DOI:10.1111/j.1750-2659.2010.00182.x
www.influenzajournal.com

Original Article

Influenza and other respiratory viruses in three Central American countries

Victor A. Laguna-Torres,[a] José F. Sánchez-Largaespada,[b] Ivette Lorenzana,[c] Brett Forshey,[a] Patricia Aguilar,[a]Mirna Jimenez,[d] Eduardo Parrales,[e] Francisco Rodriguez,[f] Josefina García,[a] Ileana Jimenez,[b] Maribel Rivera,[g] Juan Perez,[a] Merly Sovero,[a] Jane Rios,[a] María E. Gamero,[a] Eric S. Halsey,[a] Tadeusz J. Kochel[a]

[a]US Naval Medical Research Center Detachment, Lima, Perú. [b]Hospital Infantil Manuel de Jesús Rivera "La Mascota", Managua. Ministerio de Salud de Nicaragua. [c]Universidad Nacional utónoma de Honduras, Tegucigalpa, Honduras. [d]Hospital de Santa Ana, Metapán. Ministerio de Salud de El Salvador, El Salvador. [e]Centro de Salud Villa Libertad/Villa Venezuela, Managua. Ministerio de Salud de Nicaragua. [f]Hospital Humberto Alvarado Vásquez, Masaya. Ministerio de Salud de Nicaragua. [g]Instituto Hondureño de Seguridad Social, Tegucigalpa, Honduras.
Correspondence: V. A. Laguna-Torres, US Naval Medical Research Center Detachment, 3230 Lima Pl, Washington, DC 20521-3230, USA.
E-mail: alberto.laguna@med.navy.mil

Accepted 12 September 2010. Published Online 3 November 2010.

Background Despite the disease burden imposed by respiratory diseases on children in Central America, there is a paucity of data describing the etiologic agents of the disease.

Aims To analyze viral etiologic agents associated with influenza-like illness (ILI) in participants reporting to one outpatient health center, one pediatric hospital, and three general hospitals in El Salvador, Honduras, and Nicaragua

Material & Methods Between August 2006 and April 2009, pharyngeal swabs were collected from outpatients and inpatients. Patient specimens were inoculated onto cultured cell monolayers, and viral antigens were detected by indirect and direct immunofluorescence staining.

Results A total of 1,756 patients were enrolled, of whom 1,195 (68.3%) were under the age of 5; and 183 (10.4%) required hospitalization. One or more viral agents were identified in 434 (24.7%) cases, of which 17 (3.9%) were dual infections. The most common viruses isolated were influenza A virus (130; 7.4% of cases), respiratory syncytial virus (122; 6.9%), adenoviruses (63; 3.6%), parainfluenza viruses (57; 3.2%), influenza B virus (47; 2.7% of cases), and herpes simplex virus 1 (22; 1.3%). In addition, human metapneumovirus and enteroviruses (coxsackie and echovirus) were isolated from patient specimens.

Discussion When compared to the rest of the population, viruses were isolated from a significantly higher percentage of patients age 5 or younger. The prevalence of influenza A virus or influenza B virus infections was similar between the younger and older age groups. RSV was the most commonly detected pathogen in infants age 5 and younger and was significantly associated with pneumonia ($p < 0.0001$) and hospitalization ($p < 0.0001$).

Conclusion Genetic analysis of influenza isolates identified A (H3N2), A (H1N1), and B viruses. It also showed that the mutation H274Y conferring resistance to oseltamivir was first detected in Honduran influenza A/H1N1 strains at the beginning of 2008. These data demonstrate that a diverse range of respiratory pathogens are associated with ILI in Honduras, El Salvador, and Nicaragua. RSV infection in particular appears to be associated with severe disease in infants in the region.

Keywords Adenovirus, Central America, enterovirus, influenza, respiratory viruses, surveillance.

Please cite this paper as: Laguna-Torres *et al.* (2011) Influenza and other respiratory viruses in three Central American countries. Influenza and Other Respiratory Viruses 5(2), 123–134.

Introduction

Acute respiratory infections (ARI) impose a significant burden of both morbidity and mortality on children worldwide. In 2000 alone, an estimated 1·9 million children under the age of five died as the result of ARI, accounting for 14% of total mortality in the age group.[1]

While most infections are fairly mild, self-limiting, and confined to the upper respiratory tract, severe illnesses can also occur. Owing to a lack of access to adequate health care and resources, children in developing regions of the world are more susceptible to adverse sequelae. As a result, mortality levels associated with ARI far exceed those of wealthier regions.[1]

The preponderance of data on the epidemiology of the etiologic agents of ARI comes from more developed regions of the world, including the United States and Europe. By most accounts, viral pathogens are the most significant

contributors to ARI. For influenza-like illness (ILI), influenza viruses are commonly detected,[2] followed by parainfluenza viruses (PIV), respiratory syncytial viruses (RSV), and adenoviruses,[3,4] all with well-defined seasonal incidence peaks.

Much less is known about the etiology and epidemiology of ARI in tropical regions of the world, including Central America. A recent study among a cohort of young children in Managua, Nicaragua, demonstrated that ILI is common, with increasing incidence among younger age groups.[5] In that study, while a high incidence of ILI (approximately 35 ILI episodes/100 person-years) was established, the etiologic agents of the illnesses were not identified. In 2009, the Honduran Ministry of Health established a surveillance system in adults in the two main cities of the country, Tegucigalpa and San Pedro Sula. During the first quarter of 2009, a total of 254 samples were tested by indirect immunoflorescence assay (IFA). Influenza A and influenza B were identified in 2·4% and 1·2% of the total, respectively.[6]

The objective of this study was to identify the viral agents associated with ILI at five hospitals or health centers in Nicaragua, Honduras, and El Salvador. In addition, preliminary molecular characterization was conducted on the influenza A and influenza B viruses isolated to identify strains circulating in the region as well as their antiviral resistance patterns.

Material and methods

Study population and case definition

The study population included every patient with ILI, regardless of age, who sought attention or was hospitalized in participating health centers between August 2006 and April 2009 and agreed to the study. Participants (outpatients or inpatients) were recruited when reporting to any of five participating hospitals or health centers including two clinics in Managua, Nicaragua (Hospital Jesus de Rivera and Health Center Villa Libertad), one hospital in Masaya, Nicaragua (Hospital de Masaya), one hospital in Tegucigalpa, Honduras (Instituto Hondureño de Seguro Social), and one hospital in Metapán, El Salvador (Hospital Nacional de Santa Ana, Metapán) (Figure 1). At each site, trained medical personnel were responsible for properly identifying and classifying patients with ILI. The case definition was any person with a sudden onset of fever (≥38°C) and cough or sore throat fewer than 5 days in duration, with or without general symptoms such as myalgias, prostration, headache, or malaise.[7] Hospitalization was noted if the patient spent at least one night in the hospital or health center. Data on gender, age, lost work or school days, previous treatments, medical attention before enrollment, influenza vaccination status, and travel in the last 7 days were collected utilizing a case report form (CRF) from all participants who met the case definition criteria.

This ILI surveillance protocol was approved as a less than minimal risk research by the Naval Medical Research Center-Detachment (NMRCD) Institutional Review Board (IRB; Protocol NMRCD.2002.0019), and authorization was given to perform the study using an information sheet approved and stamped by the IRB. This was accepted by the Nicaraguan, Honduran, and Salvadoran institutions involved. Monthly reports were sent to Ministry of Health officers and to the site coordinators in each country.

Laboratory analysis

Sample collection

Two types of samples were obtained for diagnostic testing: a nasal swab for the Rapid Influenza Test (RIT; QUICKVUE Influenza test; Quidel, San Diego, USA) and an oropharyngeal swab for viral isolation. The RIT was processed on-site, and the results were provided to the patient. Oropharyngeal swabs were placed in transport media and stored at −70°C until they were delivered on dry ice to NMRCD in Lima, Perú for laboratory analysis.

Virus isolation and identification

Patient specimens were inoculated into four cell lines for virus isolation: Madin-Darby canine kidney (MDCK), African green monkey kidney (Vero76 and VeroE6), and Rhesus monkey kidney (LLCMK2). Upon the appearance of a cytopathic effect (CPE) or after 10 days of culture (or thirteen days in the case of Vero cells), the cells were spotted onto microscope slides. Cell suspensions were dried and fixed in chilled acetone for 15 minutes. Virus isolates were identified using a direct fluorescence antibody (DFA) assay. The Respiratory Virus Screening and Identification Kit (D3 DFA Respiratory Virus Diagnostic Hybrids; Athens, OH, USA) was utilized for the identification of adenoviruses, influenza A virus, influenza B virus, PIVs (types 1, 2, and 3), and RSV. The D3 DFA Herpes Simplex Virus (HSV) identification kit and the D3 IFA Enterovirus ID kit (Diagnostic Hybrids; Athens, OH) were utilized for the identification of HSV (both HSV-1 and HSV-2) and enteroviruses, respectively. For the isolation of metapneumovirus (hMPV), we used VERO E6 and LLC-MK2 cell lines. For the detection of hMPV antigens by direct fluorescence assay, we used an anti-hMPV mouse monoclonal antibody from Diagnostic Hybrid (Athens, OH, USA). All assays were performed following the manufacturers' instructions.

Cases with positive RIT results and negative for virus isolation were further tested for influenza A and B viruses using a one-step reverse transcriptase-polymerase chain reaction (RT-PCR) with the influenza primers described later in the text. The viral etiology of cases was determined based on the isolation of virus (CPE or IFA/DFA positive) or a positive result by both RIT and RT-PCR.

Figure 1. Map of study sites in El Salvador, Honduras, and Nicaragua.

RNA extraction and RT-PCR

For the genetic analyses of influenza viruses, viral RNA extraction was performed from the supernatant of infected MDCK cells using a QIAamp Viral RNA kit (QIAGEN; Valencia, CA, USA) following the manufacturer's protocol. The one-step RT-PCR was performed with primers that amplified the hemagglutinin (HA) gene of influenza A and influenza B viruses using the SuperScript III One-Step RT-PCR System kit (Invitrogen; San Diego, CA, USA). The following primers were used for the amplification of H1 influenza A viruses: H1F-6 (5′-AAGCAGGGGAAAATAAAA-3′) and H1R-1193 (5′-GTAATCCCGTTAATGGCA 3′); for H3 influenza A viruses: H3F-7 (5′-ACTATCATTGCTTTGAGC-3′) and H3R-1184 (5′-ATGGCTGCTTGA GTGCTT-3′); for influenza B viruses: BHAF-36 (5′-GAAGGCAATAATTGTACT-3′) and BHAR-1140 (5′-ACCAGCAATAGCTCCGAA-3′). Five microliters of the extracted RNA was added to 20 μl of master mix containing the enzyme mixture (SuperScript III RT/Platinum Taq), 2 × reaction mixture (containing 0·4 mm of each dNTP and 3·2 mm of Mg_2SO4), and 20 μm of each primer. Cycling conditions included a reverse transcription step at 50°C for 30 minutes and a denaturation step at 94°C for 2 minutes. Cycling conditions of the PCR were 40 cycles of 94°C for 15 seconds, 52°C for 30 seconds, and 68°C for 75 seconds, followed by a final incubation step at 68°C for 5 minutes.

For subtyping of the neuraminidase gene and to study the resistance to antiviral agents (amantadine and oseltamivir), the neuraminidase (NA) and matrix protein 2 genes (M2) were amplified by RT-PCR with the following specific primers: bases 617-995 (379-bp fragment) of the influenza A M2 matrix protein 2-3 (5′-CTAGTCAGGCCAGGCAAATG-3′) and M2-Rev (5′-ACTGTCGTCAGCATCCACAG-3′);

bases 449-1218 (770-bp fragment) of the influenza A NA, AN1A (5′-AGGACAGAAGCCCTTATAGG-3′) and AN1DII (5′-TTAGCTCAGGATGTTGAACG -3′); bases 299-997 (699-bp fragment) of the influenza A NA, AN2A (5′-ATTACAGGATTTGCACCTTT-3′) and H3N2-NA-2R (5′-GGGTGTGTCTCCAACAAGTCTGAGCAC-3′); bases 352-641 (290-bp fragment) of the influenza B NA, NA-RES-F (5′-GCTCTAACCCATTATGCAG-3′) and NA-RES-R (5′-CTTTCTTGTGTTCTTAGGATG-3′) as previously published.[8]

The RT-PCR products were purified using Centri-Sep Columns (Princeton Separation; Englishtown, NJ, USA) and sequenced using the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems; Foster City, CA, USA) following manufacturers' instructions. Sequences were analyzed and edited using the Sequencer 4.8 software (Applied Biosystems).

Sequencing and phylogenetic analysis

The nucleotide sequences were aligned using the Clustal program in the Mac Vector software package (Mac Vector Inc.; Cary, NC, USA), and phylogenetic analyses were performed using the neighbor joining and maximum likelihood algorithms implemented in the Phylogenetic Analysis Using Parsimony (PAUP) software.[9,10] For the neighbor-joining analyses, the HKY85 distance was used and bootstrap values were calculated based on 1000 replicates to place confidence values on groupings within trees.

Statistical analysis

The clinical–epidemiological forms were entered into a database created in Microsoft Office Access 2003. The chi-square and Fisher exact tests were used to compare means and associations using SPSS software version 10.0 (SPSS Inc., Chicago, IL, USA) and *R* version 2.8.0 (R Development Core Team, Vienna, Austria).

Results

General findings

A total of 1756 participants were enrolled in this study, 889 (50·6%) of whom were women (Table 1). The study population included patients from one pediatric hospital, three general hospitals, and one health center located in three different countries. Overall, the median age of participants was 2 years old (range 0–85), with 1195 (68·3%) age 5 or younger under the age of 5, and 1416 (80·9%) age 14 or younger. At the general hospital in El Salvador, the median age was 31, with a maximum age of 79; 77 (27·2%) of participants were age 14 or younger. At the other participating hospitals in Honduras and Nicaragua, the median age was 2 and 1, respectively, with a maximum age of 85; 1339 of 1466 (92·6%) participants were 14 or younger.

Where data were available, 192 of 1,756 (10·9%) of patients were reported as having received antibiotic treatment prior to enrollment in this study. At the time they sought medical care, 40 patients (between 5 and 65 years) reported having lost part or a full day of work or school, with an average of 1·1 days lost. A total of 183 patients were hospitalized, of whom 177 (96·7%) were 5 years of age or younger. Sixty-five (3·7%) patients reported to have received the influenza vaccine within 6 months prior to enrollment (Table 1). Of those, 28 (43%) were 5 years of age or younger. An influenza virus was isolated from three of the ILI participants who reported receiving vaccination, including two with influenza A virus infection and one co-infected with influenza B and adenovirus.

Laboratory results

Of the 1,756 specimens tested, one or more respiratory virus was isolated from 434 (24·7%) participants (Table 2). Co-infections were detected in 17 specimens. Isolations were more common from specimens in those age 5 or younger (28·5%) than from older patients (16·8%; $P < 0·0001$). Of the samples with only one virus isolated, the most common viruses isolated were influenza A virus (130; 7·4% of cases), RSV (122; 6·9%), adenoviruses (63; 3·6%), PIV 1, 2, and 3 (57; 3·2%), influenza B virus (47; 2·7%), and HSV 1 (22; 1·3%). We did not identify HSV 2 cases. Additionally, human metapneumovirus ($n = 3$) and enteroviruses ($n = 7$) were isolated. The enterovirus subtypes identified were echovirus ($n = 3$), enterovirus 70/71 ($n = 2$), and coxsackie virus ($n = 2$). Among the 17 dual infections, the most common were adenovirus-RSV ($n = 4$), influenza virus A-RSV ($n = 3$), influenza A-HSV-1 ($n = 3$), influenza B virus-RSV ($n = 2$), adenovirus–PIV ($n = 2$), and adenovirus-influenza B ($n = 2$) (Table 2). In addition, there was one patient with an adenovirus-HSV-1 co-infection.

Subtyping was performed in 83 (64%) of the influenza A positive samples; among those, a similar percentage of H1 (42/83) and H3 (41/83) viruses was identified. H3 influenza A viruses were predominant in El Salvador (13/14 of the viruses sequenced). In Nicaragua, H1 viruses were the predominant influenza A viruses in circulation during the study period (25/39; 64·1%). Among adenoviruses that were further analyzed, type C predominated (31/34 isolates); type B was the only other type of adenovirus identified (3/34).

Among identified viral infections, RSV was associated with the youngest median age (median 9 months, range 1 month–10 years), followed by PIV (median 1 year, range 2 months–8 years), adenoviruses (median 1 year, range 3 months–27 years, influenza B virus (median 3·5 years, range 2 months–24 years), influenza A virus (median age 4, range 2 months–72 years), and HSV 1 (median age 6, range 4 months–77 years). There was no statistically significant

Table 1. Characteristics of the population by country. Central America, June 2006–April 2009

Characteristics of the population	Total		Countries		
	Count	%	El Salvador	Honduras	Nicaragua
Number of samples (total patients enrolled)	1756	100·0	283	427	1046
Respiratory virus positive	434	24·7	24	128	282
Influenza virus positive (influenza A or B)	177	10·1	18	66	93
Sex					
Female	889	50·6	196	210	483
Male	867	49·4	87	217	563
Age					
Total	1749*	99·6	283	424	1042
Mean ± STD	9·43 ± 16·3		33·4 ± 21·4	5·99 ± 11·6	4·32 ± 9·1
Median [range]	2 [0·85]		31 [0·79]	2 [0·69]	1 [0·85]
0-5	1195	68·32	19	321	855
6-14	221	16·5	58	54	109
15-29	125	7·1	60	22	43
30-44	83	4·7	49	14	20
45-59	73	4·2	52	10	11
≥60	52	3·0	45	3	4
Travel (last 7 days)	75	4·3	2	57	16
Vaccination history	65	3·7	35	10	20
Hospitalized	183	10·4	5	49	129
Those losing work/school days (between 5 and 65 years)					
Total	40	2·3	24	8	8
Days lost at time of presentation					
Mean ± STD	1·09 ± 0·8		1·25 ± 0·89	0·41 ± 0·09	1·31 ± 0·58
Median [range]	1·00 [0·25,3·58]		1·00 [0·25,3·58]	0·38 [0·25,0·50]	1·50 [0·25,2·25]
<1 day	17		8	8	1
1 day	18		12	0	6
2 days	2		1	0	1
3 days	3		3	0	0
>4 days	0		0	0	0
Medical attention before enrollment	428		42	152	234
Treatment administrated before enrollment					
Antibiotics	192	10·9	29	85	78
Others	779	44·4	46	243	490
Unknown (not specified)	16	0·9	2	5	9
No treatment	607	34·6	199	71	337
Missing (no answer)	162	9·2	7	23	132
Positive rapid test					
Influenza A	71	4·0	10	32	29
Influenza B	27	1·5	2	15	10
Undifferentiated	3	0·2	1	1	1
Negative	1628	92·7	269	370	989
No test	27	1·5	1	9	17

*Gender data missing in 7. [Correction added after online publication 11 November 2010: > symbol in front of 10.4 in third column removed]

difference in the rate of influenza virus (A or B) infection between participants age 5 or younger (9·2%) and those older than 5 (11·9%; $P = 0·1$). RSV (10·5% versus 0·6%), PIV (4·5% versus 0·8%), and adenoviruses (4·7% versus 1·4%) were all significantly more commonly detected in patients 5 years of age or younger than older patients ($P < 0·001$ for each). HSV-1 was more commonly associated with participants older than 5 years of age (0·7% versus 2·3%; $P < 0·01$). When compared to the rest of the population, viruses were isolated from a significantly higher percentage of patients aged 5 or younger. (30% versus 17%; $P < 0·001$).

Respiratory viruses were isolated from 52 (28·9%) of the 183 hospitalized patients. RSV was the viral pathogen mostly commonly associated with hospitalization, accounting for 27·0% (33/122) of RSV cases identified and 33 of the 53 viruses identified (62·2%) in this group. Other

Table 2. Viral etiology of influenza-like illness cases by country. Central America, June 2006–April 2009

	Total	El Salvador	Honduras		Nicaragua	
	Count (%)	Hosp. Nac. De Santa Ana Metapan	IHSS-Instituto Hondureño De Seguro Social, Tegucigalpa	C.S Villa Libertad/Villa Venezuela, Managua	H.I.M.J.R – La Mascota Managua	Hosp. Humberto Alvarado Vasquez Masaya
Total	1756 (100)	283	427	392	310	344
Positive	434* (24·7)	24	128	81	85	116
Influenza A virus	130 (7·4)	16	47	37	17	13
H1N1	42 (2·4)	1	16	15	7	3
H3N2	41 (2·3)	13	14	3	3	8
Not subtyped	47 (2·7)	2	17	19	7	2
Influenza B virus	47 (2·7)	2	19	12	5	9
Herpes simplex virus 1	22 (1·3)	5	11	4	1	1
Respiratory syncytial virus	122 (6·9)	1	5	10	45	61
Adenovirus	63 (3·6)	1	31	6	10	15
Enteroviruses	7 (0·4)	1	0	2	2	2
Enterovirus 70/71	2 (0·1)					
Coxsackie	2 (0·1)	1	0	0	1	0
Echovirus	3 (0·2)	0	0	0	1	2
Parainfluenza viruses (1, 2 and 3)	57 (3·2)	0	18	11	9	19
Metapneumovirus	3 (0·2)	0	1	0	1	1
Negative	1322 (75·3)	259	299	311	225	228
Total patients with co-infections	17 (100)	2	4	1	5	5

*A total of 451 virus-positive specimens were obtained from 434 patients (17 patients had co-infections).

viruses found in hospitalized patients were PIV ($n = 8$), influenza B virus ($n = 5$), adenoviruses ($n = 4$), and influenza A virus ($n = 3$). One had dual infection. A total of 169 patients developed pneumonia, of whom 152 (89·9%) were 5 years of age or younger. Viruses were isolated 39 times from participants with pneumonia, including 19 RSV, 11 PIV, 8 influenza A or B, and 1 adenovirus. When comparing an RSV infection with a non-RSV infection, RSV infection was significantly associated with the development of pneumonia ($P < 0·001$).

Sequencing and phylogenetic analyses of influenza A and B isolates

Influenza A H1N1

Genetic analyses based on partial hemagglutinin gene sequence (approximately 1000 bp) of 33 H1N1 influenza A isolates from Nicaragua and Honduras revealed three distinct genotypes: (i) A/Solomon Islands/03/06-like, (ii) A/Brisbane/59/07-like, and (iii) A/New Caledonia/20/99-like (Figure 2).

Most of the isolates from Nicaragua (Figure 2, depicted in red) obtained during September through December 2006 grouped within the A/New Caledonia/20/99-like genotype. Notably, this strain was included in the recommended 2006/2007 vaccine for the Northern Hemisphere (A/New Caledonia/20/99). A single isolate from Nicaragua obtained on October 2006 grouped within the A/Solomon Islands/03/06-like genotype.

During May through June 2007, the A/New Caledonia/20/99-like genotype continued to circulate in Central America as evidenced by the placement of the Honduran isolates within this genotype (depicted in green, Figure 2). Unfortunately, Central American isolates from the 2007–2008 (December through May) were not available for sequencing.

The A/Brisbane/59/07-like genotype was detected among the isolates from Honduras and Nicaragua obtained during May through November 2008. This genotype was not included in the recommended 2007/2008 vaccine (A/Solomon Islands/03/06); however, it was included in the recommended 2008/2009 vaccine (A/Brisbane/59/07).

Influenza A H3N2

Genetic analyses based on the hemagglutinin gene of 23 H3N2 influenza A isolates from Central America revealed

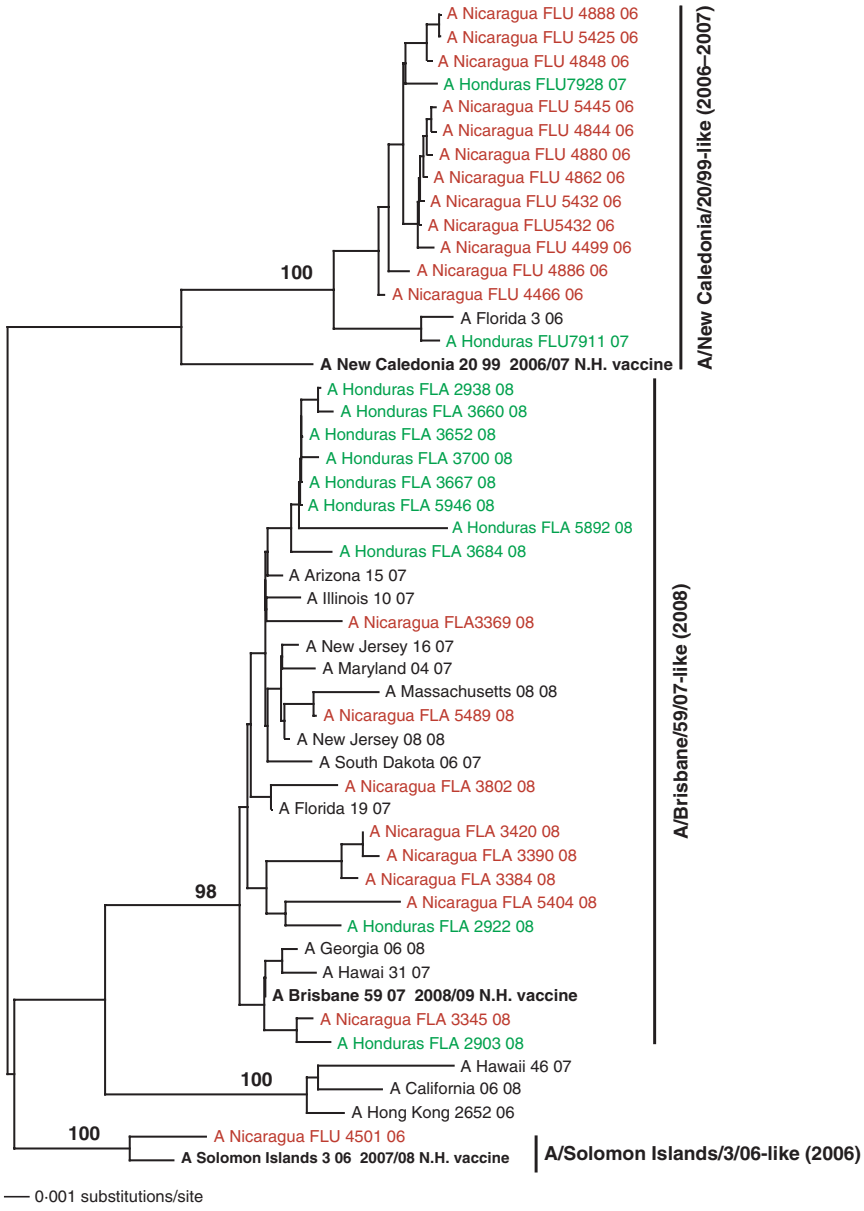

Figure 2. Neighbor-joining phylogenetic analyses based on partial hemagglutinin gene sequence (approximately 1000 bp) of 33 H1N1 influenza A isolates from Nicaragua and Honduras with three distinct genotypes: 1) A/Solomon Islands/03/06-like, 2) A/Brisbane/59/07-like, and 3) A/New Caledonia/20/99-like. Neighbor-joining and maximum likelihood analyses yielded similar tree topology.

three genotypes: (i) A/Brisbane/101/05-like, (ii) A/Honduras/AF1064/06-like, and (iii) A/Brisbane/10/07-like. Most of the isolates were obtained during 2007 and 2008 from Honduras, El Salvador, and Nicaragua. A single isolate from Honduras obtained in 2009 grouped within the A/Brisbane/10/07-like genotype. This genotype was included in the 2008/2009 vaccine for the Northern Hemisphere (A/Brisbane/10/07), but was not included in the 2006/2007 and 2007/2008 recommended vaccines (A/Wisconsin/67/05). Some isolates from Honduras and El Salvador obtained during 2007 and 2008 grouped into the A/Honduras/AF1064/06-like genotype that also includes isolates from 2006 (according to sequences available at the genbank database). A single isolate from Nicaragua obtained during 2006 grouped within the A/Brisbane/101/05-like genotype (Figure 3).

Influenza B

Phylogenetic analyses based on the HA sequence of 23 influenza B isolates revealed the presence of two genotypes in Central America: B/Malaysia/2506/07-like and B/Florida/4/06-like. The 2006 isolates from Honduras obtained during August through November grouped within the B/Malaysia/2506/07-like genotype, which was included in

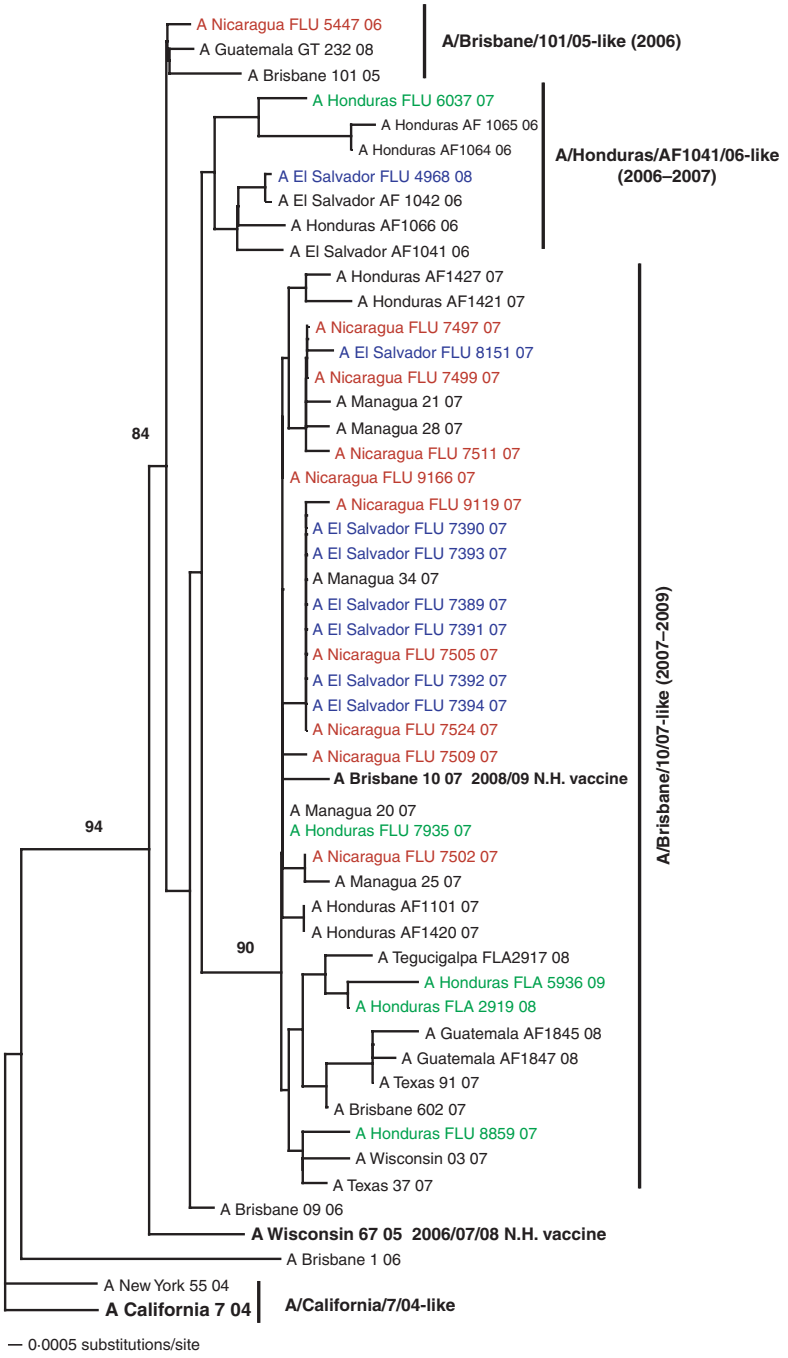

Figure 3. Neighbor-joining phylogenetic analyses based on the hemagglutinin gene of 23 H3N2 influenza A isolates from Central America revealed three genotypes: 1) A/Brisbane/101/05-like, 2) A/Honduras/AF1064/06-like, and 3) A/Brisbane/10/07-like. Neighbor-joining and maximum likelihood analyses yielded similar tree topology.

the recommended 2006/2007 and 2007/2008 vaccines for the Northern Hemisphere. The B/Florida/04/06 genotype was identified among the El Salvador, Honduras, and Nicaragua isolates obtained during May through September 2008 (Figure 4).

Influenza Resistance to Amantadine and Oseltamivir. The analyses of the amino acid sequences of the M2 and the neuraminidase proteins were carried out on randomly selected samples from El Salvador ($n = 2$), Nicaragua ($n = 12$), and Honduras ($n = 18$). The analyses show that all influenza B ($n = 10$) samples were sensitive to oseltamivir, and all influenza A/H3N2 ($n = 10$) samples were resistant to amantadine (mutation S31N) and sensitive to oseltamivir. The analyses of A/H1N1 ($n = 12$) samples show that in 2008, oseltamivir-resistant strains started to be detected in 100% of the A/H1N1 strains circulating in Central America.

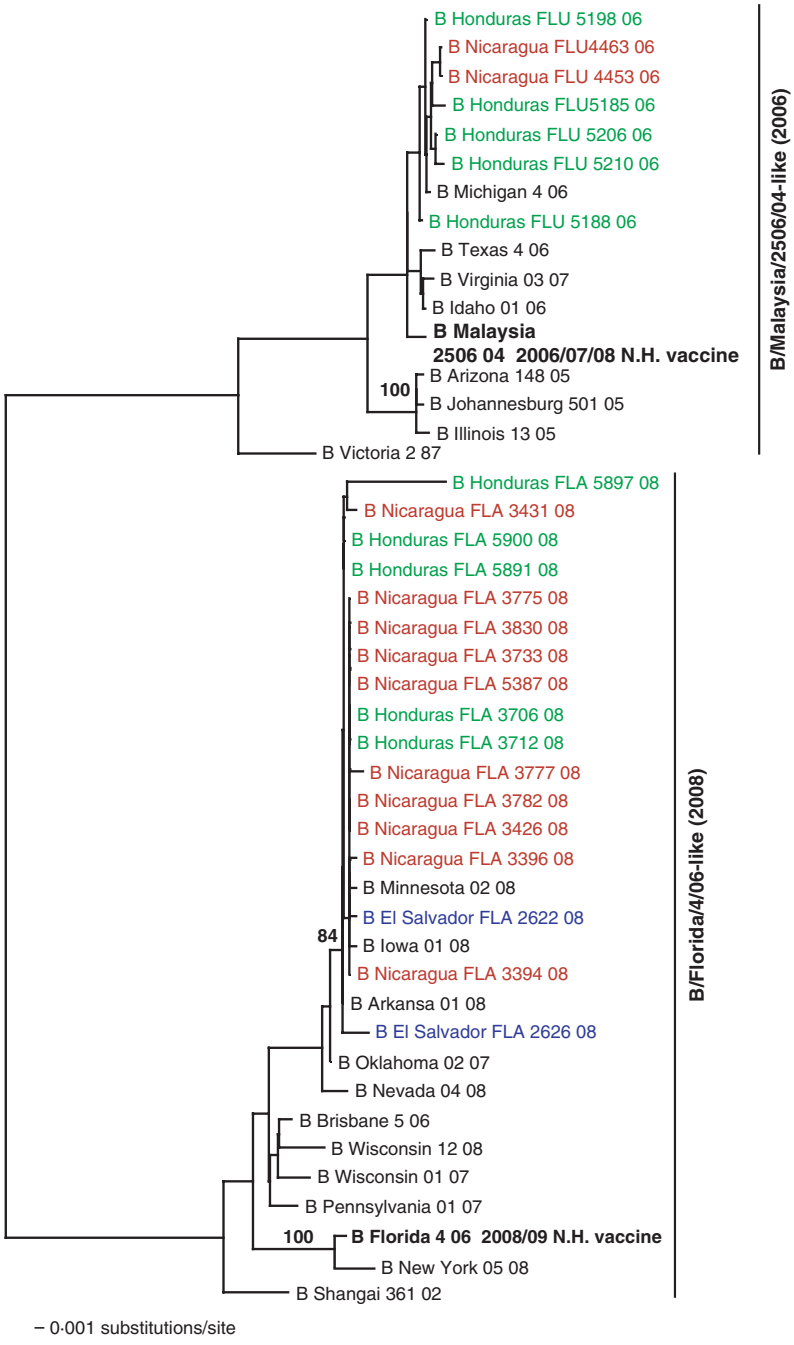

Figure 4. Neighbor-joining phylogenetic analyses based on the HA sequence of 23 influenza B isolates revealed the presence of two genotypes in Central America: B/Malaysia/2506/07-like and B/Florida/4/06-like. Neighbor-joining and maximum likelihood analyses yielded similar tree topology.

Discussion

Prior to this study, there has been a paucity of published data on the viral etiologic agents associated with ILI in Central America. One previous study conducted in the region between 1991 and 1992 involved 135 hospitalized children with acute respiratory tract infections in Honduras and El Salvador.[8] In that study, RSV was isolated from 32% of participants, influenza A and B viruses from 22%, and adenoviruses from only 3%. No PIV isolations were reported in that study. In contrast, we found that nearly 5% of ILI cases in children five and under were associated with PIV infection. In addition, we isolated HSV-1, human metapneumovirus, and enteroviruses from patients presenting with ILI in Central America.

The existence of seasonal peaks related to influenza virus transmission and ILI in tropical regions remains somewhat controversial.[11] Gordon *et al.*[5] observed a single peak of ILI

in May–June 2005 and two peaks in 2006 (June–July and October–November). Reyes *et al.*[12] reported an increase in influenza viruses and ILI concomitant with the end of the rainy season in late October and early November. These studies showed peaks in different months of the year (May–June or October–December). These studies covered short (<3 years) study periods, leaving the seasonality of specific viral pathogens in Central America an open question. Our study is still not able to show seasonality because it spans <3 years.

Several studies have suggested that ARI and ILI are significant causes of morbidity and mortality in children in Central America. In a recent population-based study of children aged 2–11 in Managua, Nicaragua, the ILI rate was 34·8/100 person-years.[5] Younger participants were at significantly higher risk than the older participants, and household density was shown to be associated with ILI. A previous survey concluded that ARI contributes to approximately 12% of mortality in children younger than 5 in El Salvador.[1] In those studies, the etiologic agents were not identified. Data from our study suggest that RSV and PIV are likely significant contributors to the deaths observed in young children in the region, as has been observed elsewhere.[13,14] We also observed that RSV was most common among young children and was statistically associated with hospitalization. In the United States, PIV and RSV infections are involved in 70 000 and 100 000 hospitalizations per year,[13–15] with annual costs of $190 million and $300 million, respectively. Similar studies are necessary to determine the economic burden caused by these and other respiratory pathogens in Central America.

In this study, viral pathogens were associated with 25% of all ILI cases, with the highest percentage of viral pathogens found in younger (age 5 and below) participants. Among adults (≥15 years of age), viral pathogens were identified in only a few cases. In a similar study in Peru that had a higher percentage of adult participants, we identified viruses in 42% of ILI episodes.[16] In two studies of children in Mexico, greater than 47% of ILI was virus associated.[17,18] It is unclear why viral pathogens were less common in our study. Etiologic pathogens will likely vary by region and by time period; however, differences in sample storage and integrity cannot be ruled out. At the El Salvador site, we observed the lowest success with virus isolation (approximately 8% of participant samples), while in the Nicaraguan sites, we found that 29% of participant specimens were positive for one or more viruses. To address the majority of ILI cases that had no pathogen identified, a wider range of both viral and bacterial pathogens needs to be considered. Nevertheless, our data suggest that viral pathogens are significant causes of ILI and hospitalization in these study sites in Central America. The development of safe and effective measurements against RSV and PIV could help significantly reduce childhood morbidity and mortality in the region.

Additionally, in this report, we found a significant prevalence of adenovirus in patients 5 years of age or younger. We have previously reported molecular characterization of adenoviruses collected from 24 ILI patients during 2006–08 in Central America.[19] In that study, subtype C was the predominant serotype identified in Nicaragua, Honduras, and El Salvador, while subtype B was identified in only three samples, and subtype A was not detected.

HSV is a viral agent not usually considered in patients with upper respiratory complaints. In this study, HSV-1 was isolated from 1·3% of ILI patients and a similar amount has been detected in other studies.[20] However, as HSV is a latent virus that may be reactivated during infections with other pathogens, we cannot conclude that HSV is the causative agent for the illnesses reported in this study. A control group without ILI symptoms will be needed to draw more definite conclusions on the role of HSV as a causative agent of ILI symptoms.

As part of the surveillance study, we also investigated the genetic diversity of the annual circulating influenza strains in Central America with the purpose of evaluating the relation to the vaccine components for the corresponding year. For influenza A H1N1, three genotypes were observed: A/Solomon Islands/03/06-like, A/Brisbane/59/07-like, and A/New Caledonia/20/99-like. During 2006, at least two genotypes were co-circulating in Nicaragua: A/Solomon Islands/03/06-like and A/New Caledonia/20/99-like. The 2006/2007 recommended vaccine strain for the Northern Hemisphere grouped within the latter as well as the majority of the 2006 isolates from Nicaragua and the 2007 isolates from Honduras. Similarly, the 2008/2009 recommended vaccine strain for the Northern Hemisphere A/Brisbane/59/07 grouped with the 2008 isolates from Nicaragua and Honduras suggesting that the vaccine component was the correct choice. The results differ from our findings in Peru where the recommended vaccine strains for the Southern Hemisphere in 2007 and 2008 were found to be genetically distinct from the circulating H1N1 influenza strains.[16]

Among the H3N2 influenza isolates, at least two genotypes were circulating in Central America in 2006, A/Brisbane/101/05-like and A/Honduras/AF1064/06-like. These genotypes were not included in the 2006/2007 or 2007/2008 recommended vaccines for the Northern Hemisphere (A/Wisconsin/67/05), suggesting that the vaccine was not completely protective against the 2006 circulating strains. A similar situation probably occurred in 2007 and partly in 2008 because of the fact that circulating strains grouped within different genotypes than the vaccine strain. In 2008, evidence of the circulation of three genotypes was

obtained, demonstrating the complexities faced during vaccine selection.

Genetic analysis of influenza B isolates revealed the presence of two genotypes in Central America: B/Malaysia/2506/07-like and B/Florida/4/06-like. The former was present in 2006, whereas the latter was present in 2008. In both years, the recommended vaccine should have protected against the circulating strain. Overall, our data highlight the need for continuous influenza virus surveillance in Central America and the need for sharing this information with World Health Organization reference laboratories for proper evaluation and better selection of future Northern Hemisphere vaccines. The limited number of isolates available for sequencing also indicates the need to reinforce our surveillance activities in Central America.

Finally, the resistance pattern analyzed by sequencing shows that all influenza B and A/H3N2 circulating strains were sensitive to oseltamivir. Our previous findings[8] showed that the mutation H274Y conferring resistance to oseltamivir began to be detected in influenza A/H1N1 strains circulating in Central America (Honduras) at the beginning of 2008. We have found that the circulating strains were rapidly replaced by an oseltamivir-resistant variant that became the most abundant A/H1N1 strain in circulation.

Acknowledgments

We express our gratitude to the Ministry of Health of Nicaragua, The University of Honduras, The Instituto Hondureño de Seguridad Social, Tegucigalpa, Honduras the Hospital de Santa Ana, El Salvador, and all personnel working at sentinel centers for supporting this surveillance study. We thank Gloria Chauca, Monica Nieto, and Ruth Centeno, professional staff of the virology department of NMRCD for invaluable laboratory and technical support in the execution of the study.

Funding

This study was funded by the United States Department of Defense Global Emerging Infections Systems Research Program, WORK UNIT NUMBER: 847705·82000·25GB.B0016.

Conflict of interest

We declare that we have no conflicts of interest.

Disclaimers

Disclaimer: The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the US Government. The study protocol was approved by the Ministry of Health of Peru and the Naval Medical Research Center Institutional Review Board (Protocol NMRCD.2002·0019) in compliance with all applicable Federal regulations governing the protection of human subjects. Disclosure: None of the authors has a financial or personal conflict of interest related to this study. The corresponding author had full access to all data in the study and final responsibility for the decision to submit this publication.

References

1 Williams BG, Gouws E, Boschi-Pinto C, Bryce J, Dye C. Estimates of world-wide distribution of child deaths from acute respiratory infections. Lancet Infect Di 2002; 2(1):25–32.
2 Puzelli S, Boros S, Affinito C *et al.* Prevalence of antibodies against A and B influenza viruses in South-Western Papua New Guinea. J Med Viro 2006; 78:820–824.
3 Gwaltney JM. Clinical significance and pathogenesis of viral respiratory infections. Am J Me 2002; 112(Suppl. 6A):13S–18S.
4 Heijnen ML, Dorigo-Zetsma JW, Bartelds AI, Wilbrink B, Sprenger MJ. Surveillance of respiratory pathogens and influenza-like illnesses in general practices - The Netherlands, winter 1997–98. Euro Surveill 1999; 4(7):81–84.
5 Gordon A, Ortega O, Kuan G *et al.* Prevalence and seasonality of influenza-like illness in children, Nicaragua, 2005–2007. Emerg Infect Dis 2009; 15(3):408–414.
6 Honduras. Secretaria de Salud Informe No 14-2009/Influenza. 2009.
7 World Health Organization (WHO). WHO Recommended Surveillance Standards.WHO/CDS/CSR/ISR/99.2 Second edition. Page 61, October 1999.
8 Garcia J, Sovero M, Laguna-Torres VA *et al.* Antiviral resistance in influenza viruses circulating in Central and South America based on the detection of established genetic markers. Influenza Other Respi Viruses 2009; 3(2):69–74.
9 Swofford DL. PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sunderland, Massachusetts: Sinauer Associates, 1998.
10 Wilgenbusch JC, Swofford D (2003) Inferring evolutionary trees with PAUP*. Curr Protoc Bioinformatics Chapter 6: Unit 6 4.
11 Viboud C, Alonso WJ, Simonsen L. Influenza in tropical regions. PLoS Med 2006; 3(4):e89.
12 Reyes M, Hedlund KO, Lorenzana I, Ehrnst A. Respiratory infection and iatrogenic diarrhea in Honduras and El Salvador during the 1991–1992 season. Am J Trop Med Hyg 1996; 54(3):260–264.
13 Weinberg GA, Hall CB, Iwane MK *et al.* Parainfluenza virus infection of young children: estimates of the population-based burden of hospitalization. J Pediatr 2009; 154(5):694–699.

14 Henrickson KJ, Kuhn SM, Savatski LL. Epidemiology and cost of infection with human parainfluenza virus types 1 and 2 in young children. Clin Infect Dis 1994; 18(5):770–779.
15 Hall CB. Respiratory syncytial virus and parainfluenza virus. N Engl J Med 2001; 344(25):1917–1928.
16 Laguna-Torres VA, Gomez J, Ocana V *et al.* Influenza-like illness sentinel surveillance in Peru. PLoS ONE 2009; 4(7):e6118.
17 Cabello C, Manjarrez ME, Olvera R, Villalba J, Valle L, Paramo I. Frequency of viruses associated with acute respiratory infections in children younger than five years of age at a locality of Mexico City. Mem Inst Oswaldo Cruz 2006; 101(1):21–24.
18 Noyola DE, Rodriguez-Moreno G, Sanchez-Alvarado J, Martinez-Wagner R, Ochoa-Zavala JR. Viral etiology of lower respiratory tract infections in hospitalized children in Mexico. Pediatr Infect Dis J 2004; 23(2):118–123.
19 Garcia J, Sovero M, Laguna-Torres VA *et al.* Molecular characterization of adenovirus circulating in Central and South America during the 2006–2008 period. Influenza Other Respi Viruses 2009; 3(6):327–330.
20 McMillan JA, Weiner LB, Higgins AM, Lamparella VJ. Pharyngitis associated with herpes simplex virus in college students. Pediatr Infect Dis J 1993; 12(4):280–284.