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Molecular characterization of influenza viruses from women and infants in Sarlahi, Nepal

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ABSTRACT

We used RT-PCR-electrospray ionization-mass spectrometry to identify subtypes and strains of influenza viruses detected during a maternal influenza immunization study in Nepal from May 2011 to April 2014. Hemagglutinin (HA) gene amino acid (aa) sequences of inferred reference strains were compared to those of the vaccines to determine impact of aa relatedness on vaccine efficacy (VE) and disease severity. Three influenza subtypes and many strains were identified. A(H3N2) strains with less than 13 aa differences in HA compared to vaccine strains (matched) showed higher VE than strains with 13 or more differences (mismatched). Yamagata lineage B strains, which were mismatched to the Victoria strain in the vaccine, demonstrated lower VE compared to Victoria strains. Differences in VE were not statistically significant. All A(H1N1pdm) matched the vaccine strain, with 10 or fewer aa differences. Except for women infected with vaccine-matched strains of influenza A, clinical signs and symptoms did not differ between vaccinated and unvaccinated participants.

1. Introduction

Influenza viruses are responsible for frequent epidemics worldwide, with varying seasonality and strain circulation. Vaccines are an effective strategy to prevent influenza disease, but rely on close antigenic similarity between the strains used for the vaccine and those circulating in the population. Because influenza subtypes, including the currently circulating A(H3N2), A(H1N1pdm), and B, are subject to antigenic drift, a lack of vaccine efficacy (VE) may arise from the circulation of strains antigenically unrelated to the vaccine strains (Durviaux et al., 2014; Tricco et al., 2013). Illness severity in vaccinated individuals who become infected with influenza may also depend on the antigenic relatedness of the infecting strain and the strain contained in the vaccine (Arriola et al., 2017).

The hemagglutination inhibition assay has been the gold-standard test for antigenic characterization and selection of influenza vaccine viruses by the World Health Organization (Ampofo et al., 2015). However, a number of limitations and difficulties are associated with the use of this assay (Ampofo et al., 2015). For example, contemporary A/H3N2 viruses do not hemagglutinate. Assessment of the genetic similarities of circulating and vaccine strains using molecular methods could lead to a more rapid prediction of VE without requiring viral culture, serology, or other advanced laboratory techniques. The ability to quickly assess potential circulating strain matches would help assure our approach to vaccination in developing countries is based on current data.

An aim of this study was to determine the diversity of subtypes and strains of influenza viruses circulating among vaccinated and unvaccinated pregnant and post-partum women, their infants from birth to 6 months old, and other family members during a 3-year study of maternal influenza immunization in the Sarlahi District of southern Nepal. Another aim was to examine the antigenic relatedness of circulating influenza strains to the vaccine strains and determine the impact on VE and disease severity. Using a rapid and high throughput molecular method to infer the identity of circulating influenza strains, we

Abbreviations: ESI-MS, Electrospray ionization-mass spectrometry; HA, Hemagglutinin; VE, Vaccine efficacy.

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phylogenetically compared the hemagglutinin (HA) gene amino acid (aa) sequences of the inferred reference strains to those of the vaccine strains used in the study.

2. Materials and methods

2.1. Study overview and specimen collection

Samples and clinical data were collected for a community-based, prospective, randomized, placebo-controlled trial of maternal influenza immunization of pregnant women and the impact on their infants in the Sarlahi District of rural Southern Nepal from April 2011 to May 2014. Detailed methods for and results of the trial have been published (Steinhoff et al., 2017; Tielsch et al., 2015). This study was approved by the IRBs of the Cincinnati Children's Medical Center, Johns Hopkins Bloomberg School of Public Health (JHBSPH), the Institute of Medicine at Tribhuvan University, Kathmandu, and the Nepal Health Research Council. IRBs at Seattle Children's Hospital, the University of Washington, and George Washington University granted oversight to the IRB at JHBSPH.

Pregnant women were continuously enrolled throughout the study and randomized to receive influenza vaccine or placebo. Northern and southern hemisphere trivalent inactivated vaccines were utilized sequentially; vaccines were administered within the recommended administration dates. Women in vaccine group 1 (A/H3N2 Perth/16/2009, A/H1N1 California/07/2009, B Brisbane/60/2008 (Victoria lineage)) were vaccinated from April 2011 to October 2012 and those in vaccine group 2 (A/H3N2 Victoria/361/2011, A/H1N1 California/07/2009, B Wisconsin/01/2010 (Yamagata lineage)) were vaccinated from October 2012 to September 2013.

At weekly home visits during pregnancy and for six months following delivery, mid-nasal nylon flocked swabs were collected if the women reported fever and one or more respiratory symptoms in the previous 7 days. Mid-nasal nylon flocked swabs were collected from their infants from birth to 6 months of age if they had any respiratory symptom reported in the previous 7 days. For this analysis, influenza positive samples from vaccinated women that were collected less than 2 weeks after vaccination were included in the placebo group. We also included influenza positive samples from infants born less than 2 weeks after mother's vaccination in the placebo group. From July 2011 to October 2012, mid-nasal swab samples were collected from family members of a subset of women enrolled in the study if they reported a respiratory symptom in the previous 7 days. Family members were adults and children less than 15 years old from households of women in vaccine group 1. Swabs were placed in PrimeStore Molecular Transport Medium (Longhorn Diagnostics LLC, Bethesda, MD) and refrigerated until shipment at room temperature to the testing laboratory where they were frozen at -80°C until testing.

2.2. Influenza detection and subtyping

Total nucleic acids were extracted from the nasal swabs and tested by a real-time reverse transcription (RT)-PCR assay targeting the influenza A and B matrix genes (Munro et al., 2013). Specimens positive for influenza A by the matrix gene assay were subtyped using real time RT-PCR assays targeting the specific HA genes of seasonal influenza A (H1N1), A(H3N2), and A(H1N1pdm) (Munro et al., 2013). Specimens positive for influenza B by the matrix gene assay were subtyped using a real-time RT-PCR assay targeting the specific HA genes of influenza B Victoria and Yamagata lineages . The influenza B lineage assay was performed using the same conditions as those used for the influenza A subtyping assays with forward primers 5′-AATCCTCAAAAGTTCA CMTCATCTG and 5′-GCCCCAGAAGTTCACCTCATC, reverse primers 5′-GGTAGTCCTCCGTCTTCTGTTTGA and 5′-TAGCCCTCCGTCTTCTGTTTGA and dual-labeled probes 5′-FAM-ATGTTTCTCAGATTGGC-MGBNFQ (Victoria) and 5′-VIC-ACGTTTCACAGATTGGT-MGBNFQ (Yamagata).

2.3. Influenza strain designation

Influenza positive samples with sufficient viral load (determined by PCR cycle threshold values <35), were tested using an RT-PCR-electrospray ionization-mass spectrometry method (RT-PCR/ESI-MS, Plex-ID, Abbott Diagnostics, Lake Forest, IL) (Tang et al., 2013). After RT-PCR, ESI-MS determined the individual molecular masses of eight PCR amplicons, including amplicons from one pan-influenza (PB1 gene), five influenza A (NP, M1, PA, NS1, and NS2 genes), and two influenza B (NP and PB2 genes) primer sets. An unambiguous base composition (BC) was derived from the mass spectral result for each amplicon and all BC results were combined to establish a BC signature (Jeng et al., 2012a; Sampath et al., 2007). The BC signature from an influenza strain in a nasal swab sample was compared to those of reference laboratory strains and influenza sequences in the NCBI nucleotide database. The influenza strain in the nasal swab was inferred to be the reference strain in the database that had a matching BC signature. Previous work has shown that the ESI-MS spectral results are strongly reflective of the influenza sequences and that this method has the ability to differentiate among genetic lineages and clades within the same subtype (Deyde et al., 2010; Sampath et al., 2007).

We determined the number of aa differences between the dictated aa sequences of the entire HA gene of the RT-PCR/ESI-MS inferred reference strains and those in the vaccines to determine their genetic relatedness. Previous studies have shown there is a rough correlation between genetic distances of the HA genes and antigenic distances of influenza variants (Koel et al., 2013; Smith et al., 2004). In one study (Smith et al., 2004), the dictated HA gene aa sequences of strains in different antigenic clusters differed by, on average, 13 aa. Based on these data, we considered influenza strains in our samples to be antigenically different from the vaccine strain if the HA gene aa sequences of the matching reference strains differed by 13 or more residues from those of the relevant vaccine strain (mismatched strain group). For each influenza subtype and the vaccine-matched and mismatched strain groups identified in samples from the women and infants enrolled in the influenza immunization trial, the relevant VE were determined using a previously published method that calculates confidence intervals by means of a Taylor series variance approximation (Hightower et al., 1988).

2.4. Illness severity

At each illness episode of the women and infants, the duration of signs and symptoms (number of days) was recorded, including cough, fever, sore throat, runny nose, and myalgia for women and cough, fever, wheezing, difficulty breathing, and drippy ear for infants. For each illness episode, the signs and symptoms were combined into a symptom score (the sum of all symptoms for all days) and an episode duration (the total duration of any symptom) (Lenahan et al., 2017). Care seeking was recorded for both women and infants and combined into a care rank score (no care=0, non-medical care only=1, medical care only=2, doctor or hospital care=3). Illness severity scores were compared between women and infants by influenza subtype, strain group, and vaccination status using Wilcoxon rank sum testing for medians. Statistical testing was performed using Stata 13.0 (College Station, TX).

3. Results

Participants in vaccine group 1 included 2726 women who received vaccine and their infants and 2709 women who received placebo and their infants. Vaccine group 2 included 941 vaccinated women and their infants and 963 women who received placebo and their infants. In addition, nasal swab samples were collected from 828 unvaccinated family members of women enrolled in the study, including 17% adults and 83% children less than 15 years old.

3.1. Influenza detection

Overall, influenza was detected in 431 samples from 118 women (31 vaccinated, 87 placebo), 201 infants (72 vaccinated, 129 placebo), and 112 family members (all unvaccinated). Influenza was detected in 26 (70%) of the 37 months in which specimens were collected (May 2011-May 2014). Influenza seasons were defined by consecutive months with at least one influenza positive sample/month separated by at least two months with no influenza positive samples. The study period could be divided into 3 influenza seasons; June 2011 through April 2012 (season 1), July 2012 through November 2012 (season 2), and February 2013 through November 2013 (season 3) with 140, 218, and 73 influenza positive samples, respectively. The number of samples positive for influenza during each season is shown in Table 1 by vaccination status, participant, and influenza subtype. Influenza A(H3N2) and influenza B of both the Victoria and Yamagata lineages were predominantly detected in season 1. In season 2, influenza A(H1N1pdm) and influenza B(Yamagata) strains were most frequently detected, while influenza A(H3N2) was again predominant in season 3.

3.2. Influenza subtyping and strain designation

Among 236 samples positive for influenza A, 40 (17%) could not be subtyped as H1 or H3, 115 (49%) were positive for influenza A(H3N2), and 81 (34%) were positive for influenza A(H1N1pdm); 83 (72%) H3N2 and 54 (67%) H1N1pdm positive samples had a strain designation by RT-PCR/ESI-MS. Among 195 samples positive for influenza B, 14 (7%) could not be subtyped regarding lineage, 49 (25%) were subtyped as Victoria, and 132 (68%) as Yamagata lineage; 141 (78%) had a strain designation by RT-PCR/ESI-MS. All RT-PCR/ESI-MS results provided the same influenza subtype as the real-time RT-PCR assays. The majority of influenza A and B positive samples that could not be further subtyped or evaluated by RT-PCR/ESI-MS had low viral loads (85% had PCR Ct values of 35 or higher). The average PCR Ct value of typed samples was 27.4 compared to 36.9 for samples with no subtype or strain designation. The proportion of samples that had results by RT-PCR/ESI-MS was similar for women, infants, and family member and for participants who received vaccine and placebo.

Over the 3-year study period, RT-PCR/ESI-MS identified multiple strains of each influenza subtype as determined by the number of unique BC signatures for each subtype. The strain number, the influenza reference strain designated by ESI-MS, and the number of samples positive for the strain in each influenza season are shown in Table 2. Most strains were detected in only one season. However, influenza B(Victoria) strain #6 and influenza B(Yamagata) strain #7 were detected in both seasons 1 and 2; influenza A(H3N2) strain #6 was detected in all 3 seasons, and influenza A(H1N1) strains #17 and #18 were detected in seasons 2 and 3.

In each season, one or two strains accounted for most detections of influenza A(H3N2) and B, with just occasional detections of several other strains. For example, influenza A(H3N2) strains #1 and #2 were identified in 81% (34 of 42) and strain #7 was identified in 87% (34 of 39) of H3N2-positive samples in seasons 1 and 3, respectively. Similarly, influenza B strains #1 and #6 were identified in 72% (31 of 43) and strain #8 was identified in 69% (68 of 98) of B-positive samples in seasons 1 and 2, respectively. The RT-PCR/ESI-MS results for influenza A (H1N1pdm)-positive samples identified 20 different BC signatures, most of which were in samples from season 2.

3.3. Strain group differences

All influenza B-positive samples were from participants in vaccine group 1. Influenza B reference strains identified by RT-PCR/ESI-MS fell into two strain groups, differing from the vaccine strain (Brisbane/60/2008, Victoria) by 6-9 (match) and 29-32 (mismatch) aa (Table 2). The strains that differed from the vaccine strain by 29-32 aa belonged to the influenza B Yamagata lineage. Vaccine 1, which contained only a Victoria lineage influenza B strain, would most likely fail to protect against influenza B Yamagata lineage strains.

Influenza A(H3N2) reference stains identified by RT-PCR/EIS-MS from participants in vaccine group 1 fell into two strain groups, differing from the vaccine strain (Perth/16/2009) by 5-10 (match) and 14-15 (mismatch) aa. Influenza A(H3N2) reference stains identified by RT-PCR/EIS-MS from participants in vaccine group 2 also fell into two strain groups, differing from the vaccine strain (Victoria/361/2011) by 9-11 (match) and 21 (mismatch) aa. All influenza A(H1N1pdm) reference strains differed from the single vaccine strain (California/07/2009) by 2-10 amino acids and were considered as one strain group that was closely related to the vaccine strain (match).

3.4. Vaccine efficacy

To examine how well the influenza vaccine protected against infection with the influenza subtypes and strains detected during the study, we calculated the relevant VE for each influenza subtype and strain

Table 1
Number of each influenza subtype detected by season, vaccination status, and participant

Season	Vaccination status	Participant	Influenza subtype						
			Influenza A			Influenza B			
			H3N2	H1N1pdm	A NT[a]	VIC[b]	YAM[b]	B NT[a]	Total
1 (June, 2011- April, 2012)	Vaccine	Women	4	0	2	1	0	1	8
		Infants	5	0	9	5	2	1	22
	Placebo	Women	17	0	4	11	2	0	34
		Infants	6	2	4	7	4	1	24
	Unvaccinated	Family members	22	1	7	19	3	0	52
	Total		54	3	26	43	11	3	140
2 (July, 2012-November, 2012)	Vaccine	Women	0	9	0	0	6	2	17
		Infants	1	5	1	2	25	5	39
	Placebo	Women	0	13	1	1	23	0	38
		Infants	1	25	6	1	29	2	64
	Unvaccinated	Family members	0	19	0	2	38	1	60
	Total		2	71	8	6	121	10	218
3 (February, 2013-November, 2013)	Vaccine	Women	5	0	1	0	0	0	6
		Infants	9	0	1	0	0	1	11
	Placebo	Women	9	6	0	0	0	0	15
		Infants	36	1	4	0	0	0	41
	Total		59	7	6	0	0	1	73

[a] Influenza A and B positive samples that could not be further subtyped.
[b] VIC, influenza B Victoria lineage; YAM, influenza B Yamagata lineage.

Table 2
Influenza strain designations for reference strains matched to the study samples by electrospray ionization-mass spectrometry (ESI-MS). The number of samples positive for the strain by influenza season and the number of hemagglutinin gene (HA) amino acid differences of each reference strain from the relevant vaccine strain HA gene are listed

			# Positive by season			# Amino acid differences from vaccine[b]	
Influenza subtype	Strain #/ Lineage[a]	ESI-MS influenza strain designation	1	2	3	Vaccine 1	Vaccine 2
B	1/V	Los Angeles/01/2002	17			9	
	2/Y	Missouri/NHRC0001/2005	4			31	
	3/V	Taiwan/71207/2004	2			9	
	4/Y	Aukland/01/2000	3			32	
	5/V	Taiwan/3246/2002	1			7	
	6/V	Tennessee/UR06-0431/2007	14	3		7	
	7/Y	Chantaburri/218/2003	2	5		29	
	8/Y	Guangzhou/01/2007		68		31	
	9/Y	Beijing/184/1993		8		29	
	10/Y	Taiwan/70513/2005		7		31	
	11/Y	ITCF-42168P2		4		NA[c]	
	12/V	Taiwan/01026/2005		2		6	
	13/Y	California/NHRC0001/2004		1		31	
	NT[d]	NA	14	39	1	NA	
A(H3N2)	1	Hanoi/TX027/2007	24			15	
	2	Czech Republic/119/2012	10			9	
	3	Taiwan/2361/2007	2			10	
	4	Brisbane/10/2007	1			9	
	5	Managua/18/2007	1			7	
	6	Arizona/WRAIR1142P/2009	4	2	3	5	11
	7	Waikato/03/2005			34	14	21
	8	Malaysia/1912341/2007			1	7	
	9	Czech Republic/121/2012			1		9
	NT[c]	NA	12		20	NA	NA
A(H1N1pdm)	1	Aalborg/INS132/2009	2			5	
	2	Managua/WRAIR/8964F/2009		9		4	
	3	Athens/INS5339/2009		5		7	
	4	Arizona/06/2009		2		3	
	5	Paris/2709/2009		2		4	
	6	Managua/WRAIR/1689P/2009		2		4	
	7	Beijing/718/2009		1		7	
	8	Argentina/8574/2009		1		4	
	9	Baden-Wurttemburg/448/2009		1		3	
	10	Boston/630/2009		1		6	
	11	California/VRDL101/2009		1		5	
	12	Canada-AB/RV2810/2009		1		4	
	13	Hubei/75/2009		1		8	
	14	Mexico/INDRE/3495/2009		1		2	
	15	Moscow/WRAIR1965P/2011		1		10	
	16	Argentina/HNRG83/2009		1		3	
	17	Bangkok/INS477/2010		13	3	8	
	18	Brussels/INS243/2009		2	1	7	
	19	Shenzhen/40/2009			2	6	
	20	Beijing/01/2009			1	2	
	NT[c]	NA	1	26		NA	

[a] V, influenza B Victoria lineage; Y, influenza B Yamagata lineage.
[b] Only strains identified in each vaccine group have the number of amino acid differences indicated. All influenza B positive samples were from participants in vaccine group 1. Participants in vaccine groups 1 and 2 received the same influenza A(H1N1pdm) vaccine strain.
[c] HA gene sequences were not available for this strain.
[d] Influenza A and B subtypes that did not have a strain designation by RT-PCR/ESI-MS.

group in samples from the women and infants enrolled in the influenza immunization trial (Table 3). Overall, the vaccines used in the study showed 65.2%, 70.2% and 37.4% efficacy for influenza subtypes A (H3N2), A(H1N1), and B, respectively.

The vaccines were less effective when the reference strains identified by RT-PCR/ESI-MS did not genetically match the vaccine strains (13 or more amino acid differences between HA genes) compared to reference strains that matched the vaccine strains more closely (less than 13 amino acid differences). For influenza B, the vaccine containing a strain belonging to the Victoria lineage was more effective against strains belonging to the Victoria lineage (match, VE=60.3%) compared to those of the Yamagata lineage (mismatch, VE=42.4%). A difference in VE was also seen between the two strain groups of influenza A(H3N2) (match, VE=66.7% vs. mismatch, VE=54.6%). Differences in VE were not statistically significant. For influenza A(H1N1pdm), all strains identified by RT-PCR/ESI-MS fell into one strain group that closely matched the vaccine strain (VE=75.8%).

Table 3
Efficacy of influenza vaccines by influenza subtype and strain group among women and infants

Influenza subtype	Strain group[a]	Vaccination status (number positive)		Vaccine efficacy % (95% CI)
		Vaccine	Placebo	
B	Match	8	20	60.3 (10.9, 82.7)
	Mismatch	33	57	42.4 (12.0, 62.4)
	Any[b]	51	81	37.4 (8.9, 54.4)
A(H3N2)	Match	5	15	66.7, 8.3, 87.9)
	Mismatch	15	33	54.6 (16.3, 75.2)
	Any[b]	24	69	65.2 (44.7, 78.1)
A(H1N1pdm)	Match	8	33	75.8 (47.5, 88.8)
	Any[b]	14	47	70.2 (45.9, 83.5)

[a] Match, <13 amino acid differences from the vaccine strain HA gene; mismatch, >13 amino acid differences from the vaccine strain.
[b] Includes samples with influenza subtypes for which ESI-MS reference strain types could not be identified.

3.5. Illness severity

To evaluate if vaccination status and subtype or strain group of influenza affected disease severity, the signs and symptoms recorded for each illness episode were scored and averaged for women and infants. The scores are shown in Table 4 by influenza subtype, vaccination status, and strain group. No differences were seen in duration of illness episode, symptom score, care rank score, or duration of fever between women or infants infected with influenza A and those infected with influenza B. Vaccinated women and their infants with either influenza A or B did not have lower illness scores than those who received placebo, with the exception of vaccinated women infected with a vaccine-matched strain of influenza A. This group had a significantly lower symptom score than women with a vaccine-matched strain of influenza A who received placebo (p=0.03, Table 4).

4. Discussion

During a 3-year prospective study of maternal influenza immunization in Sarlahi, Nepal (Steinhoff et al., 2017; Tielsch et al., 2015), all seasonal subtypes and many strains of influenza were detected in midnasal swab samples collected from pregnant and postpartum women, infants from birth to 6 months of age, and family members, often during the same influenza season. By using a combined method taking advantage of both RT-PCR and ESI-MS techniques to characterize multiple influenza strains circulating during our study and match them with reference strains, we were able to genetically compare the HA genes of the reference strains with those of the relevant vaccine strains. We found that all A(H1N1pdm) strains and some A(H3N2) and influenza B strains were closely related genetically to the HA genes of the respective vaccine strains. However, some A(H3N2) strains showed moderate genetic differences from the vaccine strains. This is not surprising since HA gene amino acid substitutions are thought to occur more frequently for influenza A(H3N2) than for influenza A(H1N1) viruses (Glatman-Freedman et al., 2017). Larger genetic differences seen between the HA genes of some influenza B reference strains and the vaccine strain, which was of the Victoria lineage, were due to circulating strains of the Yamagata lineage.

We analyzed the different strain groups of influenza A and B in relation to the participants' vaccination status. Although differences between influenza strains in the HA gene sequences do not always predict differences in antigenicity (Durviaux et al., 2014), the effectiveness of the seasonal influenza vaccines in this study could have been reduced because of influenza variants that circulated during the study. A study in Thailand that sequenced the HA genes to assess the antigenic distances between the vaccine and seasonal influenza A strains found that the emergence of multiple circulating strains in 2014 contributed to reduced VE (Tewawong et al., 2015). Suboptimal vaccine effectiveness may have arisen in our study from circulating strains that were antigenically mismatched to the vaccine strains. Specifically, genetic differences between the HA genes of some A(H3N2) and influenza B strains and the respective strains included in the vaccines may have contributed to a lower VE, although the differences were not statistically significant. We have previously reported that vaccinated women seroconverted in response to the influenza vaccine (Steinhoff et al., 2017), further suggesting that mismatched influenza strains may have been a contributing factor to lower VE.

Two lineages of influenza B circulated during the first two influenza seasons when only one lineage was included in the trivalent vaccine. The higher VE for influenza B Victoria lineage strains, which matched the strain included in the vaccine, suggests that use of a quadrivalent vaccine, containing both Victoria and Yamagata lineage influenza B, has the potential to further improve vaccine effectiveness. We estimate that the use of a quadrivalent vaccine could have provided the same protection in our study as provided by the trivalent vaccine for the influenza B Victoria strains.

Previous studies (Chi et al., 2008; Irving et al., 2012; Su et al., 2014) compared patients infected with seasonal influenza A and B in both outpatient and hospital settings and found similar clinical characteristics between the influenza A and B groups. We also saw similar illness severity in women and infants infected with influenza A and those infected with influenza B. Influenza vaccination has been shown to reduce illness severity in people who are vaccinated and subsequently become infected with influenza. One study found a reduction in death and intensive care unit admission in vaccinated versus non-vaccinated hospitalized adults during the 2013-14 influenza season (Arriola et al., 2017). Other studies showed similar reductions in illness and complications in older adult populations in hospitals and nursing homes (Casado et al., 2016; Patriarca et al., 1985) and in children in the intensive care unit setting (Ferdinands et al., 2014). In this Nepal study, we followed

Table 4
Average illness severity scores by influenza subtype, strain group, and vaccination status for women and infants

			Participants								
			Women					Infants			
Influenza subtype	Strain group[a]	Vaccination status[b]	N[c]	Episode duration[d] (days)	Symptom score[e]	Care rank score[f]	Fever duration (days)	N	Episode duration (days)	Symptom score	Care rank score
A	Any	All	69	7.2	15.3	1.0	2.9	115	4.8	6.9	0.9
		V	21	6.9	14.3	0.7	3.2	31	5.4	8.9	0.9
		P	48	7.4	15.7	1.1	2.8	84	4.6	6.1	1.0
	Match	V	8	4.8	**8.9**[g]	0.8	2.9	5	4.0	6.2	0.8
		P	21	11.0	**22.1**	1.2	3.3	26	4.9	6.4	0.7
	Mismatch	V	6	10.0	21.3	0.8	3.8	9	5.7	6.6	1.4
		P	9	3.7	10.1	1.0	2.3	24	4.4	5.8	1.1
B	Any	All	47	8.0	18.9	1.1	3.5	83	4.6	6.6	1.0
		V	10	8.3	18.2	1.1	3.3	41	4.5	6.9	0.9
		P	37	7.9	19.1	1.2	3.6	42	4.7	6.3	1.1
	Match	V	1	10.0	16.0	2.0	3.0	7	5.4	9.7	1.0
		P	12	7.4	15.4	1.1	3.3	8	7.5	6.3	1.8
	Mismatch	V	6	8.7	21.5	1.3	4.0	27	4.7	6.9	1.0
		P	25	8.1	20.9	1.2	3.7	31	3.9	6.1	0.9

[a] Match, <13 amino acid differences from the vaccine strain HA gene; mismatch, >13 amino acid differences from the vaccine strain.
[b] V, vaccine; P, placebo.
[c] Number of samples.
[d] Number of consecutive days with any sign or symptom for each illness episode.
[e] Total of all symptoms on all days.
[f] Total of care seeking including any care, medical care, doctor's care, and hospital care.
[g] p=0.03 for vaccine group compared to placebo group.

a rural population of relatively healthy women and young infants who had limited opportunities to health care and actually sought very little care during illness. We showed that, with the exception of women infected with vaccine-matched strains of influenza A, clinical signs and symptoms did not differ between influenza positive participants who were vaccinated compared to those who were not vaccinated. However, symptoms were based on maternal recall and limited examination by non-medical study personnel, which likely resulted in more misclassification of illness than if obtained through physician examination.

This study has several limitations. Assumptions were made for strain identification and genetic and antigenic relatedness. Because strain identification was performed indirectly using the RT-PCR/ESI-MS assay instead of directly by culture and sequencing the HA genes of the influenza in the samples, it is possible some misclassification of strains occurred. Nevertheless, this method has been used successfully to detect and classify influenza with clade-level resolution (Sampath et al., 2007), to identify salmonella bacteria in blood (Jeng et al., 2012b), and to detect more bacterial and fungal organisms than culture (Ozenci et al., 2018). An advantage of RT-PCR/ESI-MS over sequencing is its greater sensitive for lower amounts of RNA and possibly degraded RNA which cannot be cultured. Antigenic relatedness was assessed by assuming that 13 or more HA aa differences between vaccine and study strains represented antigenic differences. Differences of 13 aa residues in influenza A(H3N2) HA genes have previously been shown to be the average difference between strains forming distinct antigenic clusters (Smith et al., 2004). A subsequent study by the same group determined that a relatively small number of genetic changes or combinations of changes may drive changes in antigenic phenotype (Koel et al., 2013).

5. Conclusions

This study has shown that all subtypes and many strains of influenza A and B were co-circulating in rural southern Nepal between April 2011 and May 2014. The HA genes of reference strains matched to strains of influenza A (H3N2) and B by RT-PCR/ESI-MS showed sufficient differences from vaccine strains in dictated aa sequences to predict antigenic differences between the strains, which could have contributed to differences between strain groups in VE. Our results demonstrate that influenza vaccine strains that genetically match the circulating strains are needed to provide good protection from influenza illness.

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Competing Interests

Joanne Katz, Charlotte Gaydos, James Tielsch, Subarna Khatry, Mark Steinhoff, Steven LeClerq, and Jane Kuypers have no competing interests. Helen Chu has received research support from GlaxoSmithKline (GSK), Sanofi Pasteur, and Novavax. Janet Englund has been a consultant for Pfizer, a member of a Data Safety Monitoring Board for GSK influenza antiviral studies, and her institution has received research support for clinical studies from GSK, Gilead, Chimerix, and Roche.

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