Check for updates

RESEARCH ARTICLE

REVISED Novel multiplex assay for profiling influenza antibodies in breast milk and serum of mother-infant pairs [version 2; referees: 2 approved]

Kirsi M. Järvinen [iD] [1], Jiong Wang[2], Antti E. Seppo[1], Martin Zand [iD] [2,3]

[1]Department of Pediatrics, University of Rochester Medical Center, Rochester, NY, 14642, USA
[2]Department of Medicine, Division of Nephrology, University of Rochester Medical Center, Rochester, NY, 14642, USA
[3]Rochester Center for Health Informatics, University of Rochester Medical Center, Rochester, NY, 14642, USA

Abstract

Background: During early life, systemic protection to influenza is passively provided by transplacental transfer of IgG antibodies and oral and gastrointestinal mucosal protection via breast milk (BM) containing predominantly IgA. Immune imprinting, influenced by initial exposure of the infant immune system to influenza, has recently been recognized as an important determinant of future influenza immune responses.

Methods: We utilized stored frozen BM from a prospective birth cohort to assess immune factors in human milk. The earliest available BM and a paired, timed serum sample was assessed from each of 7 mothers. Paired infant serum samples were assayed at up to three time points during the first 12 months of life, one prior to assumed disappearance of transplacentally transferred IgG, and one after. We utilized a novel multiplex assay to assess mothers' and infants' IgG and IgA antibodies in serum to a panel of 30 individual recombinant hemagglutinin (rHA) proteins of influenza virus strains and chimeric rHAs. We also characterized IgA and IgG antibody levels in breast milk which provide mucosal protection.

Results: Our pilot results, analyzing a small number of samples demonstrate the feasibility of this method for studying paired maternal-infant IgG and IgA anti-influenza immunity patterns. Unlike IgG antibodies, breast milk influenza virus HA-specific IgA antibody levels and patterns were mostly discordant compared to serum. As expected, there was a steady decay of infant influenza specific IgG levels by 6 to 8 months of age, which was not, however, comparable in all infants. In contrast, most of the infants showed an increase in IgA responses throughout the first year of life

Conclusions: This new analytical method can be applied in a larger study to understand the impact of maternal imprinting on influenza immunity.

Keywords
breast milk, influenza, infants, protection, transplacental, antibody

Open Peer Review

Referee Status: ✔ ✔

	Invited Referees	
	1	**2**
REVISED **version 2** published 11 Mar 2019	✔ report	
version 1 published 20 Nov 2018	? report	✔ report

1 **Kirsty Le Doare** [iD], St George's University of London, UK

2 **David C. Dallas** [iD], Oregon State University, USA

Jiraporn Lueangsakulthai [iD], Oregon State University, USA

Any reports and responses or comments on the article can be found at the end of the article.

Corresponding author: Kirsi M. Järvinen (kirsi_jarvinen-seppo@URMC.Rochester.edu)

How to cite this article: Järvinen KM, Wang J, Seppo AE and Zand M. **Novel multiplex assay for profiling influenza antibodies in breast milk and serum of mother-infant pairs [version 2; referees: 2 approved]** *F1000Research* 2019, **7**:1822 (https://doi.org/10.12688/f1000research.16717.2)

First published: 20 Nov 2018, **7**:1822 (https://doi.org/10.12688/f1000research.16717.1)

Introduction

The immune system in neonates and young infants is initially immature, without adequate protection against infections. The dogma is that during infancy, systemic protection is passively provided by transplacental transfer of IgG antibodies and oral and gastrointestinal mucosal protection via breast milk (BM) containing predominantly IgA and some IgG. However, the clinical protection provided by maternal influenza immunization or exposure varies by season and the corresponding match against circulating influenza strains. Therefore, maternal influenza exposure, whether through immunization or natural infection, provides maternal protection and has the potential to imprint the infant immune system and significantly impact infant morbidity and mortality, as recently comprehensively reviewed[1].

Maternal influenza immunization prior to or during pregnancy provides clinical protection with 70% efficiency in the infant[2–5]. Only one study has described IgA antibody levels in human milk to influenza[6]. Their results suggested that vaccination using a single strain of influenza A (A/New Caledonia/20/1999, H1N1) induced significantly higher IgA antibody levels than those seen in non-vaccinated, and those antibodies were positively correlated with viral neutralization. In addition, higher rates of exclusive breastfeeding in the first 6 months of life were associated with protection against febrile respiratory illness in the infants of vaccinated mothers, suggesting mucosal protection against influenza by BM antibodies. However, there is little data on how influenza antibody levels, or strain-specific antibody profiles, vary between mother's serum, BM and infant serum. Data on the kinetic changes in anti-influenza IgG profiles between mother-infant pairs are also largely lacking.

In this pilot study utilizing a novel multiplex assay, we assessed infant immunity to various influenza strains reflecting maternal anti-influenza IgG levels and profiles in serum, as well as characterized IgA antibody responses in breast milk, which are distinct and reflect mucosal immunity. This new analytical method was applied to a small number of samples showing feasibility and patterns suggestive that a larger study needs to be done to understand the impact of maternal imprinting on influenza immunity.

Methods

We utilized stored frozen human foremilk collected in the morning between hours 8 and 11 and 1–2 hours after the last feeding, from a prospective birth cohort recruited in 1997–2001 in Finland to assess immune factors in human milk[7]. As part of this study, breast milk and serum samples were collected during follow-up visits, and included colostrum and breast milk at 1 month, 3 months, 6 months, 9 months, and 12 months of duration of lactation. However, a few samples were never received due to missed visits or sample timing had to be moved due to illness or other difficulties in getting to the scheduled visits, and some samples have been used up in prior studies. In the present study, the earliest available BM and a paired, timed serum sample was assessed from each of 7 mothers; ranging from 3 days to 2 months post-partum. Paired infant serum samples were assayed at up to three time points during the first 12 months of life, one prior to assumed disappearance of transplacentally transferred IgG, and one after. The samples collected in this cohort have been stored at -80°C with no recurrent freeze-thaw cycles. Aliquots have successfully been used in the past for measurement of serum and BM antibody levels with good antibody levels detected both for IgG and IgA[7]. These mothers were unvaccinated, as guidelines for maternal influenza immunization were not in place at the time samples were collected. None of the infants had been vaccinated to influenza. Clinical characteristics and timing of samples available are shown in Table 1. The study was approved by the institutional review boards of the Helsinki University Central Hospital, the City of Helsinki, and the University of Rochester Medical Center, Rochester, NY.

We have developed a multiplex assay (mPlex-Flu) that simultaneously measures absolute antibody concentrations against up to 50 influenza strains[8]. The mPlex-Flu assay has several advantages over the traditional hemagglutinin inhibition (HAI) titer assay: a linear readout over 4 logs, and high sensitivity. Our previous studies also showed that mPlex-Flu assay results highly consistent with the results from HAI and ELISA assays in human pre-and post-influenza vaccine study[8]. In the present study, a

Table 1. Mother-infant pairs, demographics and time of sampling.

Dyad	Maternal age (years)	Breast milk sample	Infant age at serum sample (month of collection)	Exclusive BF length	Total BF length
1	32	3 weeks	2 month (Nov) 8 month (May) 12 month (Aug)	4 months	nk
2	31	3 days	1 month (Feb) 13 month (Feb)	2.75 months	8.25 months
3	32	2 months	4 month (July) 7 month (Oct)	0 months	>7 months
4	32	3 days	2 month (July) 4 month (Sep) 6 month (Nov)	0 months	6 months
5	26	1 month	5 month (Sep) 7 month (Nov)	0 months	>7 months
6	23	4 days	3.5 month (Aug)	3.5 months	nk
7	30	5 days	2 month (July) 4 month (Sep) 6 month (Nov)	0 months	2 months

nk, not known; BF, breastfeeding.

panel of 30 individual recombinant hemagglutinin (rHA) proteins of influenza virus strains and chimeric rHAs were used (see Table 2). This allowed us to estimate the specific anti-influenza IgG and IgA levels against H1, H2, H3 and Flu B seasonal influenza strains, as well as HA stalk specific antibodies using chimeric rHA (i.e. head from one influenza strain and stalk from another strain), cH5/1 and cH9/1 specific for group 1 (i.e. H1, H2, H5, H6) and cH4/7 and cH5/3 for group 2 (i.e. H3, H7) influenza strains, as previously described[8]. The mPlex-Flu assay has been shown to strongly correlate with functional assays of influenza specific antibodies, including the hemagglutination inhibition (HAI) and influenza virus micro-neutralization assays[8,9]. In addition, we have previously shown that the mPlex-Flu assay has excellent strain specificity using competitive binding studies[8], which we also used here to confirm our results in human breast milk samples (Supplementary Figure 1). These data suggest minimal non-specific binding in paired samples. As we and others have previously demonstrated, there is cross-reactivity of anti-HA antibodies between antigenically similar influenza strains based on similar or shared epitopes.

In the present study, samples of maternal serum (diluted 1:500 for IgA and 1:5000 for IgG), infant serum (1:10) and BM (1:10) were diluted using PBS and incubated with rHA coupled Luminex beads (Luminex Corp, Austin, TX). IgG or IgA binding was detected with anti-human IgG or IgA specific secondary antibodies (SouthernBiotech, AL, Cat No 2040-09, 2050-09, respectively). Median fluorescence intensities (MFI) were measured using a MAGPIX multiplex reader (Luminex Co.,TX) and converted into absolute IgG concentrations (ng/mL) using a IgG standard curve generated with a human standard serum, a mixture of sera from four subjects containing high levels of anti-influenza HA IgG and IgA against multiple influenza strains[8]. Since serum IgA is monomeric, while BM secreted IgA

(SIgA) is dimeric[10], the standard curves of BM SIgA against influenza viruses are very different from that of serum standard curves generated from our human standard serum sample. We thus report the magnitude of BM IgA anti-influenza HA antibody levels in MFI units. For consistency, and to allow direct comparison, we also report IgG levels in MFI units. All data were analysis by Prism 7, and heatmap figures were generated by Mathematic 11.2.

Results

This new analytical method was applied to a small number of samples showing feasibility and several interesting patterns. BM had a pattern of IgG reactivity very similar to maternal serum. Also, the levels and strain reactivity patterns of anti-influenza IgG in mother's serum matched that of her infant, suggesting a robust transplacental transfer of antibodies. As expected, there was a steady decay of infant influenza specific IgG levels by 6 to 8 months of age (Figure 1A). This decay was, however, not comparable in all infants. Interestingly, mothers with highest anti-influenza HA IgG antibodies had infants with high initial anti-HA antibody maintained until 6 months of age (Pairs #3 and #7), compared to a mother with the lowest initial IgG (Pair #4), suggesting that initial levels attained transplacentally are directly associated with the rate of decline of passive systemic immunity. By the end of the first year, infant #1 maintained 6-month IgG antibody levels, which is likely due to new, natural exposure. Supplementary Figure 2 shows the heatmaps of IgG and IgA antibodies to influenza strains measured by multiplex array in paired mother's serum (MS), breast milk (BM) and infant serum (IS) samples. Figure 2A shows the trajectory of IgG antibodies to selected individual strains.

Unlike with IgG antibodies, BM influenza virus HA-specific IgA antibody levels and patterns differed from serum IgA

Table 2. The HA panel of the mPlex-Flu assay.

Influenza Virus Type	Subtypes	Full Name of Viruses	Abbreviation	Genbank Accession #	Bead Region
A	H1	A/South Carolina/01/1918	A/SC18	AF117241.1	35
		A/PR/8/34	A/PR8	CY148243.1	37
		A/USSR/1977	A/USSR77	DQ508897.1	55
		A/Texas/36/91	A/Tex91	DQ508889.1	43
		A/New Caledonia/20/1999	A/NewCal99	CY125100.1	18
		A/California/07/2009	A/Cali09	FJ966974.1	54
	H2	A/Japan/305/1957	A/Jap57	L20407.1	19
	H3	A/HongKong/1/1968	A/HK68	CY009348.1	53
		A/Port Chalmers/1/1973	A/PC73	CY112249.1	52
		A/Perth/16/09	A/Per09	GQ293081.1	47
		A/Victoria/361/11	A/Vic11	KM821347	56
		A/Texas/50/2012	A/Tex12	KC892248.1	36
		A/Switzerland/2013	A/Swi13	EPI537866	22
	H5	A/Viet Nam/1203/2004	A/Viet04	EF541403	30
	H6	A/chicken/Taiwan/67/2013	A/TW13	KJ162860.1	33
	H7	A/rhea/North Carolina/39482/1993	A/rheaNC93	KF695239	39
		A/Shanghai/1/2013	A/SH13	KF021597.1	44
	H9	A/guinea fowl/Hong Kong/WF10/1999	A/gfHK99	AY206676.1	42
B		**B/Malaysia/2506/2004**	B/Maly04	CY040449.1	51
		B/Brisbane/60/2008	B/Bris08	CY115343	46
		B/Wiscosin/01/2010	B/Wis10	KC306166.1	45
		B/Massachustts/2/2012	B/Mass12	KF752446.1	65
HA domains		Head of A/duck/Czech/1956	H4 Head		30
		Head of A/Shanghai/1/2013	H7 Head		25
		head of A/Indonesia/5/05	H5 Head		26
		Head of A/guinea fowl/Hong Kong/ WF10/1999	H9 head		48
Chimeric HA		cH5/1 (A/Indonesia/5/05, A/California/07/2009)	cH5/1Cal09		27
		cH9/1 (A/gf/HK/WF10/1999, A/California/07/2009)	cH9/1Cal09		20
		cH5/1 (A/Indonesia/5/05, A/Perth/16/09)	cH5/3		28
		cH4/1 (A/duck/Czech/1956, A/Shanghai/1/2013)	cH4/7		29

Seasonal Vaccine strains in Bold

Different colors determine the influenza virus type or subtypes. Influenza B virus showed in Green. Bark blue, gray, and light blue, purple and dark green present H1, H2, H5, H6 and H9 subtype influenza viruses, respectively, HA phylogenic group 1 viruses. Red and orange present H3 and H7, HA phylogenic group 2 viruses. Other black present the HA1 domains of HA or chimeric HAs.
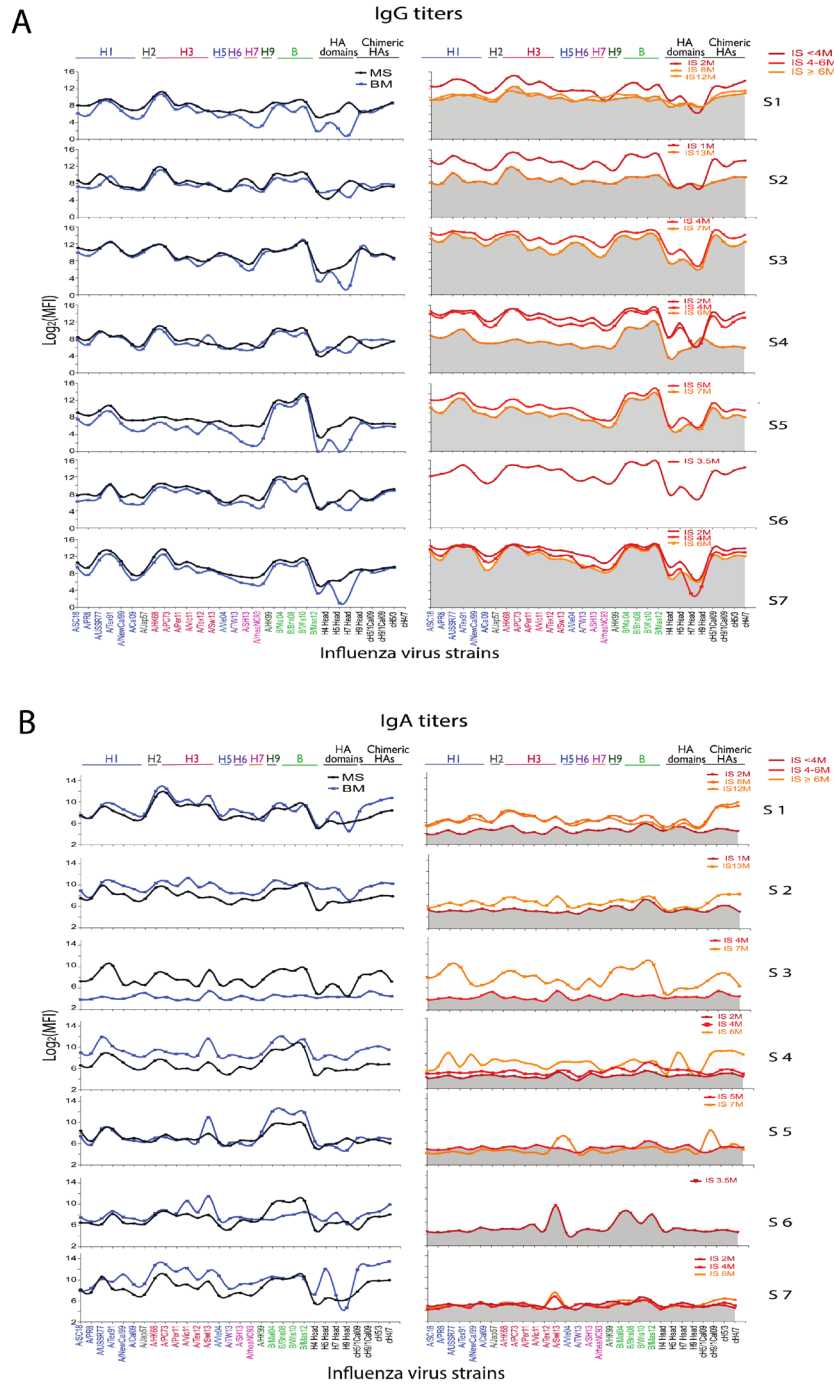
Figure 1. Influenza virus hemagglutinin (HA)-specific antibody IgG and IgA levels in paired mother-infant samples. The antibody levels against homologue and cross-reactive HA proteins from different influenza virus strains were evaluated by the mPlex-Flu assay using paired mother's serum (MS), breast milk (BM) and infant's serum (IS) at different ages expressed as months. A. The IgG antibodies against individual HA of influenza virus strains. Maternal serum was diluted 1:5000, infant serum 1:10 and breast milk 1:10. The IgG antibodies against influenza virus HA were estimated using Phycoerythrin (PE)-conjugated anti-human IgG (γ chain specific) secondary antibodies (SouthernBiotech, AL) and shown as means of median fluorescence intensity (MFI) (n=3). The antibody titers (Log$_2$MFI) against individual rHA of influenza virus strains were plotted and connected by LOWESS curves. In the panel of IgG MFI units of infant serum samples, the gray is the area under HA antibody curve of oldest sampling time point in the same subject. B. The IgA antibodies against individual HA of influenza virus strains. Maternal serum was diluted 1:500, infant serum 1:10 and breast milk 1:10. Then IgA antibodies were detected using PE-conjugated anti-human IgA (α chain specific) secondary antibodies (SouthernBiotech, AL) and shown as the mean of median fluorescence intensity (MFI) (n=3). The antibody titers (Log$_2$MFI) against individual rHA of influenza virus strains were plotted and connected by LOWESS curves. In the panel of Ig MFI units of infant serum samples, the gray is the area under the HA antibody curve of the youngest time point in the same subject.
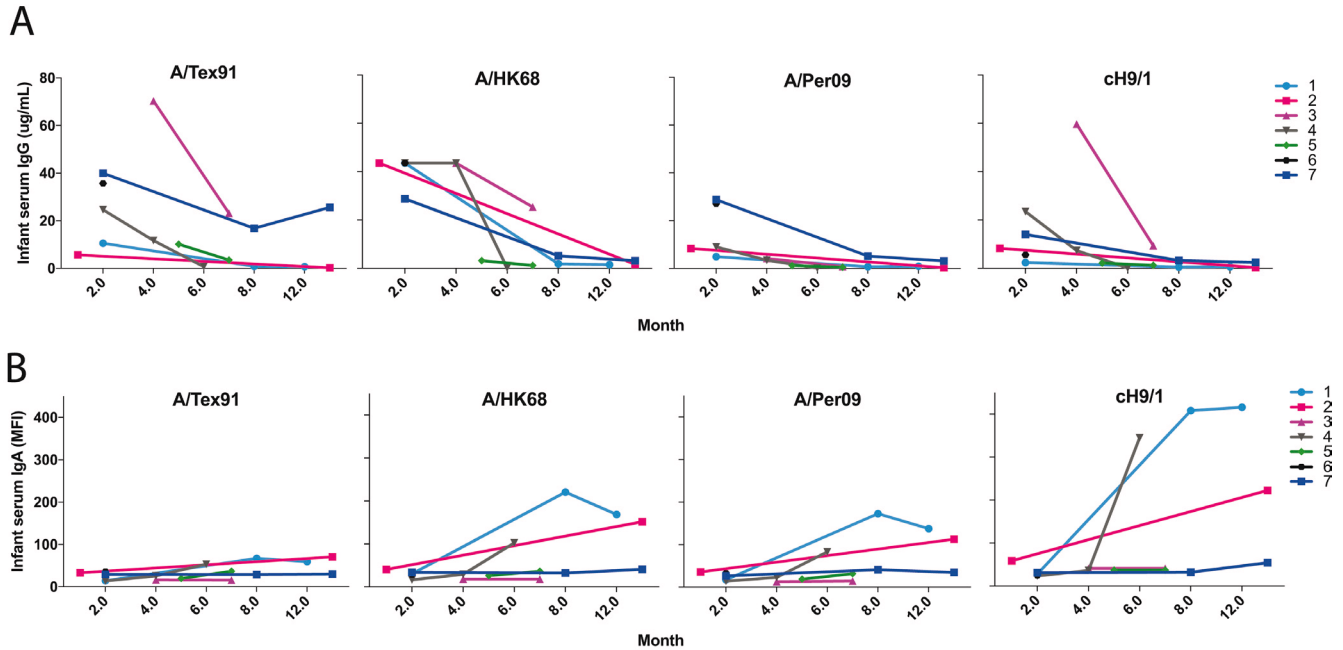
Figure 2. IgG antibody concentrations against selected influenza virus hemagglutinin (HA) in infants' sera. A. IgG antibody levels against selected influenza virus HA were plotted over time during first year (infant age in months) for 7 infant subjects from Figure 1A data. The specific antibody concentration of IgG is shown as the means for triplicates (n=3). **B.** The IgA antibody levels against selected influenza virus HA in infant sera were plotted over time during first year for 7 infant subjects from Figure 1B data. The specific antibody levels of IgA are shown as the means for MFI units (n=3).

reactivity patterns, when visually comparing the pattern of antibody reactivity. Only three mother-infant pairs showed high a degree of concordance (Pairs #1, #4, and #5). This may be due to the mucosal homing of IgA producing antibody-secreting cells to the mammary gland, resulting in a different antibody profile in breast milk from serum. Very low serum IgA antibodies in infants are consistent with the fact that IgA does not cross the placenta (Figure 1B). The pattern of IgA anti-HA antibody binding was largely similar to that of mother's serum and milk IgG, and predominantly against H1, H3 and B influenza strains. As opposed to infant IgG responses, most of the infants (Pairs #1–4) showed an increase in IgA responses throughout the first year of life (Figure 1B), whereas no matching IgG antibody response was seen. This may be due to natural, mucosal exposure to influenza inducing local responses, possibly in the absence of a systemic infection inducing IgG antibodies. Figure 2B shows the trajectory of IgA antibodies to a few individual strains. Both anti-stalk group 1 (cH5/1, cH9/1) and group 2 (cH5/3 and cH4/7) IgG (Figure 1A) and IgA antibodies (Figure 1B), which can confer cross-strain immunity, were abundant in the early months in infant serum and BM, respectively.

Dataset 1. Raw data for the present study

https://dx.doi.org/10.5256/f1000research.16717.d224137

Discussion

Our pilot data suggest feasibility for measuring antibody responses to influenza strains in maternal breast milk and paired infant serum utilizing a novel multiplex assay to assess changes over time. We show an anticipated decline in IgG responses to influenza HA in the first 5 months of life reflecting waning passive transplacentally acquired immunity, whereas the systemic IgA response in infants appears to be relatively poor, consistent with no vertical transfer. This does not exclude the possibility that such young infants might have a response at mucosal surfaces, such as in saliva upon exposure. Throughout the first year, however, a small increase in IgA antibodies, but not IgG antibodies, is seen in these unvaccinated infants, possibly suggesting that the adaptive immune response to natural exposure, in the absence of systemic infection induces initially local IgA, but not IgG antibodies. We also show that while the IgG specificity patterns were rather similar between breast milk and maternal serum, the patterns for IgA specificity were distinct and more pronounced in BM than those seen in serum. These data suggest that during this time, breast milk IgA may indeed be an important means of providing mucosal protection, which closely reflects maternal mucosal exposure to a variety of influenza strains to benefit the infant. Our previous results comparing food-specific IgA in human milk and maternal serum have shown similarly marked differences between human milk and serum IgA antibody profiles[7]. This is likely

reflecting the fact that IgA-producing cells in mammary gland originate in the gut- and bronchus-associated lymphoid tissue, which constitute an important defense mechanism of the newborn[11].

Although our study does not address the antibody profiles in vaccinated dyads, maternal influenza vaccination is recommended during pregnancy to induce infant post-partum passive immunity, and for infants after 6 months of age, although many choose to defer vaccination. As indicated by our pilot study, responses in mothers and infants are heterogeneous. At present, there is no robust literature or clinical method to optimize the vertical transfer of protective antibodies. Furthermore, the mechanisms of imprinting or maternal imprinting of the infant immune system are incompletely understood. Thus, there is a critical need for empirical data regarding maternal (serum, BM) and infant (serum) influenza-specific antibody levels over time to inform about maternal impact of influenza immunity, and to predict the individual window of infant susceptibility to influenza. Larger studies are required to further elucidate the interesting findings of this pilot study to aid in assessment of (maternal) imprinting of influenza immunity. Knowing the scope of passive immunity, both transplacental and that provided by BM, and when it vanishes, would allow for precision maternal-fetal and infant vaccination schedule design, also accounting for circulating influenza strains, seasonality, and vaccination status.

Data availability

F1000Research: Dataset 1. Raw data for the present study, https://doi.org/10.5256/f1000research.16717.d224137[12], including the following files:

- **Influenza-specific IgA antibody data as MFI.** The file contains the IgA antibody data expressed as MFI for a panel influenza strains generated by mPlex-Flu assay utilizing all breast milk and serum samples. (IgA_20160908_ MFI.xlsx)

- **Influenza-specific IgG antibody data as MFI.** The file contains the IgG antibody data expressed as MFI for a panel influenza strains generated by mPlex-Flu assay utilizing all breast milk and serum samples. (IgG_20160908_MFI.xlsx)

- **Comparison of influenza-specific IgA antibodies between paired samples.** The MFI titer comparison of IgA antibody of maternal serum (MS) vs breast milk (BM) and infant's serum (IS) over time using the Prism 7 software. (IgA version2018.pzfx)

- **Comparison of influenza-specific IgG antibodies between paired samples.** The file contains MFI unit comparison of influenza-specific IgG antibodies of maternal serum (MS) vs breast milk (BM) and infant's serum (IS) over time using the Prism 7 software. (IgG version2018.pzfx)

- Program code for IgA heatmap. The Mathematica 2 program code for generation of the heatmap figure of IgA data of maternal serum (MS), breast milk (BM) and infant's serum (IS) from mPlex-Flu assay. (IgA MFI Revised.nb)

- **Program code for IgG heatmap.** The Mathematica 2 program code for generation of the heatmap figure of IgG data of maternal serum (MS), breast milk (BM) and infant's serum (IS) from mPlex-Flu assay. (IgG MFI Revised.nb)

Supplementary material

Supplementary Figure 1. The specificity of mPlex-Flu assay for determination of IgG/IgA antibodies against influenza viruses in human breast milk samples. The specificity of mPlex-Flu assay determined by rHA protein pre-adsorption. The milk IgG or IgA antibody binding with/without rHA pre-absorption in mPlex-Flu assay is shown. The mean binding (MFI) showed in heatmap (n=4). We determine the specificity of mPlex-assay by using the antibody pre-adsorption assay as we previously described[8]. Briefly, breast milk samples (1:10 dilution, within the detectable range of mPlex assay) were pre-incubated with individual recombinant HA (rHA) protein of influenza virus 0.25 μg for 1 hour for pre-absorption of the HA specific antibodies in breast milk; then run the mPlex-Flu assay to show the antibody binding with/without pre-absorption of A/Texas/36/1991 (A/Tex91, H1), A/Victoria/361/2011 (A/Vic11, H3), B/ B/Massachusetts/2/2012 (B/Mass12, B influenza), respectively. The results showed Pre-absorption with rHAs of A/Vic11 significantly inhibited two H3 strains in IgA, and all 5 H3 strains in IgG testing of breast milk samples. Those H3 strains are antigenic similar to A/Vic11, but did not affect IgG and IgA antibody binding with other influenza virus strains. Similar results were observed when milk

samples were pre-absorbed by rHA of B/Mass12 and A/Tex91. Those results suggested a high type or subtype specificity of the mPlex-Flu assay for determination of human breast milk IgA and IgG anti-influenza strain specificity.

Click here to access the data

Supplementary Figure 2. Heatmaps of IgG and IgA antibodies to influenza strains measure by multiplex array in paired mother's serum (MS), breast milk, (BM) and infant serum (IS) samples. The antibody levels against homologue and cross-reactive HA proteins from different influenza virus strains were evaluated by the mPlex-Flu assay using paired mother's serum, breast milk (BM) and infant's serum at different ages expressed as months. A. The IgG antibodies against individual HA of influenza virus strains. MS samples were diluted 1:5000, BM samples were diluted 1:10, and IS samples were diluted 1:10. The IgG antibodies against influenza virus HA were estimated using Phycoerythrin (PE)-conjugated anti-human IgG (γ chain specific) secondary antibodies (SouthernBiotech, AL) and shown as mean median fluorescence intensity (MFI) (n=3). B. The IgA antibodies against individual HA of influenza virus strains. MS samples were diluted 1:500, BM samples were diluted 1:10 and IS samples were 1:10 diluted. Then IgA antibodies were detected using PE-conjugated anti-human IgA (α chain specific) secondary antibodies (SouthernBiotech, AL) and shown as MFI unit also (n=3).

Click here to access the data

References

1. Marchant A, Sadarangani M, Garand M, *et al.*: **Maternal immunisation: collaborating with mother nature.** *Lancet Infect Dis.* 2017; **17**(7): e197–e208.
   PubMed Abstract | **Publisher Full Text**

2. Manske JM: **Efficacy and effectiveness of maternal influenza vaccination during pregnancy: a review of the evidence.** *Matern Child Health J.* 2014; **18**(7): 1599–609.
   PubMed Abstract | **Publisher Full Text**

3. Zaman K, Roy E, Arifeen SE, *et al.*: **Effectiveness of maternal influenza immunization in mothers and infants.** *N Engl J Med.* 2008; **359**(15): 1555–64.
   PubMed Abstract | **Publisher Full Text**

4. Madhi SA, Cutland CL, Kuwanda L, *et al.*: **Influenza vaccination of pregnant women and protection of their infants.** *N Engl J Med.* 2014; **371**(10): 918–31.
   PubMed Abstract | **Publisher Full Text**

5. Tapia MD, Sow SO, Tamboura B, *et al.*: **Maternal immunisation with trivalent inactivated influenza vaccine for prevention of influenza in infants in Mali: a prospective, active-controlled, observer-blind, randomised phase 4 trial.** *Lancet Infect Dis.* 2016; **16**(9): 1026–35.
   PubMed Abstract | **Publisher Full Text** | **Free Full Text**

6. Schlaudecker EP, Steinhoff MC, Omer SB, *et al.*: **IgA and neutralizing antibodies to influenza a virus in human milk: a randomized trial of antenatal influenza immunization.** *PLoS One.* 2013; **8**(8): e70867.
   PubMed Abstract | **Publisher Full Text** | **Free Full Text**

7. Seppo AE, Savilahti EM, Berin MC, *et al.*: **Breast milk IgA to foods has different epitope specificity than serum IgA-Evidence for entero-mammary link for food-specific IgA?** *Clin Exp Allergy.* 2017; **47**(10): 1275–84.
   PubMed Abstract | **Publisher Full Text**

8. Wang J, Hilchey SP, Hyrien O, *et al.*: **Multi-Dimensional Measurement of Antibody-Mediated Heterosubtypic Immunity to Influenza.** *PLoS One.* 2015; **10**(6): e0129858.
   PubMed Abstract | **Publisher Full Text** | **Free Full Text**

9. Wang J, Hilchey SP, DeDiego M, *et al.*: **Broad cross-reactive IgG responses elicited by adjuvanted vaccination with recombinant influenza hemagglutinin (rHA) in ferrets and mice.** *PLoS One.* 2018; **13**(4): e0193680.
   PubMed Abstract | **Publisher Full Text** | **Free Full Text**

10. Fagarasan S, Kinoshita K, Muramatsu M, *et al.*: ***In situ* class switching and differentiation to IgA-producing cells in the gut lamina propria.** *Nature.* 2001; **413**(6856): 639–43.
    PubMed Abstract | **Publisher Full Text**

11. Peri BA, Theodore CM, Losonsky GA, *et al.*: **Antibody content of rabbit milk and serum following inhalation or ingestion of respiratory syncytial virus and bovine serum albumin.** *Clin Exp Immunol.* 1982; **48**(1): 91–101.
    PubMed Abstract | **Free Full Text**

12. Järvinen KM, Wang J, Seppo AE, *et al.*: **Dataset 1 in: Novel multiplex assay for profiling influenza antibodies in breast milk and serum of mother-infant pairs.** *F1000Research.* 2018.
    http://www.doi.org/10.5256/f1000research.16717.d224137

Open Peer Review

Current Referee Status: ✔ ✔

Version 2

Referee Report 13 March 2019

✔ **Kirsty Le Doare** iD

St George's University of London, London, UK

The queries have been answered.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Vaccine immunity

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Referee Report 04 February 2019

✔ **David C. Dallas** iD [1], **Jiraporn Lueangsakulthai** iD [2]

[1] Nutrition Program, School of Biological and Population Health Sciences, College of Public Health and Human Sciences, Oregon State University, Corvallis, OR, USA
[2] Nutrition Program, School of Biological and Population Health Sciences, College of Public Health and Human Sciences, Oregon State University, Corvallis, OR, USA

Overall, this is a very interesting method and an interesting application. I have a few comments.

Page 1, Methods: This should be "which can provide mucosal protection".

Page 3, Methods: Shouldn't it be -80 Celsius? Not Fahrenheit.
Page 3, Methods: Please specify how the dilutions used for each sample type were determined.

Page 4, Table 2: Need to specify the meaning of the coloring of the text. It is not clear why some are colored and some are not. The table only says that "seasonal vaccine strains are in bold" but does not mention the colors.

Page 5, Results, paragraph 2: How do you defined "concordance"? What are your thresholds for how similar it needs to be? Pair 4 (IgA titers) could also be argued to be concordant.

Page 6, Figure 1 legend: curvel -> curve? Curvel is written twice—not sure if this is a word I'm not familiar with or a misspelling of "curve".
Page 6, Figure 1: The text is very small. Can you figure out how to enlarge please?
Page 6, Figure 1 legend: MS abbreviation needs to be defined in the legend.
Page 6, Figure 1 legend: "IS", infant serum needs to be defined in legend.
Page 6, Figure 1 legend: Need to explain why Log2 was selected for this display.
Page 6, Figure 1 legend: Need to explain what "net" MFI refers to. What is the "net" part?
Page 6, Figure 1 legend: Unclear why Log2(MFI + 1) is used. What is the + 1 for?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: milk protein digestion and peptidomics

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 11 Mar 2019
Kirsi Jarvinen, University of Rochester Medical Center, USA

Overall, this is a very interesting method and an interesting application. I have a few comments.

COMMENT: Page 1, Methods: This should be "which can provide mucosal protection".
REPLY: corrected.

COMMENT: Page 3, Methods: Shouldn't it be -80 Celsius? Not Fahrenheit.
REPLY: corrected.

COMMENT: Page 3, Methods: Please specify how the dilutions used for each sample type were

determined.

REPLY: Dilutions were determined by pilot testing to ensure IgG and IgA influenza virus-specific antibody binding within the mPlex-Flu assay range, i.e. the detectable range of the mPlex-Flu assay (the lower to the upper limits of quantification (LLOQ and ULOQ) concentrations for each influenza HA and each Ig isotype) (Wang et al 2018, ref #8). The pilot testing had been performed to confirm the dilution of samples enables all samples in this detectable range. Usually, the dilutions of samples are not laborious to be determined since mPlex-Flu can detect four Log10 scales.

COMMENT: Page 4, Table 2: Need to specify the meaning of the coloring of the text. It is not clear why some are colored and some are not. The table only says that "seasonal vaccine strains are in bold" but does not mention the colors.
REPLY: The color determines the subtype of influenza viruses. We added the notes under the table to make it clear.

COMMENT: Page 5, Results, paragraph 2: How do you defined "concordance"? What are your thresholds for how similar it needs to be? Pair 4 (IgA titers) could also be argued to be concordant.
REPLY: By visually comparing the patterns of antibody reactivity. We agree that #4 is also concordant and have revised the manuscript accordingly.

COMMENT: Page 6, Figure 1 legend: curvel -> curve? Curvel is written twice—not sure if this is a word I'm not familiar with or a misspelling of "curve".
REPLY: should say curve. Corrected.

COMMENT: Page 6, Figure 1: The text is very small. Can you figure out how to enlarge please?
REPLY: We have increased the font on those figures. Thank you for pointing out.

COMMENT: Page 6, Figure 1 legend: MS abbreviation needs to be defined in the legend.
REPLY: Done

COMMENT: Page 6, Figure 1 legend: "IS", infant serum needs to be defined in legend.
REPLY: Done

COMMENT: Page 6, Figure 1 legend: Need to explain why Log2 was selected for this display.
REPLY: Log2 is customarily used for antibody titers to indicate fold-change.

COMMENT: Page 6, Figure 1 legend: Need to explain what "net" MFI refers to. What is the "net" part?
REPLY: It should say MFI, not net MFI. We have removed "net".

COMMENT: Page 6, Figure 1 legend: Unclear why Log2(MFI + 1) is used. What is the + 1 for?
REPLY: It should be Log_2MFI. Corrected in the manuscript.

Competing Interests: No competing interests were disclosed.

? Kirsty Le Doare iD

St George's University of London, London, UK

This interesting pilot study investigates the association between anti-influenza antibodies measured by Luminex in blood and breast milk.

Breast milk is a much understudied body fluid. However, the numbers in this study and the fact that the timing of breast milk collection varies so greatly means that the results are merely descriptive. Breast milk changes throughout feeding, by time of day and by time of collection relative to feeds. Thus the sampling, although I understand the rationale of convenience, means that the results are not interpretable as they currently are described.

It would be useful to add more detail from the original study on timing of sample collection etc. How do you account for different rates of decline? IgG rate of decline is uniform across many pathogens and I am unsure why this should be any different in your study. What was the half life and how does it compare to the other studies you cite?

Could the differences you see between pairs also be due to non-specific binding within your assay?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Vaccine immunity

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 28 Jan 2019
Kirsi Jarvinen, University of Rochester Medical Center, USA

COMMENT: Breast milk is a much understudied body fluid. However, the numbers in this study and the fact that the timing of breast milk collection varies so greatly means that the results are merely descriptive. Breast milk changes throughout feeding, by time of day and by time of collection relative to feeds. Thus the sampling, although I understand the rationale of convenience, means that the results are not interpretable as they currently are described.

REPLY: As mentioned under methods, we utilized stored frozen foremilk collected in the morning. The sample was not a convenience sample but taken according to the study protocol using strict criteria in a prospective manner. Specifically samples were foremilk (not mixed sampling or hindmilk) taken always between hours 8 and 11, and 1-2 hours after the last feeding.

COMMENT: It would be useful to add more detail from the original study on timing of sample collection etc.

How do you account for different rates of decline? IgG rate of decline is uniform across many pathogens and I am unsure why this should be any different in your study. What was the half life and how does it compare to the other studies you cite?

REPLY: As part of the prospective study, breast milk and serum samples were collected during followup visits, and included colostrum and breast milk at 1 month, 3 months, 6 months, 9 months, and 12 months of duration of lactation. However, a few samples were never received due to missed visits or sample timing had to be moved due to illness or other difficulties in getting to the scheduled visits, and some samples have been used up in prior studies. Therefore, not all sample time points are available for all subjects. Regarding decline in IgG, we agree that the levels decline throughout the lactation, as seen in Fig 2a. Due to the small number of samples, no statistical modeling or halflife calculations have been done.

COMMENT: Could the differences you see between pairs also be due to non-specific binding within your assay?

REPLY: The mPlex-Flu assay is a novel assay to estimate the specific antibody binding to recombinant hemagglutinin (rHA) of multiple influenza virus strains. It has been shown to strongly correlate with all functional assays of influenza specific antibodies, such as hemagglutination inhibition (HAI) assay and influenza virus micro-neutralization assay (Wang J et al 2015 Ref 8, Wang J et al 2018 (new reference added #9)). In addition, in our previous study, mPlex-Flu assay had been shown to have excellent specificity for each influenza virus strains using the HA specific bind blocking in multiple plex assay (Ref 8), and we also used this method to confirmed the specificity of mPlex-Flu assay using human milk samples is similar to using human serum samples (New Supplementary Fig 1). These data suggest minimal non-specific binding between pair samples. As we have demonstrated before, there is cross-reactivity of anti-HA antibodies between antigenically similar influenza strains, which is directed against specific B cell epitopes with similar or homologous sequences and antigenic similarity. This type of cross reactivity, while it may be broad, is specific and based on shared epitopes.

Competing Interests: None

The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias

- You can publish traditional articles, null/negative results, case reports, data notes and more

- The peer review process is transparent and collaborative

- Your article is indexed in PubMed after passing peer review

- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com

F1000Research