volume 12 months prior to the start of the study and the planned blood collection volume for the study:
—males: 1,200 mL
—females: 800 mL
Systolic blood pressure of 180 mmHg or higher and/or diastolic blood pressure of 110 mmHg or higher
Others who have been determined ineligible by principal/sub investigator

candidates, and blood biochemical and hematological tests were conducted. Finally, a total of 64 subjects were selected for the study. All subjects were confirmed as negative for hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), and syphilis by the infection test that detect antibodies or antigens in the serum. All subjects asserted that they had no history of coronavirus disease 2019 (COVID-19). This study was conducted in accordance with the Declaration of Helsinki, and all procedures involving human subjects were approved by the Institutional Review Board of Chiyoda Paramedical Care Clinic (Tokyo, Japan). The study was registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) as number UMIN000045264.

Subjects were asked to avoid alcoholic beverages and to fast from 9:00 PM on the night prior to the day of saliva collection, which took place between 10:00 AM and 12:00 AM the following day. Subjects had an assigned meal, after which they rinsed their mouths with tap water. They then rested for 90 min prior to saliva collection, before which they rinsed their mouths with tap water again. They were asked to collect non-stimulated saliva produced over a period of 10 min. Saliva samples were frozen at −80°C until analysis.

Microbial protein microarrays

We selected 12 subjects for salivary IgA profiling, keeping the numbers of males and females and the numbers of severe acute respiratory syndrome coronavirus 2 of the genus Betacoronavirus (SARS-CoV-2) vaccinated and non-vaccinated subjects equal. Saliva samples were confirmed to be negative for COVID-19 using VisCheck SARS-CoV-2 (VisGene, Osaka, Japan). Salivary IgA profiling was performed using microbial protein microarrays containing 2,280 samples of protein extracts and 1,367 samples of recombinant proteins of viruses, bacteria, and fungi (Fukushima Translational Research Project, Fukushima, Japan). In brief, the microarrays were incubated with diluted saliva samples and Goat Reference Antibody Mixture I (Fukushima Protein Factory, Inc., Fukushima, Japan) after blocking and stained with Alexa Fluor 647-conjugated anti-human IgA and Cy3-conjugated anti-goat IgG antibodies. The microarrays were then scanned with a GenePix 4000B (Molecular Devices, San Jose, CA, USA). Microarrays incubated without saliva samples served as negative controls. To compare between microarrays, the fluorescence intensity ratios (Alexa Fluor 647/Cy3) were normalized, and then relative values against negative controls were calculated to exclude cross-reactions of secondary antibodies. Values are indicated as relative $\log_2$ ratios. Antigen binding at relative $\log_2$ ratios >0.5849 (i.e., 1.5-fold the negative control) was recorded in at least 7 of the 12 subjects.

Statistical analysis

Relative $\log_2$ ratios against SARS-CoV-2 recombinant proteins were compared between SARS-CoV-2 vaccinees and non-vaccinees by unpaired t-test using the SAS® 9.4 Software (SAS Institute Japan, Tokyo, Japan). A two-tailed p-value <0.05 was considered significant for all tests.

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RESULTS

Characteristics of study participants

Characteristics of the 12 subjects are shown in Table 2. None of the subjects had a history of COVID-19 infection. Six were SARS-CoV-2 vaccinees and 6 were non-vaccinees. Saliva was collected from all subjects more than 30 days after the vaccinees received their second vaccinations.

Viruses

Fifty-six different viruses were found to be bound by salivary IgA in at least 7 of the 12 subjects (Table 3). They included pathogenic viruses such as SARS-CoV-2, severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), other human coronaviruses (HCoVs), influenza virus, respiratory syncytial virus, rotavirus, human alpha herpesvirus 1 (heterotypic synonym of herpes simplex virus type 1), human papillomavirus, dengue virus, and Zika virus. In addition, they also included three species of ebolavirus. Table 4 shows the species and the recombinant proteins of coronaviruses which were bound by salivary IgA. A variety of different proteins were bound by salivary IgA, especially the spike proteins of SARS-CoV-2 (Wuhan-Hu-1 strain), SARS-CoV, MERS-CoV, and HCoV-OC43. Relative $\log_2$ ratios were not significantly different between SARS-CoV-2 vaccinees and non-vaccinees. Seventy-nine strains of influenza

Table 2. Characteristics of participants

	All	Non-vaccinated	SARS-CoV-2 vaccinated
Number of subjects (male/female)	12 (6/6)	6 (3/3)	6 (3/3)
Age (years)	50.2 ± 4.6	42.8 ± 6.9	57.5 ± 5.1
Height (cm)	162.4 ± 3.4	161.9 ± 4.2	162.9 ± 6.4
Body weight (kg)	56.6 ± 3.3	52.8 ± 1.6	61.5 ± 6.9
History of SARS-CoV-2 infection	no	no	no
Vaccination (BNT162b2 /mRNA-1273)	-	-	6 (2/4)
Days from the second vaccination (d)	-	-	61.2 ± 6.1

Mean ± SE. SE: standard error.

Table 3. Species of viruses bound by salivary IgA

Family	Species
Anelloviridae	*Chicken anemia virus*
Arenaviridae	*Lassa mammarenavirus*, *Lymphocytic choriomeningitis mammarenavirus*
Arteriviridae	*Porcine reproductive and respiratory syndrome virus*
Baculoviridae	*Autographa californica multiple nucleopolyhedrovirus*
Caliciviridae	*Norwalk virus*
Coronaviridae	*Human coronavirus*, *Middle East respiratory syndrome* (MERS) *coronavirus*, *Severe acute respiratory syndrome* (SARS) *coronavirus*, *Severe acute respiratory syndrome coronavirus* 2 (SARS-CoV-2)
Filoviridae	*Marburg marburgvirus*, *Reston ebolavirus*, *Sudan ebolavirus*, *Zaire ebolavirus*
Flaviviridae	*Dengue virus* 2, *Hepacivirus C*, *Japanese encephalitis virus*, *West Nile virus*, *Zika virus*
Geminiviridae	*African cassava mosaic virus*
Hepadnaviridae	*Hepatitis B virus*
Hepeviridae	*Hepatitis E virus*
Herpesviridae	*Human alphaherpesvirus* 1 (*Herpes simplex virus* 1), *Human alphaherpesvirus* 2 (*Herpes simplex virus* 2), *Human betaherpesvirus* 5 (*Human cytomegalovirus*), *Human gammaherpesvirus* 4 (*Epstein-Barr virus*)
Kolmioviridae	*Hepatitis delta virus*
Myoviridae	*Escherichia virus*
Orthomyxoviridae	*Influenza A virus*, *Influenza B virus*
Papillomaviridae	*Human papillomavirus* type 16, *Human papillomavirus* type 18, *Human papillomavirus* type 52
Paramyxoviridae	*Avian avulavirus* 1, *Canine morbillivirus*, *Human respirovirus* 1 (*Human parainfluenza virus* 1), *Murine respirovirus*
Parvoviridae	*Human parvovirus*
Peribunyaviridae	*La Crosse virus*
Phenuiviridae	*Rift Valley fever virus*
Picornaviridae	*Coxsackievirus* A16, *Human rhinovirus* A89
Pneumoviridae	*Bovine orthopneumovirus*, *Human respiratory syncytial virus* A, *Human respiratory syncytial virus* B
Polyomaviridae	*Human polyomavirus* 1 (*BK polyomavirus*), *Human polyomavirus* 2 (*JC polyomavirus*)
Poxviridae	*Vaccinia virus*
Reoviridae	*Human rotavirus* B219, *Rotavirus* A
Retroviridae	*Human immunodeficiency virus* 2, *Moloney murine leukemia virus*, *Simian immunodeficiency virus*
Rhabdoviridae	*Rabies lyssavirus*
Siphoviridae	*Enterobacteria phage*
Totiviridae	*Ustilago maydis virus* P6

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Table 4. Species and the recombinant protein of coronavirus to which salivary IgA bound

Virus	Recombinant protein	Fluorescence intensity ratio with negative control ($\log_2$)		p value (Vaccinated vs. non-vaccinated)
		Non-vaccinated (n=6)	SARS-CoV-2 vaccinated (n=6)	
Human coronavirus HKU1	Non-structural protein 4	2.29 ± 0.41	1.69 ± 0.38	0.3027
Human coronavirus NL63	Envelope small membrane protein E	1.03 ± 0.24	0.94 ± 0.45	0.8758
	non-structural protein 3	1.20 ± 0.47	0.86 ± 0.30	0.5541
	Nucleoprotein	2.08 ± 0.69	2.63 ± 0.70	0.5853
Human coronavirus OC43	Spike glycoprotein S1	2.59 ± 0.43	1.90 ± 0.28	0.2069
Middle East respiratory syndrome (MERS) coronavirus	Spike glycoprotein (extracellular domain)	1.14 ± 0.46	1.38 ± 0.73	0.7829
	Spike glycoprotein S1	1.54 ± 0.28	0.87 ± 0.38	0.1849
	Spike glycoprotein S2	1.22 ± 0.49	1.39 ± 0.66	0.8399
Severe acute respiratory syndrome (SARS) coronavirus	Envelope small membrane protein E	2.17 ± 0.52	1.72 ± 0.38	0.4961
	Matrix glycoprotein M	1.21 ± 0.46	1.51 ± 0.49	0.6640
	Nucleoprotein (C-terminal domain)	1.30 ± 0.43	1.30 ± 0.44	0.9961
	Nucleoprotein (N-terminal domain)	2.11 ± 0.64	1.70 ± 0.52	0.6337
	Nucleoprotein (N-terminal/middle domains)	2.60 ± 0.58	2.21 ± 0.72	0.6850
	Papain-like protease	0.75 ± 0.40	1.00 ± 0.49	0.7047
	Spike glycoprotein (C-terminal domain)	1.36 ± 0.33	0.78 ± 0.40	0.2863
	Spike glycoprotein (middle domain)	2.78 ± 0.45	2.36 ± 0.48	0.5431
	Spike glycoprotein S1	0.85 ± 0.56	1.34 ± 0.56	0.5504
	Spike glycoprotein (receptor-binding domain)	2.52 ± 0.49	2.11 ± 0.38	0.5235
	Spike glycoprotein S2	1.65 ± 0.35	0.83 ± 0.28	0.0941
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)	3C-like protease	1.17 ± 0.40	0.40 ± 0.20	0.1134
	Non-structural protein Nsp1	0.78 ± 0.38	1.11 ± 0.50	0.6124
	Nsp7 replicase	1.16 ± 0.54	0.68 ± 0.34	0.4678
	Nsp8 replicase	1.67 ± 0.57	1.58 ± 0.31	0.8982
	Spike glycoprotein (extracellular domain)	1.78 ± 0.62	1.41 ± 0.44	0.6371
	Spike glycoprotein S1	0.73 ± 0.49	0.58 ± 0.35	0.8050
	Spike glycoprotein S2 (extracellular domain)	1.97 ± 0.55	1.65 ± 0.60	0.6982

Mean ± SE. SE: standard error.

A viruses and 16 strains of influenza B viruses were bound (Table 5). Many subtypes and strains other than those included in seasonal influenza vaccines since 2001 in Japan were detected as being bound by salivary IgA.

Bacteria and fungi

The families of bacteria and fungi detected are shown in Table 6, and the results showed that large numbers of species were bound (208 and 58 for bacteria and fungi, respectively; see Supplementary Table 1 for bacteria and Supplementary Table 2 for fungi). The results showed that a great variety of pathogenic bacteria were bound by salivary IgA. This included bacteria such as *S. pneumoniae*, *Mycobacterium tuberculosis*, and *Haemophilus influenzae*, which are typical infectious bacteria of the respiratory tract, and also *Helicobacter pylori*, which is well known to infect the stomach. Many intestinal bacteria species that are infectious or related to food poisoning, such as *Escherichia coli*, *S. enterica*, and *Staphylococcus aureus*, were also bound. Table 7 shows the pathotypes, strains, and serogroups of *E. coli* to which salivary IgA bound. All 5 pathotypes and most of the serogroups were included. Four subspecies and 34 serovars of *S. enterica* were found to be bound by salivary IgA (Table 8).

DISCUSSION

The present study investigated the salivary IgA microorganism-binding profile in 12 healthy adults. It is clear that salivary IgA binds numerous species of pathogenic viruses, bacteria, and fungi. Salivary IgA not only bound to a variety of species but also many different subtypes and strains of influenza virus, pathotypes and serogroups of *E. coli*, and serovars of *S. enterica*.

It has been reported that IgA antibodies against SARS-CoV-2 are present in the saliva of individuals who have never been infected with COVID-19 [24]. In the present study, we also documented the presence of salivary IgA binding to various different human coronaviruses in both SARS-CoV-2 vaccinees and non-vaccinees (Table 4). This suggests that IgA produced against coronaviruses during past infections also binds to new coronaviruses with similar molecules. More than half of the 12 subjects possessed salivary IgA that bound a variety of viral proteins, especially SARS-CoV-2, SARS-CoV, MERS-CoV, and HCoV spike proteins (Table 4). Because binding of the spike protein to the ACE2 receptor of target cells is the first step in coronavirus infection, IgG antibodies against the spike protein of SARS-CoV-2 have been developed as therapeutic antibody drugs [25]. However, various strains of coronaviruses with mutations in the spike protein have been reported [26, 27]. This implies that

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Table 5. Influenza virus strains bound by salivary IgA

Type and Subtype	Viral strains (total 95 strains)
Influenza A virus H1N1	A/Beijing/262/1995, A/Bellamy/1942, A/California/06/2009, **A/California/07/2009**, A/Denver/1957, A/England/195/2009, A/Fort Monmouth/1/1947, A/Fort Warren/1/1950, A/Hickox/1940, A/Malaya/302/1954, A/Memphis/1/1987, **A/New Caledonia/20/1999**, A/New Jersey/8/1976, A/New York/1/18, A/New York/18/2009, A/Phila/1935, A/Puerto Rico/8/1934, **A/Solomon Islands/3/2006**, A/USSR/90/1977, A/WSN/1933 (20 strains)
Influenza A virus H1N3	A/duck/NZL/160/1976
Influenza A virus H1N9	A/mallard/Ohio/265/1987
Influenza A virus H2N2	A/Canada/720/2005, A/Guiyang/1/1957, A/mallard/New York/6750/1978 (3 strains)
Influenza A virus H3N1	A/swine/Korea/PZ72-1/2006
Influenza A virus H3N2	A/Bangkok/1/1979, A/Brisbane/10/2007, A/England/878/1969, A/Fujian/411/2002, A/Guangdong-Luohu/1256/2009, A/Hanoi/EL134/2008, A/Hanoi/EL201/2009, A/Hong Kong/1/1968, A/Hong Kong/CUHK31987/2011, A/Johannesburg/33/1994, A/Missouri/09/2014, A/Nanchang/933/1995, **A/Panama/2007/1999**, A/Perth/16/2009, A/Philippines/472/2002, A/Port Chalmers/1/1973, A/Shandong/9/1993, **A/Switzerland/9715293/2013**, **A/Texas/50/2012**, A/Victoria/208/2009, **A/Victoria/361/2011**, A/Wisconsin/67/2005, A/Wuhan/359/1995, **A/Wyoming/03/2003**, A/X-31 (25 strains)
Influenza A virus H4N2	A/duck/Hunan/8-19/2009
Influenza A virus H4N4	A/mallard duck/Alberta/299/1977
Influenza A virus H4N6	A/mallard/Ohio/657/2002
Influenza A virus H5N1	A/Anhui/1/2005, A/Duck/Hong Kong/p46/97, A/Viet Nam/1203/2004 (3 strains)
Influenza A virus H5N2	A/chicken/Iowa/04-20/2015
Influenza A virus H5N6	A/black swan/Akita/1/2016, A/Sichuan/26221/2014 (2 strains)
Influenza A virus H5N8	A/broiler duck/Korea/Buan2/2014, A/chicken/Netherlands/14015526/2014, A/turkey/Germany-MV/R2472/2014 (3 strains)
Influenza A virus H5N9	A/chicken/Italy/22A/1998
Influenza A virus H7N7	A/equine/Prague/1/1956
Influenza A virus H7N9	A/Anhui/1/2013, A/Shanghai/02/2013 (2 strains)
Influenza A virus H9N2	A/Hong Kong/1073/99, A/turkey/Wisconsin/1966 (2 strains)
Influenza A virus H9N5	A/shorebird/DE/261/2003
Influenza A virus H10N3	A/mallard/Minnesota/Sg-00194/2007
Influenza A virus H10N8	A/Jiangxi/IPB13/2013
Influenza A virus H10N9	A/duck/Hong Kong/562/1979
Influenza A virus H11N2	A/duck/Yangzhou/906/2002
Influenza A virus H12N5	A/duck/Alberta/60/1976
Influenza A virus H13N6	A/black-headed gull/Sweden/1/1999
Influenza A virus H14N5	A/mallard/Astrakhan/263/1982
Influenza A virus H17N10	A/little yellow-shouldered bat/Guatemala/164/2009
Influenza A virus H18N11	A/flat-faced bat/Peru/033/2010
Influenza B virus	B/Brigit, **B/Florida/4/2006**, B/Florida/7/2004, B/Lee/1940, **B/Malaysia/2506/2004**, B/Maryland/1/1959, **B/Massachusetts/02/2012**, B/Massachusetts/3/1966, **B/Phuket/3073/2013**, B/Qingdao/102/91, B/R22 Barbara, B/R5, B/R75, B/Singapore/222/1979, B/Utah/02/2012, B/Victoria/504/2000 (16 strains)

Viral strains which had been included in IFV vaccine since 2001 in Japan are indicated in bold.

Table 6. Families of bacteria and fungi bound by salivary IgA

Class	Families
Bacteria (60 families, 208 species)	*Actinomycetaceae* (4), *Aerococcaceae* (6), *Aeromonadaceae* (5), *Alcaligenaceae* (2), *Bacillaceae* (1), *Bacteroidaceae* (4), *Brevibacteriaceae* (1), *Brucellaceae* (1), *Bruguierivoracaceae* (1), *Burkholderiaceae* (1), *Campylobacteraceae* (2), *Carnobacteriaceae* (1), *Chlamydiaceae* (3), *Clostridiaceae* (5), *Corynebacteriaceae* (4), *Deinococcaceae* (1), *Dermabacteraceae* (1), *Enterobacteriaceae* (29), *Enterococcaceae* (1), *Eubacteriales incertae sedis* (1), *Flavobacteriaceae* (4), *Fusobacteriaceae* (1), *Gordoniaceae* (1), *Hafniaceae* (1), *Helicobacteraceae* (3), *Lachnospiraceae* (6), *Lactobacillaceae* (4), *Legionellaceae* (3), *Leptotrichiaceae* (1), *Listeriaceae* (1), *Micrococcaceae* (1), *Moraxellaceae* (12), *Morganellaceae* (4), *Mycobacteriaceae* (12), *Mycoplasmataceae* (3), *Neisseriaceae* (2), *Paenibacillaceae* (1), *Pasteurellaceae* (11), *Pectobacteriaceae* (4), *Porphyromonadaceae* (1), *Prevotellaceae* (4), *Pseudomonadaceae* (6), *Rhizobiaceae* (1), *Rhodobacteraceae* (1), *Ruminococcaceae* (1), *Segniliparaceae* (1), *Shewanellaceae* (1), *Sphingobacteriaceae* (1), *Sphingomonadaceae* (2), *Spirochaetaceae* (1), *Staphylococcaceae* (2), *Streptococcaceae* (9), *Streptomycetaceae* (7), *Succinivibrionaceae* (1), *Tannerellaceae* (1), *Tissierellaceae* (1), *Vibrionaceae* (1), *Victivallaceae* (1), *Weeksellaceae* (3), *Yersiniaceae* (13)
Fungi (28 families, 58 species)	*Arthrodermataceae* (3), *Aspergillaceae* (18), *Chaetomiaceae* (1), *Cladosporiaceae* (3), *Cryptococcaceae* (1), *Debaryomycetaceae* (4), *Didymellaceae* (1), *Dipodascaceae* (1), *Filobasidiaceae* (1), *Herpotrichiellaceae* (1), *Hypocreaceae* (1), *Massarinaceae* (1), *Metschnikowiaceae* (1), *Microascaceae* (2), *Mucoraceae* (1), *Myxotrichaceae* (1), *Ophiostomataceae* (1), *Pichiaceae* (1), *Pleosporaceae* (3), *Rhizopodaceae* (1), *Saccharomycetales incertae sedis* (4), *Saccotheciaceae* (1), *Sarocladiaceae* (1), *Sclerotiniaceae* (1), *Sporidiobolaceae* (1), *Trichocomaceae* (1), *Trichomonascaceae* (1), *Trichosporonaceae* (1)

The number in parentheses after the family name indicates the number of species.

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Table 7. *E. coli* pathotypes, strains, and serogroups bound by salivary IgA

Pathotypes	Enterohemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC)
Strains	55989, K-12, MDR, UTI89
Serogroups	O1, O2, O3, O4, O5, O6, O8, O9, O11, O15, O18, O21, O25, O26, O28, O29, O39, O45, O48, O51, O52, O53, O55, O56, O59, O60, O61, O62, O63, O64, O65, O68, O69, O70, O71, O73, O76, O77, O78, O79, O80, O82, O83. O84, O85, O86, O87, O88, O91, O92, O93, O95, O96, O97, O98, O99, O100, O101, O102, O103, O104, O109, O111, O112, O113, O114, O115, O116, O119, O121, O123, O124, O126, O127, O128, O131, O132, O135, O136, O137, O138, O139, O140, O141, O142, O143, O145, O146, O149, O152, O153, O155, O156, O157, O158, O159, O161, O162, O164, O167, O168

Table 8. *Salmonella* species and serovars bound by salivary IgA

Species	Serovars
Salmonella enterica subsp. *arizonae*	-
Salmonella enterica subsp. *enterica*	Aberdeen, Anatum, Brandenburg, Cerro, Choleraesuis, Decatur, Derby, Deversoir, Durban, Enteritidis, Essen, Gallinarum, group III, Halle, Java, Kentucky, Landau, Lomita, London, Minnesota, Montevideo, Muenchen, Nairobi, Newport, Niarembe, Onderstepoort, Oranienbur, Panama, Paratyphi B, Pullorum, Reading, Taksony, Typhi, Typhimurium
Salmonella enterica subsp. *houtenae*	-
Salmonella enterica subsp. *salamae*	-

IgG antibody drugs with high specificity for ancestral SARS-CoV-2 spike proteins may not be effective against new mutant strains. Indeed, both vaccines inducing IgG antibodies and antibody cocktail therapies have been reported to have decreased efficacy against SARS-Cov-2 mutant strains [28, 29]. On the other hand, secretory IgA is polymeric, and polymerization enhances cross-reactive properties, suggesting that mutations in the spike protein may have little effect on the binding of secretory IgA to SARS-CoV-2 targets. In a computational comparison study of spike proteins in coronaviruses, it was shown that SARS-CoV-2 and the endemic human coronaviruses are very similar [30]. Given this, it may be that polymeric secretory IgA, with its low specificity and high cross-reactivity, recognizes similarities of the antigen that specific IgG cannot. Mahla and Dustin stated that in order to respond to viral mutations, the next generation of antibody cocktail therapy requires polymerization of antibodies [29], and recently, the ability of dimeric IgA to neutralize SARS-CoV-2 has been investigated [19, 20, 31, 32]. In addition, it was reported that salivary IgA concentrations were low in COVID-19 patients and in those who had recovered, relative to asymptomatic controls negative for SARS-CoV-2 by polymerase chain reaction (PCR) [33]. Taken together, the findings suggest that salivary IgA may provide cross-protection against SARS-CoV-2 infection regardless of mutation due to its high propensity to cross-react. No differences in the values of IgA binding to each antigen of coronaviruses between vaccinees and non-vaccinees were observed, which may be due to the cross-reactive IgA in non-vaccinees acquired by previous coronavirus infections.

In addition to coronaviruses, outbreaks of various other viruses and bacteria have been recorded as a result of mutations. Since the 20th century, different subtypes or strains of influenza with mutations have repeatedly been the prevailing influenza viruses worldwide, such as H1N1 in 1918 H2N2 in 1957, H3N2 in 1968, and H1N1 in 2009 [34]. The shift in dominant viral strains is due to the fact that these viruses are constantly changing in two different ways: antigenic drift and antigenic shift [35]. Because mucosal IgA provides protection against influenza virus infection [36], the finding that salivary IgA reacted with multiple subtypes and strains of influenza virus regardless of the epidemic strain or vaccine strain (Table 5) indicates its importance for protecting against viral infection.

Food-borne disease resulting in intestinal bacterial infection also requires attention. *E. coli*, *S. enterica*, *Campylobacter* spp., *Yersinia* spp., and *Listeria monocytogenes* are the most common cause of food-poisoning in OECD countries [37]. Interestingly, salivary IgA also bound these pathogenic bacteria (Supplementary Table 1). *E. coli* is a commensal bacterium in the human intestine and is usually non-pathogenic. However, highly adapted *E. coli* clones may acquire virulence and cause a broad spectrum of disease [38]. Pathogenic *E. coli* are distinguished as enterohemorrhagic, enterotoxigenic, enteropathogenic, enteroinvasive, and enteroaggregative based on six characteristics. These different pathotypes of *E. coli* are also defined as serogroups according to their shared O (lipopolysaccharide) antigens, such as O26, O111, and O157. These pathogenic variants of *E. coli* result in much morbidity and mortality worldwide by causing significant diarrheal and extraintestinal diseases [39]. Even though there is such a wide variety of *E. coli*, salivary IgA was found to bind many of them (Table 7). Similarly, *Salmonella enterica* subsp. *enterica* serovars Enteritidis and Typhimurium are the most common serovars worldwide [40], and salivary IgA also bound multiple species or serovars of them (Table 8). Currently, the presence of antimicrobial-resistant bacteria, which cause serious disease outbreaks, is becoming an increasing public health concern worldwide [40]. Such antimicrobial resistance occurs in a variety of bacteria, including *E. coli*, *Salmonella* spp., *Klebsiella pneumoniae*, *L. monocytogenes*, *S. pneumoniae*, and *S. aureus* [41, 42]. We found that salivary IgA still binds the above bacteria (Supplementary Table 1). In addition, methicillin-resistant and methicillin/vancomycin-resistant strains of *S. aureus* exhibited relative $\log_2$ ratios >0.5849 (1.5-fold the negative control) in 11 and 7 of the 12 subjects, respectively (data not shown). This indicates that the binding of salivary IgA is not affected by antimicrobial resistance. Antibodies are thought to be useful in

controlling the spread of antimicrobial-resistant bacteria, and a variety of vaccines are being developed [43, 44].

Vaccines have also been developed against viruses with the aim of producing neutralizing antibodies, but in the case of influenza, for example, there is a constant threat of the emergence of a highly pathogenic virus with a high fatality rate, or a pandemic, due to the occurrence of new pathogenic reassortants. However, current vaccines against influenza are likely to provide little protection against such viruses in the event of an epidemic or pandemic because of the nature of the antigens presented to adaptive immunity, which induces highly specific IgG antibodies [45]. Therefore, a universal influenza vaccine that would cover most or all seasonal strains and also provide protection during a pandemic is highly desirable [46]. Development of pan-coronavirus vaccines is also being attempted to provide protection not only from SARS-CoV and SARS-CoV-2 but also from a potential “SARS-CoV-3”, which is expected to arise sometime in the future [47, 48]. Mucosal vaccines inducing secretory IgA have been successfully used against poliovirus and are also being developed against influenza virus and SARS-CoV-2 [49–52]. Because there are so many subtypes and strains of pathogenic microorganisms, which are often transmitted through mucosal surfaces, such as the respiratory and intestinal tracts, it may be useful to modulate mucosal immunity and increase cross-protective IgA.

The viruses bound by salivary IgA in the present study included those to which the participants had little or no previous exposure, such as human immunodeficiency virus 2, dengue virus, Zika virus, and ebolavirus (Table 3). B cells produce antibodies with the same antigen recognition site as the B-cell receptor (BCR) expressed on the cell surface, which varies from cell to cell. Thus, the diversity of the BCR repertoires results in a variety of antibodies that bind to different antigens. Regarding the diversity of BCR repertoires, antibodies produced by infections or vaccines are initially produced by B cells with low specificity and cross-reactive BCR repertoire, after which repeated exposure to the antigen leads to the production of antibodies with high specificity and affinity [53]. In combination with the results of the present study, it can be inferred that IgA-producing cells differentiate from IgA-positive cells with a low specificity and cross-reactive BCR and secrete IgA in mucosal tissues. Although further research is needed, we believe that increasing secretory IgA in mucosal tissues may inhibit not only pathogens that have infected people in the past but also new mutant strains and emerging infections.

Gut microbiota dysbiosis is known to be exhibited in IgA-deficient subjects [54]. Sugahara *et al.* reported that the gut microbiota of the elderly has higher proportions of *Clostridiaceae* and *Enterobacteriaceae*, to which pathogenic and inflammation-causing bacterial groups belong, than healthy adults and that the fecal IgA of the elderly is less responsive to these bacteria [55]. It has also been suggested that high-fat diet-induced gut microbiota dysbiosis is caused by a decrease of intestinal IgA [56]. These reports indicate that mucosal IgA may regulate the gut microbiota by excluding pathogenic microorganisms. The present study demonstrates that salivary IgA reacts with a large number of microorganisms, suggesting that mucosal IgA antibodies may modulate not only the gut but also the oral and respiratory tract microbiota.

This study was conducted on a small number of subjects, and the results were obtained by qualitative microbial protein microarray assays. Therefore, although the salivary IgA-binding antigens were identified, the amount of IgA antibodies against each antigen was not evaluated. The details of the binding sites of homologous antigen to which IgA binds are also unknown. Previous infection or vaccination may induce IgA antibodies in saliva. However, pathogenic microorganisms other than SARS-CoV-2, HBV, HCV, HIV, and syphilis were not surveyed in this study. Further investigation is required to understand the importance of secretory IgA, including salivary IgA, in infectious diseases.

In conclusion, the saliva of healthy adults contains IgA antibodies that bind to a variety of pathogenic microorganisms, some of which would not have been previously encountered by them. Polymeric secretory IgA has higher cross-reactivity than other subclasses of immunoglobulin, which may be one of the reasons why it binds so many subtypes and strains of pathogens. With such broad cross-reactivity, secretory IgA is an essential element of mucosal immunity at the forefront of defense, providing protection against infection not only from previously encountered pathogens but also from novel pathogens, such as mutant strains. Therefore, increasing secretory IgA, such as salivary IgA, may be important for preventing infection.

CONFLICT OF INTEREST

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