奨用量によるワクチンの有効性と安全性の評価を行うことを目的として，2007/08シーズンに三重県内11カ所の調査対象施設の小児科を受診した6カ月～13歳未満の小児を対象に，欧米諸国と同じ接種量である0.25mL接種群，0.5mL接種群に分け，わが国の現行のインフルエンザHAワクチンとして一般財団法人阪大微生物病研究会製フルービックHA（以下；ビケン製），対照群としてサノフィパスツール社製VAXIGRIP（以下；サノフィ製）のワクチンを用い，接種によるHI抗体価上昇と副反応発現の状況を比較し，ワクチン効果と安全性について検討した．HI抗体価の結果を補完するため，中和抗体価を測定した．また，サノフィ製ワクチンの日本人小児での有効性・安全性についても検討した．

対象と方法

1. 調査対象者

三重県内の小児科11カ所（1カ所：桑名市，四日市市，亀山市，松阪市，伊勢市，鳥羽市，名張市，2カ所：鈴鹿市，津市）を対象に，医療機関ごとに受診した小児の保護者のインフォームドコンセントを得た上で，6カ月～3歳未満児300人，3歳～13歳未満児300人を目標に協力者を募り，協力が得られた6カ月～3歳未満児253人，3歳～13歳未満児307人，合計560人を調査対象とした．

2. ワクチン接種

使用したワクチンは，ビケン製はA/Solomon lslands/3/2006 (H1N1) 15.6μg，A/Hiroshima/52/2005 (H3N2) 15.3μg，B/Malaysia/2506/2004 (B) 16.1μg，サノフィ製はA/Solomon lslands/3/2006 IVR-145 (H1N1)，A/Wisconsin/67/2005 NYMC X-161B (H3N2)，B/Malaysia/2506/2004 like strains (B) 各15μgを含む不活化スプリットワクチンであった．接種方法は，サノフィ製の添付文書に沿い，6カ月～3歳未満児に対しては0.25mL，3歳～13歳未満児に対しては0.5mLを4週間隔で2回皮下注射した．なお，サノフィ製はわが国では承認されていないため研究者が個人輸入して使用した．

3. 調査情報

対象者の属性は，保護者記入用調査票により性，生年月日，体重，通園状況，家族状況，過去3シーズンの接種歴，前シーズン（2006/07シーズン）のインフルエンザ罹患歴を，医療機関記入用調査票により当該シーズン（2007/08シーズン）の接種年月日，採血年月日，基礎疾患，受診時のインフルエンザ迅速診断キット使用状況および臨床症状等の情報を得た．また，接種後の副反応（接種後48時間以内の全身副反応と局所副反応）および調査期間中（2007年12月17日～2008年3月30日）のインフルエンザ罹患状況は，調査票返信葉書により情報を得た．

4. HI抗体測定

1）採血時期

対象者（ビケン製接種群，サノフィ製接種群）の採血は，接種前と2回接種後（2回目接種から4週間後）に実施した．

2）HA抗原

ビケン製接種群には2007/08シーズンワクチン株（デンカ生研）を用い，サノフィ製接種群にはサノフィパスツール社から提供された2007/08シーズンWHO推奨ワクチン株を用いた．なお，HA抗原同士の凝集塊形成を除去する目的で超音波処理を行った後に使用した．

3）赤血球凝集抑制（HI）抗体測定

RDE処理後，10倍から2倍階段希釈した血清25μLにHA抗原4HA単位/25μLおよび0.5%ニワトリ赤血球50μLを添加するWHO方式により行い，HIを示した血清の最大希釈倍数を抗体価とした．この測定は三重県保健環境研究所で実施した．

5. 中和抗体測定

マイクロ中和試験法に準じて実施した[3]．RDE処理後，10倍から4倍段階希釈した血清30μLに至適濃度に調製したウイルス（2007/08シーズンインフルエンザHAワクチン株）を等量添加し，96穴プレートにて37℃，60分中和反応を行い，あらかじめ前日に96穴平底プレートに培養しておいたMDCK細胞に中和反応液30μLを添加し，CO_2インキュベータ内で37℃，16～24時間培養した．培養後，エタノールにて感染細胞を固定し，PAP染色を行い，染色された細胞（フォーカス）の数を鏡顕下でカウントし，ウイルスコントロールの数の50%のフォーカス数を基準に中和抗体価を算出するPAP法にて測定した．この測定は阪大微生物病研究会で実施した．

6. 解析方法

1）インフルエンザ発症に関連する要因

インフルエンザ発症に関連する要因を検討するため，当該シーズンのインフルエンザ発症を目的変数に，ワクチン種別，性別，過去3年間の予防接種歴の有無，前シーズンA型インフルエンザ発症の有無，前シーズンB型インフルエンザ発症の有無，ワクチン接種量，アレルギー（喘息又はアトピー）の有無，通園状況，兄弟姉妹の有無，2回接種後HI抗体価について感染防御水準[4)～8)]とされている40倍以上のHI抗体価の有無の12項目を説明変数として多重ロジスティックモデルによる解析を行った．分析に際し，調査参加者560人から1回目と2回目の接種量が異なる7人，毎週の臨床症状情報に欠落のあった3人，2回目の接種時期が臨床症状調査を開始した2007年第51週以降

Table 1 Stydy subjects broken down by age and the vaccine administered

	age														total
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	
male	12	68	53	25	36	31	23	18	15	7	11	4	5	1	309
female	14	61	45	19	29	13	21	8	11	14	9	5	2		251
BIKEN	13	65	49	24	37	23	24	13	12	7	10	6	2		285
Sanofi Pasteur	13	64	49	20	28	21	20	13	14	14	10	3	5	1	275
total	26	129	98	44	65	44	44	26	26	21	20	9	7	1	560

となった50人を除く500人のうち，前記12項目の情報に欠落がない480人を対象とした．また，接種前のA/H1N1型に対するHI抗体価が10倍以下の者を対象とした分析は，480人から接種前HI抗体価20倍以上の96人を除く384人を対象とした．

2）ワクチン接種前後のHI抗体価推移の解析

ワクチン種別，接種量別に接種前と2回接種後でのHI抗体価の推移をウイルス型ごとにまとめるとともに，欧州医薬品庁（EMEA）の基準[9]に基づく幾何平均抗体価変化率（Geometric mean fold rise以下GMFR），抗体陽転率（Seroconversion rate以下SCR）を求め，比較した．HI抗体価の結果を補完するため中和抗体価を測定し，GMFRおよびHI抗体価に対するSpearmanの順序相関係数を算出した．さらに，接種によるHI抗体上昇に影響する要因を検討するため，接種前HI抗体価が10倍以下の者を対象として，接種後にHI抗体価が40倍以上に上昇したことを目的変数に，ワクチン種別，接種量別，性別，過去3年間の接種歴の有無，前シーズンA型インフルエンザ発症の有無，前シーズンB型インフルエンザ発症の有無，アレルギー（喘息またはアトピー）の有無の7項目を説明変数として多重ロジスティックモデルによる解析を行った．分析に際しては，500人のうち前記7項目の情報に欠落がないことに加えて，接種前抗体価が10倍以下の者と，A/H1N1型については385人，A/H3N2型は287人，B型は482人を対象とした．

3）ワクチン接種後の副反応発現状況の解析

1回目接種後および2回目接種後の48時間以内の副反応について，全身副反応として37.5℃以上の発熱，発疹，局所副反応として接種部位の発赤，腫脹，硬結の発現率をワクチン種別，接種量別に比較した．

7．倫理審査

本研究は，2007年10月16日に開催の国立病院機構三重病院倫理審査委員会において承認を得た（受付番号19-22，題名「小児におけるインフルエンザHAワクチンの接種量と効果に関する研究」）．

結　果

1．調査対象者

県内のほぼ全域から，6カ月～3歳未満児253人，3歳～13歳未満児307人（13歳1人を含む）の合計560人の協力が得られ，性別内訳は男309人，女251人であった．年齢別内訳をTable 1に示した．

2．ワクチン接種状況

対象者には1回目と2回目の接種量が異なるものが含まれており，0.25mLを2回接種したものはビケン製126人，サノフィ製125人，0.50mLを2回接種したものはビケン製155人，サノフィ製147人，合計553人であった．なお，接種時期については，1回目は2007年第42週から第48週，2回目は2007年第46週から2008年第4週であり，2回目の接種が臨床症状の調査開始週（第51週）以降となったのは調査対象者560人中50人であった．

3．調査対象者のインフルエンザ発症状況

三重県における感染症発生動向調査の結果，2007/08シーズンのインフルエンザの流行は，2008年第4週をピークとするA/H1N1型のほぼ単独流行であった．各調査対象機関でインフルエンザと診断された人数は調査対象者560人中47人（8.4%）で，診断週は2008年第4週から第6週に集中しており，感染症発生動向調査と同様の傾向であった．なお，罹患者47人の内訳は，ビケン製0.25mL接種者11人，同0.50mL接種者14人，サノフィ製0.25mL接種者8人，同0.50mL接種者14人であった．

4．解析

1）インフルエンザ発症に関連する要因

2007/08シーズンのインフルエンザ発症に関連する要因について多重ロジスティックモデルによる解析を行ったところ，発症リスクを高める要因として有意となったのは「2回接種後のA/H1N1型に対するHI抗体価が20倍以下」（オッズ比4.10，p＝0.0004）のみであった．なお，HI抗体保有に係る調査年の流行による影響を避けるため，解析に際しては2007年第51週の前に2回接種が完了した者を解析対象とした（Table 2）．

平成25年3月20日

Table 2 Factor analysis for influenza contraction by the univariate analysis (χ^2 test) and the multivariate (multiple logistic model) analysis

Qualitative explanatory variable	Category	Number of occurrence	/	Number of subjects*1	χ^2 test		Multiple logistic model		
					RRs*2	p value	ORs*3	(95% CI)	p value
(1) Vaccine	BIKEN	18	/	242	1		1		
	Sanofi Pasteur	19	/	238	1.07	0.958	1.02	(0.48-2.17)	0.951
(2) Sex	Male	18	/	268	1		1		
	Female	19	/	212	1.33	0.457	1.35	(0.67-2.71)	0.396
(3) Vaccination record for the past 3years	−	8	/	148	1		1		
	+	29	/	332	1.62	0.281	1.35	(0.48-3.78)	0.568
(4) Last winter type A patients	−	36	/	439	1		1		
	+	1	/	41	0.30	0.309	0.33	(0.04-2.60)	0.292
(5) Last winter type B patients	−	33	/	449	1		1		
	+	4	/	31	1.76	0.439	2.39	(0.70-8.22)	0.166
(6) Vaccinated dose (age)	0.25mL (<3 years)	15	/	214	1		1		
	0.50mL (≧3 years)	22	/	266	1.18	0.732	1.66	(0.57-4.84)	0.352
(7) Asthma or atopic dermatitis	−	31	/	419	1		1		
	+	6	/	61	1.33	0.682	1.31	(0.49-3.48)	0.595
(8) Preschool	−	14	/	164	1		1		
	+	23	/	316	0.85	0.757	0.47	(0.17-1.33)	0.157
(9) Siblings	−	4	/	50	1		1		
	+	33	/	430	0.96	1.000	0.98	(0.30-3.15)	0.970
(10) HI Antibody titer after the vaccination A/H1N1	≧40-fold	17	/	354	1		1		
	≦20-fold	20	/	126	3.31	0.0001	4.10	(1.87-9.00)	0.0004***
(11) HI Antibody titer after the vaccination A/H3N2	≧40-fold	25	/	324	1		1		
	≦20-fold	12	/	156	1.00	1.000	0.65	(0.27-1.57)	0.338
(12) HI Antibody titer after the vaccination B	≧40-fold	6	/	106	1		1		
	≦20-fold	31	/	374	1.46	0.491	1.23	(0.46-3.29)	0.681

*1: Number of analysis subjects 480
*2: RR is risk ratio (Ratio of incidence)
*3: OR is adjusted odds ratio by multiple logistic model
Annotation *2 and *3 are the same in Table 3, 6, 7 and 8
***p<0.001

この結果から，さらに解析対象を接種前のHI抗体価が10倍以下の者とし，説明変数のうちHI抗体価はA/H1N1型のみとして解析を行ったところ，発症リスクを高める要因として有意となったのは「2回接種後のA/H1N1型に対するHI抗体価が20倍以下（オッズ比3.12，p=0.003)」，「前シーズンB型インフルエンザ発症（オッズ比3.85，p=0.043)」であった（Table 3).

2) 抗体価

抗体価の解析は，2007/08シーズンのインフルエンザ流行がA/H1N1型のほぼ単独流行であったことから，この影響を避けるため，A/H1N1型については2007年第51週の前に2回接種が完了した者を対象として行った．A/H1N1型のHI抗体価は0.25mL接種群，0.50mL接種群でビケン製，サノフィ製ともに欧州医薬品庁（EMEA）の基準を満たす良好な上昇傾向(0.25mL接種群ビケン製：GMFR 6.8，SCR 60.7%，サノフィ製：GMFR 13.4，SCR 76.6%，0.50mL接種群ビケン製：GMFR 7.0，SCR 68.6%，サノフィ製：GMFR 5.1，SCR 60.9%）を示し，中和抗体価も同様の傾向（0.25mL接種群ビケン製：GMFR 30.4，サノフィ製：GMFR 57.2, 0.50mL接種群ビケン製：GMFR 14.6，サノフィ製：GMFR 11.0）を示した．A/H3N2型のHI抗体価は0.25mL接種群，0.50mL接種群とも，ビケン製に比べサノフィ製が良好な上昇傾向（0.25mL接種群ビケン製：GMFR 2.3，SCR 23.0%，サノフィ製：GMFR 8.4, SCR 73.9%, 0.50mL接種群ビケン製：GMFR 1.9, SCR 16.8%，サノフィ製：GMFR 2.4, SCR 31.3%）を示したが，中和抗体価はこれと異なり，ビケン製，サノフィ製とも良好な上昇傾向（0.25mL接種群ビケン製：GMFR 23.2，サノフィ製：GMFR 16.0，0.50mL接種群ビケン製：GMFR 2.7，サノフィ製：GMFR 1.9）を示した．B型では，両接種群でビケン製，サノフィ製ともに顕著な上昇傾向は認められなかった（0.25mL接種群ビケン製：GMFR 2.2，SCR 16.7%，サノフィ製：GMFR 2.0，SCR 10.4%，0.50mL

Table 3 Factor analysis for influenza contraction of subjects with an antibody titer of 10-fold or less before vaccination by the univariate analysis (χ^2 test) and the multivariate (multiple logistic model) analysis

Qualitative explanatory variable	Category	Number of occurrence	/	Number of subjects*1	χ^2 test		Multiple logistic model		
					RR*2	p value	OR*3	(95% CI)	p value
(1) Vaccine	BIKEN	16	/	194	1		1		
	Sanofi Pasteur	19	/	190	1.21	0.675	1.37	(0.66-2.87)	0.399
(2) Sex	Male	17	/	211	1		1		
	Female	18	/	173	1.29	0.537	1.38	(0.67-2.85)	0.377
(3) Vaccination record for the past 3 years	−	8	/	134	1		1		
	+	27	/	250	1.81	0.167	1.39	(0.49-3.94)	0.537
(4) Last winter type A patients	−	34	/	357	1		1		
	+	1	/	27	0.39	0.505	0.41	(0.05-3.37)	0.404
(5) Last winter type B patients	−	31	/	367	1		1		
	+	4	/	17	2.79	0.093	3.85	(1.04-14.24)	0.043*
(6) Vaccinated dose (age)	0.25mL (<3 years)	15	/	203	1		1		
	0.50mL (≧3 years)	20	/	181	1.50	0.286	1.79	(0.61-5.27)	0.287
(7) Asthma or atopic dermatitis	−	30	/	340	1		1		
	+	5	/	44	1.29	0.785	1.23	(0.42-3.61)	0.703
(8) Preschool	−	14	/	157	1		1		
	+	21	/	227	1.04	0.945	0.51	(0.18-1.43)	0.201
(9) Siblings	−	4	/	44	1		1		
	+	31	/	340	1.00	1.000	0.89	(0.27-2.92)	0.846
(10) HI Antibody titer after the vaccination A/H1N1	≧40-fold	15	/	259	1		1		
	≦20-fold	20	/	125	2.76	0.002	3.12	(1.46-6.69)	0.003**

*1: Number of analysis subjects 384
*p<0.05
**p<0.01

接種群ビケン製：GMFR 2.0，SCR 17.4%，サノフィ製：GMFR 1.8，SCR 12.4%）が，中和抗体価は，A/H1N1型，A/H3N2型に比較するとやや低いものの，ともに良好な上昇傾向（0.25mL接種群ビケン製：GMFR 14.4，サノフィ製：GMFR 11.4，0.50mL接種群ビケン製：GMFR 4.3，サノフィ製：GMFR 4.3）を示した（Table 4，5）．なお，HI抗体価と中和抗体価は良好な相関（Spearmanの順序相関係数：0.711～0.915）を示した（Table 4）．

次に，2回接種後にHI抗体価が40倍以上に上昇したことと関連する要因について多重ロジスティックモデルにより解析した．A/H1N1型では，抗体上昇にプラスに影響する要因として有意となったのは「サノフィ製ワクチンを接種したこと（オッズ比1.72，p=0.019）」，「前シーズンA型インフルエンザ発症（オッズ比2.93，p=0.044）」，「接種量0.50mL（オッズ比2.12，p=0.007）」であり，抗体上昇にマイナスに影響する要因として有意となったのは「過去3年間にワクチン接種歴があること（オッズ比0.25，p=0.000）」，「アレルギーがあること（オッズ比0.44，p=0.016）」であった（Table 6）．A/H3N2型では，抗体上昇にプラスに影響する因子として「サノフィ製ワクチンを接種すること（オッズ比10.07，p=0.000）」のみで有意となった（Table 7）．B型では，抗体上昇にプラスに影響する要因として「前シーズンB型インフルエンザ発症（オッズ比3.75，p=0.006）」のみで有意となった（Table 8）．

さらに，多重ロジスティックモデルによる分析の結果，発症リスクを下げる要因として「2回接種後のA/H1N1型に対するHI抗体価が40倍以上」が有意となったことから，2007年第50週以前に2回のワクチン接種が完了している500人について，A/H1N1型に対する2回接種後のHI抗体価レベル別，発症時の最高発熱レベル別発熱者数，発熱率をまとめた（Table 9）．「2回接種後のA/H1N1型に対するHI抗体価が40倍以上」を指標に38.5℃以上の発熱に対する相対危険（RR）を算出したところ，40倍以上群に対する20倍以下群のRRは2.9（p=0.0010）と有意に高く，「2回接種後のA/H1N1型に対するHI抗体価が80倍以上」を指標とすると，その傾向はより強くなった（RR=3.5，p=0.0007）．

3）ワクチン接種後の副反応発現状況

1回目接種後および2回目接種後の副反応について，ワクチン種別，接種量別の37.5℃以上の発熱，発疹，発赤，腫脹，硬結，疼痛の発現数および発現率（%）を取りまとめたところ，重篤な副反応は1回目，2回

平成25年3月20日

Table 4 Change of geometric mean in the antibody titer

Dose	Method	Vaccine	A/H1N1 mean antibody titer						A/H3N2 mean antibody titer						B mean antibody titer					
			Subjects*1	Before vaccination	R*2	After the second vaccination	R	GMFR*3	Subjects	Before vaccination	R	After the second vaccination	R	GMFR	Subjects	Before vaccination	R	After the second vaccination	R	GMFR
0.25mL (<3 years)	HI test	BIKEN	107	6.0	0.711	40.8	0.830	6.8	126	7.6	0.836	17.1	0.817	2.3	126	5.1	0.734	11.4	0.842	2.2
		Sanofi Pasteur	111	6.1	0.722	82.0	0.868	13.4	125	11.1	0.915	93.4	0.814	8.4	125	5.2	0.730	10.2	0.835	2.0
	NT test	BIKEN	106	7.3	0.711	221.9	0.830	30.4	125	40.9	0.836	948.8	0.817	23.2	125	7.5	0.734	107.9	0.842	14.4
		Sanofi Pasteur	111	8.4	0.722	480.2	0.868	57.2	125	63.4	0.915	1,014.1	0.814	16.0	125	8.1	0.730	92.4	0.835	11.4
0.50mL (≧3 years)	HI test	BIKEN	140	11.4		79.6		7.0	155	19.4		36.4		1.9	155	7.7		15.4		2.0
		Sanofi Pasteur	135	13.3		68.2		5.1	144	75.5		177.9		2.4	144	8.0		14.6		1.8
	NT test	BIKEN	138	26.5		387.3		14.6	153	1,388.8		3,762.5		2.7	153	44.4		190.9		4.3
		Sanofi Pasteur	136	46.6		511.4		11.0	144	2,609.8		4,926.6		1.9	144	45.1		194.0		4.3
0.25mL (2 years)	HI test	BIKEN	45	6.7		43.9		6.6	48	8.3		22.8		2.7	48	5.1		14.6		2.9
		Sanofi Pasteur	44	6.9		55.7		8.1	49	18.4		85.9		4.7	49	5.4		11.4		2.1
	NT test	BIKEN	45	10.8		235.2		21.8	48	127.0		1,890.3		14.9	48	10.9		164.7		15.1
		Sanofi Pasteur	44	12.1		357.3		29.5	49	224.7		1,582.6		7.0	49	12.0		125.8		10.5
0.50mL (3 years)	HI test	BIKEN	20	6.8		49.2		7.2	23	13.5		26.2		1.9	23	5.5		11.6		2.1
		Sanofi Pasteur	20	6.8		42.9		6.3	20	26.4		117.1		4.4	20	5.2		10.0		1.9
	NT test	BIKEN	20	13.2		251.1		19.0	23	584.7		2,638.3		4.5	23	13.9		122.0		8.8
		Sanofi Pasteur	20	11.5		278.6		24.2	20	485.0		3,151.7		6.5	20	10.0		121.3		12.1

*1: Analysis of A/H1N1 mean antibody titer was perfomed in the subjects who received the second vaccination before 50th week in 2007 (non-epidemic periods of A/H1N1 subtype virus)

*2: Spearman rank correlation coefficient between HI antibody titer and NT antibody titer (0.25mL + 0.50mL)

*3: Geometric mean fold rise

Table 5 Seroconversion rate of the HI antibody titer

Dose	Vaccine	A/H1N1 HI antibody titer after the second vaccination					A/H3N2 HI antibody titer after the second vaccination					B HI antibody titer after the second vaccination				
		Subjects*1	NSC*2	SCR (%)*3	RR*4	p value	Subjects	NSC	SCR (%)	RR	p value	Subjects	NSC	SCR (%)	RR	p value
0.25mL (<3 years)	BIKEN	107	65	(60.7)	1		126	29	(23.0)	1		126	21	(16.7)	1	
	Sanofi Pasteur	111	85	(76.6)	1.26	0.012	119	88	(73.9)	3.21	0.000	125	13	(10.4)	0.62	0.147
0.50mL (≧3 years)	BIKEN	137	94	(68.6)	1		155	26	(16.8)	1		155	27	(17.4)	1	
	Sanofi Pasteur	133	81	(60.9)	0.89	0.185	128	40	(31.3)	1.86	0.004	145	18	(12.4)	0.71	0.225
0.25mL (2 years)	BIKEN	45	26	(57.8)	1		48	14	(29.2)	1		48	12	(25.0)	1	
	Sanofi Pasteur	44	27	(61.4)	1.06	0.730	46	26	(56.5)	1.94	0.007	49	6	(12.2)	0.49	0.106
0.50mL (3 years)	BIKEN	20	13	(65.0)	1		23	6	(26.1)	1		23	5	(21.7)	1	
	Sanofi Pasteur	20	13	(65.0)	1.00	1.000	17	11	(64.7)	2.48	0.015	20	3	(15.0)	0.69	0.571

*1: Analysis of A/H1N1 mean HI antibody titer was performed in the subjects who received the second vaccination before 50th week in 2007 (non-epidemic periods of A/H1N1 subtype virus)

*2: Number of seroconversions or significant increase (Standards by The European Agency for the Evaluation of Medicinal Products)

*3: Seroconversion rate

*4: Relative risk of "Sanofi Pasteur SCR" was set to 1 if the "BIKEN SCR"

Table 6 Factor analysis for acquisition of an A/H1N1 HI antibody titer of 40-fold or more after the second vaccination by the univariate analysis (χ^2 test) and the multivariate (multiple logistic model) analysis

Qualitative explanatory variable	Category	Number of occurrence / Number of subjects*1	χ^2 test		Multiple logistic model		
			RR*2	p value	OR*3	(95% CI)	p value
(1) Vaccine	BIKEN	122 / 195	1		1		
	Sanofi Pasteur	138 / 190	1.16	0.045	1.72	(1.09-2.70)	0.019*
(2) Sex	Male	140 / 212	1		1		
	Female	120 / 173	1.05	0.559	1.23	(0.78-1.93)	0.375
(3) Vaccination record for the past 3 years	−	107 / 134	1		1		
	+	153 / 251	0.76	0.000	0.25	(0.14-0.45)	0.000***
(4) Last winter type A patients	−	238 / 358	1		1		
	+	22 / 27	1.23	0.164	2.93	(1.03-8.34)	0.044*
(5) Last winter type B patients	−	248 / 368	1		1		
	+	12 / 17	1.05	0.992	0.87	(0.28-2.65)	0.801
(6) Vaccinated dose (age)	0.25mL (<3 years)	138 / 203	1		1		
	0.50mL (≧3 years)	122 / 182	0.99	0.929	2.12	(1.23-3.67)	0.007**
(7) Asthma or atopic dermatitis	−	238 / 341	1		1		
	+	22 / 44	0.72	0.014	0.44	(0.23-0.86)	0.016*

*1: Number of analysis subjects 385

目ともに認められなかった（Table 10, 11）．37.5℃以上の発熱は，概して0.50mL接種者（3歳以上）に比べ，0.25mL接種者（3歳未満）で発現率が高かったが，0.25mL接種者についてワクチン種別にみると，ビケン製の1回目での発現が少なく，2回目での発現率はビケン製，サノフィ製でほとんど差がみられなかった．局所反応は，発疹を除き，0.25mL接種者に比べ，0.50mL接種者で発現率が高く，1回目と2回目でほぼ同じ傾向がみられた．

考　察

本調査は，6カ月～13歳未満の小児を対象に，わが国のインフルエンザHAワクチンをWHO推奨用量に増量して使用した場合の有効性，安全性を検討することにより，わが国の接種規定を見直す基礎資料を得ることを目的として実施した．

接種後のHI抗体価の幾何平均値をもとに有効性をみると，A/H1N1型については，ビケン製，サノフィ製ともに良好な上昇傾向が認められた．A/H3N2型については，サノフィ製がビケン製を上回る傾向がみられたが，この一因として，ビケン製接種群のHI抗体測定に使用したHA抗原（A/Hiroshima/52/2005株）のインヒビター感受性に変異が生じている可能性が指摘されており[10]，本研究に用いたRDE処理によるインヒビターの除去が不十分となり，低値となった

平成25年3月20日

Table 7 Factor analysis for acquisition of an A/H3N2 HI antibody titer of 40-fold or more after the second vaccination by the univariate analysis (χ^2 test) and the multivariate (multiple logistic model) analysis

Qualitative explanatory variable	Category	Number of occurrence / Number of subjects*1	χ^2 test RR*2	χ^2 test p value	Multiple logistic model OR*3	Multiple logistic model (95% CI)	Multiple logistic model p value
(1) Vaccine	BIKEN	34 / 168	1		1		
	Sanofi Pasteur	85 / 119	3.53	0.000	10.07	(5.69-17.82)	0.000***
(2) Sex	Male	57 / 158	1		1		
	Female	62 / 129	1.33	0.054	1.72	(0.98-3.04)	0.061
(3) Vaccination record for the past 3 years	−	67 / 142	1		1		
	+	52 / 145	0.76	0.068	0.78	(0.42-1.46)	0.434
(4) Last winter type A patients	−	116 / 280	1		1		
	+	3 / 7	1.03	1.000	2.29	(0.41-12.72)	0.345
(5) Last winter type B patients	−	116 / 281	1		1		
	+	3 / 6	1.21	0.992	2.37	(0.36-15.74)	0.372
(6) Vaccinated dose (age)	0.25mL (<3 years)	96 / 203	1		1		
	0.50mL (≧3 years)	23 / 84	0.58	0.003	0.69	(0.33-1.43)	0.313
(7) Asthma or atopic dermatitis	−	108 / 252	1		1		
	+	11 / 35	0.73	0.270	0.65	(0.26-1.62)	0.360

*1: Number of analysis subjects 287

Table 8 Factor analysis for acquisition of a B HI antibody titer of 40-fold or more after the second vaccination by the univariate analysis (χ^2 test) and the multivariate (multiple logistic model) analysis

Qualitative explanatory variable	Category	Number of occurrence / Number of subjects*1	χ^2 test RR*2	χ^2 test p value	Multiple logistic model OR*3	Multiple logistic model (95% CI)	Multiple logistic model p value
(1) Vaccine	BIKEN	42 / 246	1		1		
	Sanofi Pasteur	29 / 236	0.72	0.176	0.65	(0.39-1.10)	0.108
(2) Sex	Male	41 / 268	1		1		
	Female	30 / 214	0.92	0.791	0.89	(0.53-1.50)	0.673
(3) Vaccination record for the past 3 years	−	25 / 169	1		1		
	+	46 / 313	0.99	1.000	0.88	(0.46-1.68)	0.699
(4) Last winter type A patients	−	65 / 445	1		1		
	+	6 / 37	1.11	0.981	1.02	(0.40-2.63)	0.963
(5) Last winter type B patients	−	63 / 460	1		1		
	+	8 / 22	2.66	0.009	3.75	(1.47-9.56)	0.006**
(6) Vaccinated dose (age)	0.25mL (<3 years)	33 / 241	1		1		
	0.50mL (≧3 years)	38 / 241	1.15	0.607	1.12	(0.59-2.12)	0.721
(7) Asthma or atopic dermatitis	−	61 / 419	1		1		
	+	10 / 63	1.09	0.933	1.02	(0.48-2.16)	0.955

*1: Number of analysis subjects 482

可能性が考えられる．このことは，中和抗体価ではビケン製，サノフィ製ともに良好な上昇傾向を示したことからも窺えた．B型については両接種群でビケン製，サノフィ製ともに顕著なHI抗体価の上昇傾向は認められなかったが，前シーズンの主流株がワクチン株と同じVictoria系統であり，多重ロジスティックモデルによる解析から前シーズンに罹患歴のある児でのブースター効果を認め，ワクチン接種の有効性が示された．また，2007/08シーズンのインフルエンザ発症リスクを高める要因として，多重ロジスティックモデルによる解析から2回目接種後のA/H1N1型に対するHI抗体価が20倍以下であることが有意となった．併せて，2回接種後のHI抗体価が40倍以上上昇群に対する同20倍以下群の発熱に関する相対危険が有意に高く，抗体価の高い群で発熱の程度が抑制される傾向がみられた．このことは，ワクチンメーカーの違いにかかわらず，今までの文献と同様に，HI抗体価40倍以上の保有が発症予防効果を示すことを支持する結果であった．一方で，40倍以上のHI抗体価上昇が認められたにもかかわらず，38.5℃以上の発熱を呈した

Table 9 Highest fever at the time of influenza contraction according to the A/H1N1 HI antibody titer after the second vaccination

Highest fever at the time of influenza contraction	A/H1N1 HI antibody titer after the second vaccination									Total
	≧1,280	640	320	160	80	40	20	10	<10	
Number of subjects in whom no fever developed	17	22	49	80	90	95	55	40	12	460
37.0 ～ 37.9℃					1					1
38.0 ～ 38.4℃							2	1		3
38.5 ～ 38.9℃			1	4		4	3		2	14
39.0 ～ 39.4℃			1		2	4	1	2	3	13
39.5 ～ 39.9℃					1	1	1	4		7
≧40℃							1		1	2
Number of patients 39.0℃ or more	0	0	1	0	3	5	3	6	4	22
(%)	(0.0)	(0.0)	(2.0)	(0.0)	(3.2)	(4.8)	(4.8)	(12.8)	(22.2)	(4.4)
Number of patients 38.5℃ or more	0	0	2	4	3	9	6	6	6	36
(%)	(0.0)	(0.0)	(3.9)	(4.8)	(3.2)	(8.7)	(9.5)	(12.8)	(33.3)	(7.2)
Number of patients 37.0℃ or more	0	0	2	4	4	9	8	7	6	40
(%)	(0.0)	(0.0)	(3.9)	(4.8)	(4.3)	(8.7)	(12.7)	(14.9)	(33.3)	(8.0)
Total	17	22	51	84	94	104	63	47	18	500

1: The square indicates those patients who ran a fever of 38.5℃ or more despite having an A/H1N1 antibody titer of 40-fold or greater.
Analysis of A/H1N1 was performed in the subjects who received the second vaccination before the 50th week in 2007.
2: The relative risk was 2.9 with a p value of 0.0010 with the Yates corrected chi-square analysis, representing a significant probability of generation of a fever of 38.5℃ or more in those subjects whose A/H1N1 HI antibody titer was less than 40-fold.
3: The relative risk was 3.5 with a p value of 0.0007 with the Yates corrected chi-square analysis, representing a significant probability of generation of a fever of 38.5℃ or more in those subjects whose A/H1N1 HI antibody titer was less than 80-fold.

Table 10 Adverse reactions after the first vaccination

Dose	Vaccine	Adverse reactions (Number of generation/Nnumber of answers) (%)					
		Fever ≧37.5℃	Rash	Redness	Swelling	Induration	Pain
0.25mL (<3 years)	BIKEN	1/126	8/127	11/127	3/127	12/127	1/127
	(%)	(0.8)	(6.3)	(8.7)	(2.4)	(9.4)	(0.8)
	Sanofi Pasteur	6/125	1/125	6/125	5/125	7/125	3/124
	(%)	(4.8)	(0.8)	(4.8)	(4.0)	(5.6)	(2.4)
	Subtotal	7/251	9/252	17/252	8/252	19/252	4/251
	(%)	(2.8)	(3.6)	(6.7)	(3.2)	(7.5)	(1.6)
0.50mL (≧3 years)	BIKEN	3/157	2/158	21/158	23/158	21/158	29/158
	(%)	(1.9)	(1.3)	(13.3)	(14.6)	(13.3)	(18.4)
	Sanofi Pasteur	5/147	0/149	23/149	36/149	22/149	41/149
	(%)	(3.4)	(0.0)	(15.4)	(24.2)	(14.8)	(27.5)
	Subtotal	8/304	2/307	44/307	59/307	43/307	70/307
	(%)	(2.6)	(0.7)	(14.3)	(19.2)	(14.0)	(22.8)
Total		15/555	11/559	61/559	67/559	62/559	74/558
(%)		(2.7)	(2.0)	(10.9)	(12.0)	(11.1)	(13.3)

No answer and unknown answers are excluded

者がみられたことにも留意する必要はある[8]. なお, 当該シーズンがA/H1N1型の単独流行であったことから, A/H1N1型に対する発症リスクに関する臨床効果は確認したが, A/H3N2型, B型に対する効果は検証できていないことを付記する.

副反応については, 発熱の発現率はビケン製がサノフィ製よりやや低く, 腫脹, 硬結, 疼痛はビケン製, サノフィ製とも0.50mL接種群でやや高めであったが, いずれも重篤なものではなく, 同程度の安全性が認められた.

また, 接種量について, 著者らが過去6シーズン(1999/2000～2004/05)に従来の用法・用量により実施した調査で, 0歳児に対する接種量0.1mLが十分でない可能性を指摘してきた[11)~14)]. 伊藤らはWHO推奨用量による(H1N1) 2009pdmワクチンの免疫原性について既承認用量接種群と比較した同一試験内におけ

平成25年3月20日

Table 11 Adverse reactions after the second vaccination

Dose	Vaccine	Adverse reactions (Number of generation/Nnumber of answers) (%)					
		Fever 37.5℃ or more	Rash	Redness	Swelling	Induration	Pain
0.25mL (<3 years)	BIKEN	8/128	5/128	9/128	5/128	5/127	1/128
	(%)	(6.3)	(3.9)	(7.0)	(3.9)	(3.9)	(0.8)
	Sanofi Pasteur	8/125	2/124	7/125	5/125	4/125	6/125
	(%)	(6.4)	(1.6)	(5.6)	(4.0)	(3.2)	(4.8)
	Subtotal	16/253	7/252	16/253	10/253	9/252	7/253
	(%)	(6.3)	(2.8)	(6.3)	(4.0)	(3.6)	(2.8)
0.50mL (≧3 years)	BIKEN	4/155	2/156	17/156	21/156	22/154	37/156
	(%)	(2.6)	(1.3)	(10.9)	(13.5)	(14.3)	(23.7)
	Sanofi Pasteur	2/145	1/146	18/145	26/146	24/146	34/144
	(%)	(1.4)	(0.7)	(12.4)	(17.8)	(16.4)	(23.6)
	Subtotal	6/300	3/302	35/301	47/302	46/300	71/300
	(%)	(2.0)	(1.0)	(11.6)	(15.6)	(15.3)	(23.7)
Total		22/553	10/554	51/554	57/555	55/552	78/553
(%)		(4.0)	(1.8)	(9.2)	(10.3)	(10.0)	(14.1)

No answer and unknown answers are excluded

る医師主導治験を実施し，3歳以上13歳未満の年齢区分では既承認用量接種群における抗体産生が低かったことを報告している[15]．田村らはわが国の従来の規定により1歳未満の乳児と1歳児にワクチンを接種しHI抗体価変動を比較し，40倍以上の抗体価獲得の割合はいずれの型も乳児では有意に低値で，4倍以上の抗体価上昇の割合，平均抗体価ともにA香港型では有意差を認めなかったが，Aソ連型およびB型においては乳児では有意に低値であったことから，乳児と1歳児での抗体反応の差は年齢差ではなくワクチン接種量の差を反映したものとして，乳児に対する接種量増量の必要性を指摘している[2]．本研究では，WHO推奨用量のみにより3歳未満を0.25mL接種群，3歳以上を0.5mL接種群として比較分析しており，従来法との直接比較及び年齢の影響は確認できていない．しかしながら，少なくともビケン製とサノフィ製とでWHO推奨用量接種による免疫原性，安全性に差がないことから，わが国のインフルエンザHAワクチンの効果が低いとされる原因の一つに接種量が関係していることが示唆された．

以上のことから，わが国における小児に対するインフルエンザHAワクチンの効果を高めるために接種量を増量することは妥当であると考えられた．

謝辞：本稿は，平成18～20年度に実施された厚生労働科学研究費補助金 創薬基盤推進研究事業：政策創薬総合研究事業「新型インフルエンザ用ワクチンの有効性・安全性確保に関する研究」の分担研究（分担研究者：国立病院機構三重病院名誉院長 神谷齊）の成果を基に作成を進めたものであり，本来であれば神谷先生を筆頭著者として投稿すべきものですが，2011年2月末に急逝されたことから，それが叶わなくなりました．ご冥福をお祈り申し上げます．

また，本調査にご協力いただきました，うめもとこどもクリニック 梅本正和先生，安田小児科内科 安田尚樹先生，はね小児科 羽根靖之先生に深謝します．

利益相反自己申告：著者前田一洋は（財）阪大微生物病研究会の職員，奥野良信は同理事である．その他は，利益相反として申告すべきものなし．

文 献

1) Fiore AE, Shay DK, Haber P, Iskander JK, Uyeki TM, Mootrey G, *et al.*：Prevention and Control of Influenza. Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR 2007；56：1—54.
2) 田村大輔，三浦琢磨，上原里程，菅谷憲夫：0歳児及び1歳児におけるインフルエンザワクチン接種後の血清抗体価の推移と接種量に関する検討．感染症誌 2005；79：427—32.
3) Okuno Y, Tanaka K, Baba K, Maeda A, Kunita N, Ueda S：Rapid focus reduction neutralization test of influenza A and B viruses in microtiter system. J. Clin. Microbiol. 1990；28：1308—13.
4) 廣田良夫：インフルエンザワクチンの有効性と疫学的考察．インフルエンザ 2000；1：35—40.
5) 池松秀之：インフルエンザワクチンの接種回数．インフルエンザ 2001；2：237—43.
6) 清水一史：インフルエンザワクチン．臨床と微生物 1997；24：137—41.
7) Gross PA, Quinnan GV, Rodstein M, LaMontagne JR, Kaslow RA, Saah AJ, *et al.*：Association of influenza immunization with reduction in mortality in an elderly population. A prospective study. Arch Intern Med. 1988；148：562—5.
8) Plotkin SA：Correlates Vaccine-Induced Immunity. VACCINES・CID 2008；47：401—9.
9) The European Agency for the Evaluation of

Medicinal Products : Committee for proprietary medicinal products (CPMP). Note for guidance on harmonization of requirements for influenza vaccines 1997 ; CPMP/BMW/214/96 : 1—18.
10) 前田章子, 森川佐依子, 加瀬哲男, 入江 伸, 廣田良夫 : インフルエンザウイルス抗体価測定に関する問題点—2006/07 シーズンワクチン株 A/広島/52/2005 (H4N2) の非特異的凝集抑制物質 (nonspecific inhibitor) 感受性に関する検討—. 感染症誌 2012 ; 86 : 400—4.
11) 大熊和行, 松村義晴, 矢野拓弥, 杉山 明, 中山 治, 中野貴司, 他 : 2001/2002 年の三重県における乳幼児に対するインフルエンザ HA ワクチンの有効性と安全性. 小児感染免疫 2004 ; 16 (1) : 11—20.
12) 大熊和行, 松村義晴, 矢野拓弥, 杉山 明, 中山 治, 中野貴司, 他 : 2002/2003 年の三重県における乳幼児に対するインフルエンザ HA ワクチンの有効性と安全性. 小児感染免疫 2005 ; 1 : 3—16.
13) 大熊和行, 松村義晴, 矢野拓弥, 杉山 明, 中山 治, 中野貴司, 他 : 1999/2000～2002/2003 年の三重県における幼児に対するインフルエンザ HA ワクチンによる HI 抗体産生と副反応. 小児感染免疫 2006 ; 18 : 239—54.
14) 神谷 齊, 中野貴司, 庵原俊昭, 高橋裕明, 矢野拓弥, 大熊和行, 他 : 小児におけるインフルエンザワクチンの接種量と効果に関する研究 (その 2). 第 13 回日本ワクチン学会学術集会抄録集. 2009 ; p. 1A-A-8.
15) 伊藤澄信, 堀部敬三, 岩田 敏 : BK-FUL の免疫原性に関する小児臨床試験. 国立病院機構医師主導治験総括報告書. 2010 ; p. 1—202.

平成25年 3 月20日

A Study on the HA Amount of HA Influenza Vaccination on Efficacy and Safety in Infants

Hiroaki TAKAHASHI[1], Takuya YANO[1], Miwa FUKUTA[1], Akinori YAMAUCHI[1], Kazuyuki OKUMA[1], Toshiaki IHARA[2], Takashi NAKANO[2], Tadashi MATSUDA[3], Sadayoshi TORIGOE[4], Ritsue NII[5], Machiko ISAJI[5], Masahiro WATANABE[6], Hitoshi OCHIAI[7], Hiroyuki SAKATOKU[8], Takashi KATO[9], Kazuhiro MAEDA[10], Yoshinobu OKUNO[10] & Hitoshi KAMIYA[2]

[1]Mie Prefecture Health and Environment Research Institute,
[2]National Hospital Organization Mie National Hospital,
[3]Matsuda Pediatrics Clinic,
[4]Aquair Medical Station,
[5]Shiroko Clinic,
[6]Suzuka Pediatrics Clinic,
[7]Ochiai Pediatrics Clinic,
[8]Sakatoku Pediatrics Clinic,
[9]Kato Pediatrics Clinic,
[10]The Research Foundation for Microbial Diseases of Osaka University

We examined the efficacy and safety of inactivated influenza vaccine when the amount of HA influenza vaccination in children was increased to the dose recommended by the WHO. The purpose of this study was to obtain basic evidence to review the vaccination dose in Japanese children. HA influenza vaccine produced by the Research Foundation for Microbial Diseases of Osaka University (Biken) licenced in Japan was administered through vaccination at the international dose, and split HA influenza vaccine produced by Sanofi Pasteur corp. (Sanofi) was used as control. Children from 6 months to less than 13 years of age were registered, and vaccinated with doses of 0.25mL or 0.5mL. Clinical symptoms during the influenza season were monitored to investigate vaccine efficacy, and information on adverse reactions was collected to evaluate safety profile. Paired serum HI and NT antibody titers were measured at pre first dose and post second dose of vaccination.

Both HI and NT antibody titers for H1N1 subtype were satisfactory elevated after administration of both vaccines. Elevation of the NT antibody titer for the H3N2 subtype was observed for both vaccines, but the H3N2 HI antibody titer for the Biken vaccine was not so high. For the subtype B virus, the NT titer had a better response than the HI titer for both vaccines. As only the H1N1 virus was prevalent in the area during the study period, we performed factor analysis concerning influenza contraction only for the H1N1 antibody titer. An HI titer of 1 : 40 or more at post-vaccination was a significant factor to lower the risk of influenza contraction. The relative risk for fever among children with an HI titer of 1 : 20 or less was significantly higher than those with an HI titer of 1 : 40 or more. Children with a higher HI titer had better prevention against fever, so that both vaccines were considered to be effective.

As for the appearance of adverse reactions, both vaccines were considered to be safe. From the abovementioned results, vaccination with the Japanese Biken vaccine at an international dose was thought to be an effective and safe procedure.

Journal of Clinical Virology 51 (2011) 170–174

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Genetic diversity and antiviral drug resistance of pandemic H1N1 2009 in Lebanon

Hassan Zaraket[a,*], Hiroki Kondo[b], Carelle Tabet[c], Rima Hanna-Wakim[c], Yasushi Suzuki[b], Ghassan S. Dbaibo[c], Reiko Saito[b], Hiroshi Suzuki[b]

[a] Division of Virology, Department of Infectious Diseases, St Jude Children's Research Hospital, 262 Danny Thomas Place, Mail Stop 330, Memphis, TN 38105, USA
[b] Division of Public Health, Department of Infectious Disease Control and International Medicine, Niigata University, Graduate School of Medical and Dental Sciences, Niigata 951-8510, Japan
[c] Division of Pediatric Infectious Diseases, Department of Pediatrics and Adolescent Medicine, American University of Beirut, Beirut, P.O. Box 113/6044/B21, Lebanon

ARTICLE INFO

Article history:
Received 11 January 2011
Received in revised form 3 April 2011
Accepted 6 April 2011

Keywords:
Influenza virus
H1N1 pdm
Lebanon
Oseltamivir resistance

ABSTRACT

Background: In June 2009, the World Health Organization announced the 21st century's first influenza pandemic caused by pandemic influenza H1N1 2009 (H1N1 pdm).
Objectives: Our goal was to analyze antiviral drug resistance and the phylogenetic relationships among hemagglutinin (HA) and neuraminidase (NA) genes of H1N1 pdm samples in Lebanon.
Study design: Nasopharyngeal swabs were collected from 197 patients with influenza-like illness from May 2009 through January 2010. Of the 50 influenza A-positive samples, 30 were analyzed for antiviral drug resistance by using in vitro susceptibility assays and cycling-probe real-time PCR. The HA and NA genes were also analyzed.
Results: The results of hemagglutination-inhibition assays confirmed that all 30 analyzed samples were H1N1 pdm. In July 2009, community transmission of H1N1 pdm was detected in Lebanon, and an outbreak occurred in October 2009. The outbreak cases were caused by a strain with 4 mutations in the NA gene (i.e., V42I, N68T, N248D, and E462K) and 2 mutations in the HA gene: 1 in the Ca1 antigenic site (i.e., S206T) and 1 in the Ca2 antigenic site (i.e., D225E). This strain was closely related to a major H1N1 pdm cluster that was isolated worldwide. All 30 samples were amantadine-resistant, and none were zanamivir-resistant. The 1 oseltamivir-resistant sample appeared to be from a community-transmitted case in an otherwise healthy 2-year-old child.
Conclusion: Continuous monitoring of oseltamivir susceptibility among H1N1 pdm is essential to guide the effective use of this drug.

1. Background

Influenza virus infection is a great burden on health, causing seasonal outbreaks that affect approximately 20% of the population each year.[1] Pandemic viruses emerge periodically when an influenza virus acquires genes to which humans have not been exposed, resulting in increased morbidity and mortality. Pandemics can result in high morbidity and mortality rates. For example, the 1918 Spanish influenza, the 1957 Asian influenza, and the 1968 Hong Kong influenza pandemics killed 25–55 million people worldwide.[1,2]

In April 2009, a new influenza virus emerged in Mexico and North America[3,4] and rapidly spread to more than 70 countries, causing approximately 28,000 infections and 144 deaths before the World Health Organization announced it to be the 21st century's first pandemic, known as pandemic influenza H1N1 2009 (H1N1 pdm). This virus combines gene segments from North American avian and classical swine influenza, human influenza, and Eurasian swine influenza[5]; thus, it is distinct from the seasonal H1N1 influenza virus that was circulating in humans, enabling it to spread in the thus-far naive population.

The first 3 cases of H1N1 pdm in Lebanon were detected at Beirut International Airport on May 30, 2009.[6] Although there is no national influenza surveillance system in Lebanon, our group has been conducting influenza surveillance in the country since 2007, which enabled us to collect samples during the first few months of the pandemic.

2. Objective

Our goal was to characterize antiviral drug susceptibility of H1N1 pdm isolates in Lebanon and to determine the phylogenetic

* Corresponding author. Tel.: +1 901 595 8757; fax: +1 901 595 8559.
E-mail address: Hassan.Zaraket@stjude.org (H. Zaraket).

1386-6532/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jcv.2011.04.001

relationships among the hemagglutinin (HA) and neuraminidase (NA) genes of these isolates and their relationship to isolates from other countries.

3. Study design

3.1. Sample collection

Between May 2009 and January 2010, two nasopharyngeal swabs were collected from each patient at the infectious diseases outpatient clinic at the American University of Beirut, who presented with at least 1 of the following influenza-like illness (ILI) symptoms: fever of at least 38 °C, cough, rhinorrhea, headache, or abdominal symptoms (i.e., diarrhea and vomiting). One swab was used to screen for influenza A by using a rapid influenza detection kit, Quick S-Influ A/B "Seiken" (Denka Seiken Tokyo, Japan). The second swab was placed in virus transport medium and stored at −80 °C until further analysis. A representative portion of the influenza A-positive swabs was shipped to the Division of Public Health, Niigata University, Japan, for further analysis. The Institutional Review Board of the American University of Beirut approved this study, and informed consent was obtained from each patient or patient's guardian before sample collection.

3.2. Virus isolation and characterization

Madin-Darby Canine Kidney (MDCK) cells were inoculated with the swab suspension (100 μL) and incubated until the characteristic cytopathic effect could be observed. To determine the type and subtype of each isolate, we performed hemagglutination-inhibition (HI) assays using A/California/07/09 (H1N1 pdm), A/Victoria/210/2009 (H3N2), B/Brisbane/60/2008 (influenza B) antisera (Denka Seiken Tokyo, Japan), and guinea pig red blood cells. Reference antigens for the antisera were purchased from Denka Seiken (Tokyo, Japan) and included as controls.

3.3. RNA extraction, PCR, and sequencing

RNA isolation was performed by using Extragen II (Kainos, Tokyo, Japan). First-strand complementary DNA was then generated by reverse transcription with U12 primers.[7,8] HA and NA genes were amplified and sequenced as described previously.[9] Phylogenetic tree analyses were performed by using the MEGA program (version 4.0).[10] Reference strains in the tree analyses included some of the closest BLAST search hits for which both the HA and NA genes' sequences were available on the Influenza Virus Resource Data Base (http://www.ncbi.nlm.nih.gov/genomes/FLU/). Sequences generated in this study were deposited in the Japan Genbank database (http://www.ddbj.nig.ac.jp/; accession numbers: AB603583–AB603642).

3.4. Antiviral drug susceptibility testing

In vitro amantadine susceptibility was tested by using 50% tissue culture inhibitory dose ($TCID_{50}$) assays. The 50% inhibitory concentrations (IC_{50}) of oseltamivir and zanamivir were assayed by using a fluorescent-based neuraminidase inhibition assay with methylumbelliferone N-acetylneuraminic acid (MUNANA) as the substrate.[11] Single-nucleotide polymorphisms at codons encoding S31N in the M2 gene fragment and H275Y (N1 numbering) in the NA gene, which confer resistance to amantadine and oseltamivir, respectively, were analyzed by using cycling-probe real-time PCR.[9,12]

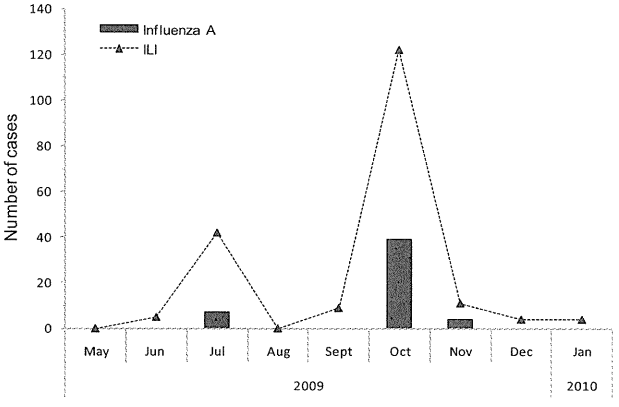

Fig. 1. Graphical illustration of the influenza-like illness (ILI) cases and the influenza A rapid test-positive cases detected in Lebanon from May 2009 through January 2010.

4. Results

4.1. Virus isolation

From May 2009 through January 2010, a total of 197 cases of ILI symptoms were screened in Beirut, Lebanon (Fig. 1). Of these, 50 were influenza A-positive by rapid influenza detection kit; 7 were isolated in July, 39 in October, and 4 in November 2009 (Fig. 1). Of these 50 influenza A-positive swabs, only 30 samples were shipped to Japan for further analysis due to the high shipping cost of the pandemic virus. All 30 samples were confirmed to be influenza A H1N1 pdm viruses by HI assay and HA-specific PCR and sequencing upon virus isolation in MDCK cells.

4.2. Phylogenetic analysis of the HA and NA genes

The HA and NA genes of the 30 virus isolates were sequenced to determine their phylogenetic relationships. In the HA phylogeny, the Lebanese isolates and the reference strains could be divided into 2 main clusters that could be distinguished by a Ser to Thr mutation at residue 206 (H3 numbering) (Fig. 2).[13] Cluster 1 included viruses isolated in July and 1 virus each from October and November. Cluster 2 harbored 25 of the 30 Lebanese isolates, and its subcluster possessed an additional D225E mutation that was detected only in the 21 Lebanese isolates obtained in October and November. Isolates in this subcluster shared high sequence homology (99.7–100%). The S206T and D225E mutations occur in the HA receptor-binding domain and in antigenic sites Ca1 and Ca2, respectively (Fig. 3).

Three of the isolates belonging to cluster 1 in the HA tree, including one Lebanese isolate (09L-06), belonged to cluster 2 in the NA tree. Cluster 2's lineage and a subset of cluster 1's isolates (based on the HA tree) had V42I and N248D mutations in their NA genes. Therefore, these 2 mutations in NA were selected before the S206T mutation in HA. Of the 21 Lebanese isolates with the HA-D225E mutation, 18 clustered together in the NA gene tree and were nearly identical (>99% homology). These isolates shared 2 characteristic mutations, N68T and E462K, in their NA gene. A BLAST search of these isolates' sequence showed that these mutations were unique (at least among the published sequences) to the Lebanese isolates, suggesting that this strain evolved locally or regionally and caused a local outbreak in October and November. Distribution of isolates among clusters 1 and 2 (for either gene) did not relate to the month of isolation, and isolates from different months intermingled in both clusters in the HA and NA trees.

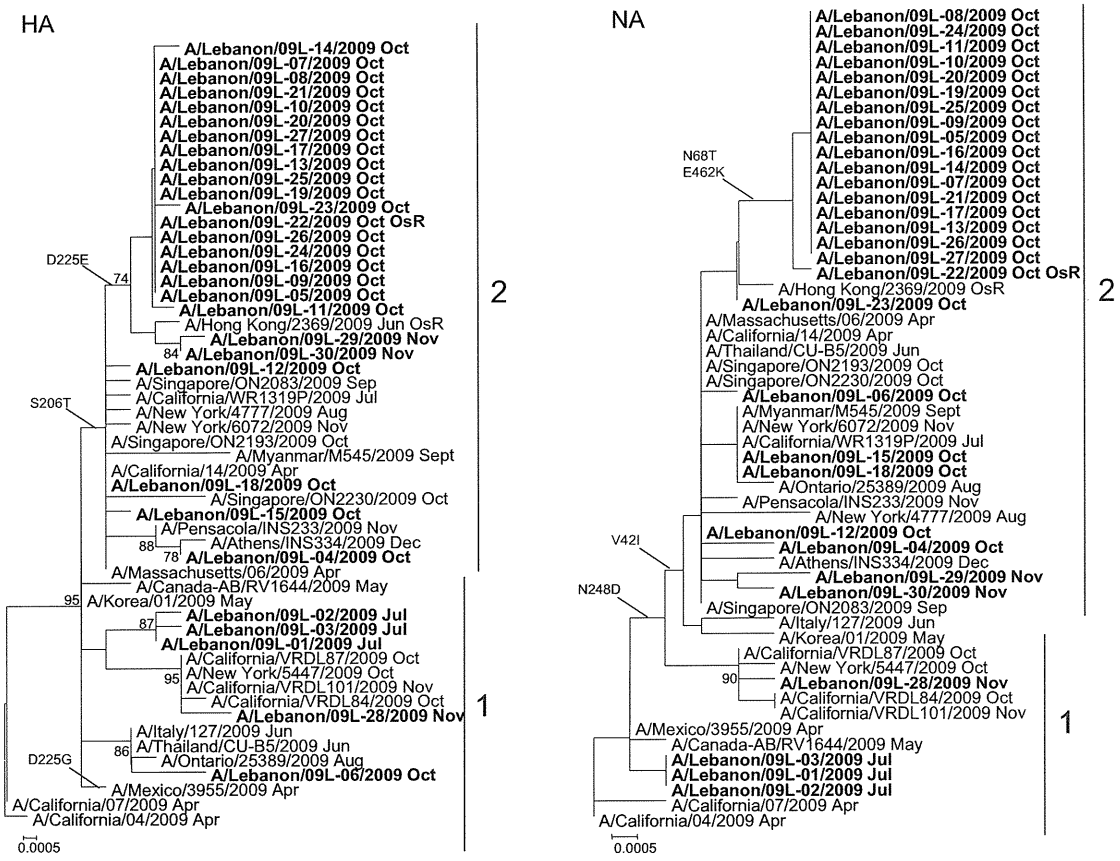

Fig. 2. Phylogenetic relationships of reference strains to the hemagglutinin (HA) and neuraminidase (NA) genes of influenza H1N1 pdm isolates collected in Lebanon from May 2009 through January 2010. Viruses collected in this study are in bold. The trees are rooted at A/California/04/09, and bootstrap values greater than 70% are shown. Characteristic amino acid mutations for main branches are indicated.

4.3. Phenotypic and genotypic antiviral drug-susceptibility

All of the isolates in this study were resistant to amantadine in the $TCID_{50}$ assay, and this was confirmed by the presence of the S31N amantadine-resistance mutation in their M2 genes detected by using the cycling-probe real-time PCR assay. One of the 30 isolates (3.4%) was oseltamivir-resistant, having an IC_{50} value of 240.1 nM; IC_{50} values of susceptible viruses were 1.6–3.0 nM (data not shown). The oseltamivir-resistant isolate had an H275Y mutation in its NA gene in addition to the N68T and E462K mutations detected in the oseltamivir-susceptible isolates. This unique isolate was collected in October from a 2-year-old patient, who was not receiving antiviral-drug treatment when the sample was collected. All iso-

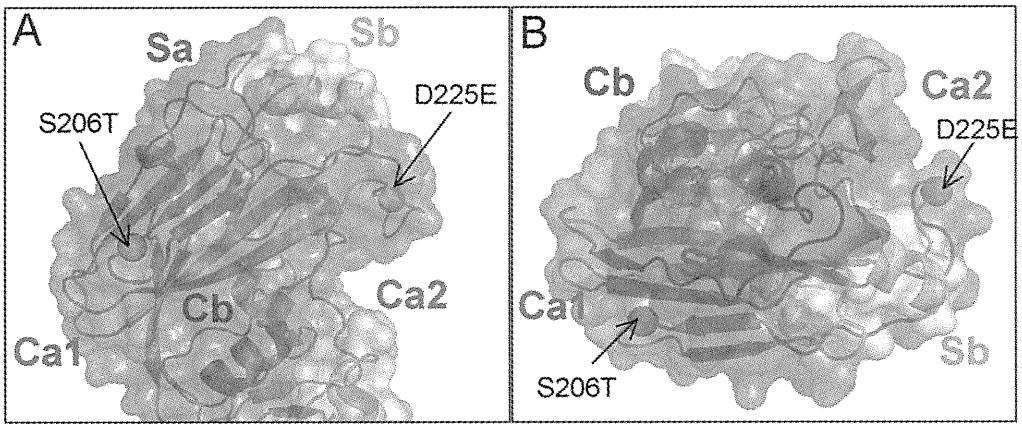

Fig. 3. Structural overview of the receptor-binding domain of the HA protein of H1N1 pdm. Side view (A) and top view (B) of the surface-filling representation of the domain. Antigenic sites are highlighted as follows: Sa (red), Sb (yellow), Ca1 and Ca2 (green), and Cb (blue). The 2 major amino acid mutations detected in the H1N1 pdm's HA are indicated in spheres in the Ca1 and Ca2 sites. The structure was rendered in Pymol (www.Pymol.org) using the H1N1 pdm published structure (pdb: 3AL4; Zhang et al., 2010).

lates were sensitive to zanamivir (IC_{50}, 0.4–2.5 nM; data not shown).

5. Discussion

We detected community-transmitted cases of H1N1 pdm in July 2009, nearly 6 weeks after the first pandemic cases arrived in Lebanon.[6] The Ca1 antigenic site in the majority of the Lebanese isolates had an S206T mutation, which has been reported as a characteristic mutation of a widespread H1N1 pdm strain.[13–15] We showed that this strain also had 2 amino acid mutations in NA: V42I and N248D. The phylogenetic history of these viruses suggested that these NA mutations evolved prior to the HA S206T mutation. This strain was successful in continuous circulation, suggesting that it has a better transmission and infection efficiency than older viruses do, but the mechanisms responsible for this are still unknown.[15] Most of the Lebanese isolates also had a D225E mutation in their Ca2 antigenic sites. Viruses with this mutation were highly homologous, were all isolated in October and November 2009, and had identical NA genes that shared 2 mutations (N68T and E462K), suggesting that this strain was the cause of a local outbreak in Lebanon during these months. It is noteworthy that older viruses without the N68T and E462K mutations in NA were also detected in Lebanon during October but at a lower frequency.

A BLAST search of the HA sequences revealed that the D225E mutation was detected in a virus isolated in Hong Kong in June 2009; however, that isolate did not possess N68T and E462K NA gene mutations. The HA D225E mutation was also detected in an Egyptian isolate during October 2009, but no NA sequence was available from this isolate to compare with those from our study.[16] A D225G mutation in the HA protein was associated with severe and fatal H1N1 pdm cases.[17,18] The increased disease severity could be attributed to enhanced binding of 225G HA to α2-3-linked sialyl receptors of a type expressed in the bronchial and lung epithelial cells, allowing deeper virus infection.[18] However, 225D and 225E variants of the HA protein have low-to-moderate binding affinities to α2-3-linked sialyl receptors.[18] None of the patients with D225E-mutated viruses in this study had severe symptoms, in agreement with previous reports.[17,18] Additionally, none of our patients had the D225G variant. The S206T and D225E mutations are in receptor-binding and antigenic sites of the HA protein and could thus improve the adaptation of H1N1 pdm to human hosts. In a report of analyzed serum samples from pre-H1N1 pandemic years, 6–9% of those aged 18–64 years and 33% of those aged over 60 years had cross-reactive antibodies.[19] Therefore, selection of mutations in the antigenic sites of the HA protein of the pandemic virus might also suggest immune selection during the early phase of the pandemic because of pre-existing cross-reactive antibodies. The N68T and E462K mutations in the NA are located far from the enzyme pocket's active site or framework, and therefore are not expected to affect the enzyme's activity. However, whether or not these mutations contribute to virus adaptation or pathogenicity is unknown.

All of the H1N1 pdm viruses isolated in this study were amantadine-resistant and zanamivir-sensitive, consistent with previously published data.[3] However, the only oseltamivir-resistant isolate was obtained from a child who was not previously treated with oseltamivir, indicating community transmission of oseltamivir-resistant H1N1 pdm, which could be due to the wide use of oseltamivir in Lebanon during the pandemic. Most of the reported oseltamivir-resistant H1N1 pdm cases were isolated from immunocompromised or hospitalized patients receiving prophylactic or treatment oseltamivir,[20–22] and very few cases of community transmission of resistant viruses have been reported.[23,24] The results of in vivo studies in mice and ferret models showed that oseltamivir-resistant viruses are pathogenic and can be efficiently transmitted through direct contact but not via respiratory droplets.[25–27] Inefficient droplet transmission of oseltamivir-resistant viruses could explain the very low prevalence of these viruses. Therefore, continuous monitoring of antiviral drug-susceptibility of influenza is important to guide the effective use of currently available anti-influenza drugs.

Conflict of interest

GD has received support to conduct clinical trials of oseltamivir.

Funding

U.S.-Japan Acute Respiratory Infection Panel (The Ministry of Health, Labor, and Welfare, Japan).

Ethical approval

The Institutional Review Board, American University of Beirut.

Acknowledgements

We are very thankful for the excellent technical assistance of Akemi Watanabe and the extensive secretarial work of Yoshiko Kato. We also thank Richard Webby for reviewing the manuscript and Cherise Guess for editing the manuscript.

References

1. Nicholson KG, Wood JM, Zambon M. Influenza. *Lancet* 2003;**362**:1733–45.
2. Schnitzler SU, Schnitzler P. An update on swine-origin influenza virus A/H1N1: a review. *Virus Genes* 2009;**39**:279–92.
3. Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, et al. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. *N Engl J Med* 2009;**360**:2605–15.
4. Neumann G, Noda T, Kawaoka Y. Emergence and pandemic potential of swine-origin H1N1 influenza virus. *Nature* 2009;**459**:931–9.
5. Smith G, Vijaykrishna D, Bahl J, Lycett S, Worobey M, Pybus O, et al. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature* 2009;**25**:1122–5.
6. Ministry of Public Health Lebanon. Press Release May 2009. http://www.moph.gov.lb/Media/Pages/SwineFluOutbreak.aspx.
7. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. Universal primer set for the full-length amplification of all influenza A viruses. *Arch Virol* 2001;**146**:2275–89.
8. Dapat C, Saito R, Kyaw Y, Naito M, Hasegawa G, Suzuki Y, et al. Epidemiology of human influenza A and B Viruses in Myanmar from 2005 to 2007. *Intervirology* 2009;**52**:310–20.
9. Suzuki Y, Saito R, Sato I, Zaraket H, Nishikawa M, Tamura T, et al. Identification of oseltamivir resistance among pandemic and seasonal influenza A(H1N1) viruses by His275Tyr genotyping assay using cycling probe method. *J Clin Microbiol* 2011;**49**:125–30.
10. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 2007;**24**:1596–9.
11. Hurt AC, Barr IG, Hartel G, Hampson AW. Susceptibility of human influenza viruses from Australasia and South East Asia to the neuraminidase inhibitors zanamivir and oseltamivir. *Antiviral Res* 2004;**62**:37–45.
12. Suzuki Y, Saito R, Zaraket H, Dapat C, Caperig-Dapat I, Suzuki H. Rapid and specific detection of amantadine-resistant influenza A viruses with a Ser31Asn mutation by the cycling probe method. *J Clin Microbiol* 2010;**48**:57–63.
13. Fereidouni SR, Beer M, Vahlenkamp T, Starick E. Differentiation of two distinct clusters among currently circulating influenza A(H1N1)v viruses, March–September 2009. *Euro Surveill* 2009:14.
14. Goni N, Moratorio G, Ramas V, Coppola L, Chiparelli H, Cristina J. Phylogenetic analysis of pandemic 2009 influenza A virus circulating in the South American region: genetic relationships and vaccine strain match. *Arch Virol* 2010;**156**:87–94.
15. Shiino T, Okabe N, Yasui Y, Sunagawa T, Ujike M, Obuchi M, et al. Molecular evolutionary analysis of the influenza A(H1N1)pdm, May–September, 2009: temporal and spatial spreading profile of the viruses in Japan. *PLoS ONE* 2010;**5**:e11057.
16. Adwan G. Molecular characterization and phylogenetic analysis of Middle East 2009 H1N1 pdm isolates. *Asian Pacific J Trop Med* 2010;**3**:624–8.
17. Chen H, Wen X, To KK, Wang P, Tse H, Chan JF, et al. Quasispecies of the D225G substitution in the hemagglutinin of pandemic influenza A(H1N1) 2009

virus from patients with severe disease in Hong Kong, China. *J Infect Dis* 2010;**201**:1517–21.
18. Liu Y, Childs RA, Matrosovich T, Wharton S, Palma AS, Chai W, et al. Altered receptor specificity and cell tropism of D222G hemagglutinin mutants isolated from fatal cases of pandemic A(H1N1) 2009 influenza virus. *J Virol* 2010;**84**:12069–74.
19. Centers for Disease Control and Prevention. Serum cross-reactive antibody response to a novel influenza A (H1N1) virus after vaccination with seasonal influenza vaccine. *MMWR Morb Mortal Wkly Rep* 2009;**58**: 521–4.
20. Centers for Disease Control and Prevention. Oseltamivir-resistant novel influenza A (H1N1) virus infection in two immunosuppressed patients – Seattle, Washington, 2009. *MMWR Morb Mortal Wkly Rep* 2009;**58**: 893–6.
21. Centers for Disease Control and Prevention. Oseltamivir-resistant 2009 pandemic influenza A (H1N1) virus infection in two summer campers receiving prophylaxis – North Carolina, 2009. *MMWR Morb Mortal Wkly Rep* 2009;**58**:969–72.
22. World Health Organization. Oseltamivir resistance in immunocompromised hospital patients (2009 December). http://www.who.int/csr/disease/swineflu/notes/briefing_20091202/en/index.html.
23. Gulland A. First cases of spread of oseltamivir resistant swine flu between patients are reported in Wales. *Br Med J* 2009;**339**:b4975.
24. Le QM, Wertheim HF, Tran ND, van Doorn HR, Nguyen TH, Horby P. A community cluster of oseltamivir-resistant cases of 2009 H1N1 influenza. *N Engl J Med* 2009;**362**:86–7.
25. Duan S, Boltz DA, Seiler P, Li J, Bragstad K, Nielsen LP, et al. Oseltamivir-resistant pandemic H1N1/2009 influenza virus possesses lower transmissibility and fitness in ferrets. *PLoS Pathog* 2010;**6**:e1001022.
26. Kiso M, Shinya K, Shimojima M, Takano R, Takahashi K, Katsura H, et al. Characterization of oseltamivir-resistant 2009 H1N1 pandemic influenza A viruses. *PLoS Pathog* 2010;**6**:e1001079.
27. Seibert CW, Kaminski M, Philipp J, Rubbenstroth D, Albrecht RA, Schwalm F, et al. Oseltamivir-resistant variants of the 2009 pandemic H1N1 influenza A virus are not attenuated in the guinea pig and ferret transmission models. *J Virol* 2010;**84**:11219–26.

G Model
VIRUS-95928; No. of Pages 5
ARTICLE IN PRESS

Virus Research xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Proteins of duck influenza virus responsible for acquisition of pathogenicity in chickens

Naoki Yamamoto[a], Kosuke Soda[a,1], Yoshihiro Sakoda[a], Masatoshi Okamatsu[a], Hiroshi Kida[a,b,*]

[a] Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo, Hokkaido 060-0818, Japan
[b] Research Center for Zoonosis Control, Hokkaido University, Kita 20, Nishi 10, Kita-ku, Sapporo, Hokkaido 001-0020, Japan

ARTICLE INFO

Article history:
Received 8 October 2012
Received in revised form 13 January 2013
Accepted 4 February 2013
Available online xxx

Keywords:
Highly pathogenic avian influenza virus
H5N1
Pathogenicity factors

ABSTRACT

Influenza virus rgVac1sub-P0 (H5N1) (rgVac1-P0), in which a pair of dibasic amino acid residues was introduced at the cleavage site of the HA of a reassortant of H5N2 and H7N1 viruses of duck origin, was low pathogenic in chickens. Vac1sub-P3 (H5N1) (Vac1-P3) was selected as a highly pathogenic avian influenza virus by 3 consecutive passages in chickens from low pathogenic strain rgVac1-P0. Comparison of amino acid sequences of the virus proteins and experimental infection of chickens with a series of recombinant viruses demonstrated that in addition to the HA, each of the PA, NP, M1, and M2 of Vac1-P3 are responsible for the acquisition of pathogenicity in chickens. These 4 proteins of Vac1-P3 synergistically contributed to efficient virus replication in chickens.

1. Introduction

Influenza A virus belongs to the *Orthomyxoviridae* family and has been isolated from birds and mammals. Highly pathogenic avian influenza viruses (HPAIVs) are selected in chicken populations (Ito et al., 2001). The hemagglutinin (HA) of HPAIV has a pair of dibasic amino acid residues at the cleavage site (Senne et al., 1996), which permits ubiquitous proteases such as furin and PC6 to cleave the HA, leading to systemic infection in chickens (Horimoto et al., 1994). In contrast, the HA of low pathogenic avian influenza virus (LPAIV) is cleaved only by trypsin-like proteases, which are expressed in the cells lining the respiratory or intestinal tracts, so that the viruses cause only localized infections, resulting in mild or asymptomatic diseases (Klenk and Garten, 1994). LPAIV did not acquire high pathogenicity in chickens only by introduction of a pair of dibasic amino acid residues at the HA cleavage site (Bogs et al., 2010; Soda et al., 2011; Stech et al., 2009; Veits et al., 2012), indicating that other factors should be required for viruses to acquire high pathogenicity in chickens.

rgVac1sub-P0 (H5N1) (rgVac1-P0) was generated by introduction of a pair of dibasic amino acid residues at the HA cleavage site of non-pathogenic avian influenza virus A/duck/Hokkaido/Vac-1/2004 (H5N1) (Vac1), which is a reassortant of H5N2 and H7N1 viruses isolated from wild ducks (Soda et al., 2011). rgVac1-P0 was low pathogenic in chickens, although it replicated in MDCK cells without the addition of trypsin (Soda et al., 2011). On the other hand, Vac1sub-P3 (H5N1) (Vac1-P3) was selected by consecutive passages of rgVac1-P0 in chickens and was highly pathogenic in chickens.

In the present study, putative amino acid sequences of the proteins of the passaged virus, Vac1-P3, were compared with those of the parental virus, rgVac1-P0. In order to identify the virus proteins responsible for pathogenicity in chickens in addition to the HA, a series of recombinant viruses was generated, and the pathogenicity of these viruses was assessed by experimental infection of chickens. Function of the virus proteins was investigated by in vitro assay.

2. Materials and methods

2.1. Viruses

Non-pathogenic avian influenza virus Vac1 is a reassortant of H5N2 and H7N1 viruses isolated form wild duck. To generate reverse genetic viruses, the vectors of the PA, HA, NP, M1, and M2 genes were constructed by site-directed mutagenesis with primers, QuikChange Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Germany), and pHW2000 vectors containing the PB2, PB1, PA, NP, NA, M, and NS segments of Vac1 and the HA segment of rgVac1-P0 (Hoffmann et al., 2000; Soda et al., 2008, 2011). Recombinant viruses rgVac1-P3/0PA, rgVac1-P3/0NP, rgVac1-P3/0M1, rgVac1-P3/0M2, and rgVac1-P3/0M1,2 were generated by replacing the

* Corresponding author at: Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan. Tel.: +81 11 706 5207; fax: +81 11 706 5273.
E-mail address: kida@vetmed.hokudai.ac.jp (H. Kida).
[1] Present address: Avian Zoonosis Research Center, Faculty of Agriculture, Tottori University, Tottori, 101 Minami 4, Koyama-cho, Tottori 680-8553, Japan.

0168-1702/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.virusres.2013.02.001

Please cite this article in press as: Yamamoto, N., et al., Proteins of duck influenza virus responsible for acquisition of pathogenicity in chickens. Virus Res. (2013), http://dx.doi.org/10.1016/j.virusres.2013.02.001

PA, NP, M1, and M2 genes of rgVac1-P3 with those of rgVac1-P0, respectively. Madin–Darby canine kidney (MDCK) cells and human embryonic kidney (HEK) 293T cells were co-cultured and transfected with 1.0 μg of each of the 8 plasmids and 16 μl of TransIT-293 (Panvera, Madison, WI, U.S.A.) in 150 μl Opti-MEM I (Invitrogen, Carlsbad, CA, U.S.A.). After 30 h of incubation, 1.0 ml Opti-MEM I was added to each well. After 48 h of incubation at 35 °C, 100 μl supernatant was injected into the allantoic cavity of 10-day-old chicken embryos. After 48 h of incubation at 35 °C, allantoic fluid was harvested as the virus stock.

2.2. Cells

MDCK cells were maintained in Minimum Essential Medium (MEM, Nissui, Tokyo, Japan) supplemented with 0.3 mg/ml L-glutamine (Wako Chemicals, Tokyo, Japan), 10% calf serum (Sanko Junyaku, Tokyo, Japan), 100 U/ml penicillin G (Meiji Seika, Tokyo, Japan), 0.1 mg/ml streptomycin (Meiji Seika) and 8.0 μg/ml gentamicin (Merck Sharp and Dohme, Rahway, NJ, U.S.A.). HEK 293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 0.3 mg/ml L-glutamine, 10% fetal calf serum (Cambrex, Walkersville, MD, U.S.A.) and antibiotics. Chicken embryonic fibroblast (CEF) cells were prepared from 10-day-old chicken embryos and were cultured in MEM supplemented with L-glutamine, calf serum, and antibiotics.

2.3. Sequence analysis

Virus RNA was extracted from the allantoic fluid of chicken embryos infected with the isolates by TRIzol LS Reagent (Invitrogen) and reverse-transcribed with the Uni12 primer (Hoffmann et al., 2001) and M-MLV Reverse Transcriptase (Invitrogen). The full-length of each gene segment was amplified by polymerase chain reaction with gene-specific primer sets (Hoffmann et al., 2001). Each gene segment was sequenced using 3500 Genetic Analyzer (Applied Biosystems, Foster city, CA, U.S.A.).

2.4. Experimental infection of chickens

To assess the pathogenicity in chickens, rgVac1-P0, rgVac1-P3, rgVac1-P3/0PA, rgVac1-P3/0NP, rgVac1-P3/0M1, rgVac1-P3/0M2, and rgVac1-P3/0M1,2 of $10^{6.5}$ 50% egg infectious dose (EID_{50}) were inoculated into 9 4-week-old chickens (Boris Brown). Three of 9 chickens were euthanized 3 days post-inoculation (dpi), and the brain, trachea, lung, and colon were collected aseptically for virus recovery. The remaining 6 chickens were observed daily for disease signs for 14 days after inoculation. The pathogenicity score was calculated according to the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2012 of World Organization for Animal Health. Briefly, the score was calculated by dividing the sum of daily scores by the number of observation days (0: normal, 1: sick, 2: severely sick, 3: dead). Sera were collected on the day of inoculation and 14 dpi to test for antibodies against H5N1 virus. Specific antibodies against Vac1 were detected in the sera of chickens infected with the viruses on 14 dpi by the hemagglutination inhibition (HI) test as described previously (Kida et al., 1982). The tissue homogenates were inoculated into 10-day-old chicken embryos and the titers were calculated and expressed as the EID_{50}/g.

Chickens were housed in a self-contained isolator unit (Tokiwa Kagaku, Tokyo, Japan) at a BSL-3 facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan. The institutional animal care and use committee of the Graduate School of Veterinary Medicine authorized this animal experiment (approval number: 1112) and all experiments were performed according to the guidelines of this committee.

2.5. Replicon assay

Replicon assay was performed according to Salomon et al. (2006). Briefly, subconfluent monolayers of CEF cells were transfected with 1.0 μg the viral UTR-driven firefly luciferase plasmid containing chicken polymerase I promoter pHW-luc2CP, pRL-TK (Int-) renilla luciferase plasmid (Promega, Madison, WI, Germany), a mixture of 0.2 μg of the PB2, PB1, and PA plasmids and 0.4 μg of the NP plasmid, and 4.0 μg polyethyleneimine (Polyscience, Warrington, PA, U.S.A.). Forty-eight hours post-transfection, cell extracts were prepared using 200 μl Passive Lysis Buffer (Promega). Luciferase activities were quantified by Dual-Glo Luciferase Assay System (Promega) and measured by POWER SCAN 4 (DS Pharma Biomedical, Japan). Firefly luciferase activities were normalized by renilla luciferase activity.

2.6. Electron microscopy

rgVac1-P0, rgVac1-P3, rgVac1-P3/0M1, rgVac1-P3/0M2, and rgVac1-P3/0M1,2 were inactivated with 0.1% (v/v) formaldehyde and centrifuged at 10,000 × *g* for 1.5 h, and the pellets were resuspended in phosphate-buffered saline (PBS). Purified viruses were spread on mesh coated with collodion (Nisshin EM, Tokyo, Japan), and negatively stained with 2% phosphotungstic acid (pH 5.8). The specimens were examined under the transmission electron microscope JEM-1210 (Japan Electron Optics Laboratory, Tokyo, Japan) operated at an accelerating voltage of 80 kV.

3. Results

3.1. Amino acid sequences of the proteins of rgVac1-P0 and Vac1-P3

In comparison of putative amino acid sequences of the proteins of Vac1-P3 with those of rgVac1-P0, a total of 6 substitutions and an insertion of amino acid were found in the PA, HA, NP, M1, and M2 of Vac1-P3, as shown in Table 1.

3.2. Pathogenicity of recombinant viruses in chickens

To identify the virus proteins responsible for the acquisition of pathogenicity in chickens, rgVac1-P3 and recombinant viruses, in which the PA, NP, M1, or M2 of rgVac1-P3 were substituted with those of rgVac1-P0, were prepared by reverse genetics (Table 1). These viruses were intranasally inoculated into 9 4-week-old chickens with $10^{6.5}$ EID_{50}, respectively. Any clinical symptoms such as depression and poor feeding were observed in the chickens infected with rgVac1-P3 after 2 dpi and all of the chickens died by 9 dpi. Four of 6 chickens inoculated with rgVac1-P3/0PA and rgVac1-P3/0M1 showed clinical symptoms after 2 dpi and 2 of 6 chickens inoculated with rgVac1-P3/NP showed clinical symptoms after 4 dpi. Pathogenicity scores of rgVac1-P3/0PA, rgVac1-P3/0NP, and rgVac1-P3/0M1 were lower than that of rgVac1-P3 (Table 1). Infectivity rates of rgVac1-P3/0M2 and rgVac1-P3/0M1,2 were lower than that of rgVac1-P3.

3.3. Growth of recombinant viruses in chickens

To examine the growth of viruses, rgVac1-P0, rgVac1-P3, and recombinant viruses were intranasally inoculated into chickens. Viruses were recovered from the organs of chickens infected with each virus on 3 dpi. Infectivity titers of the recovered viruses from the tissue samples of chickens infected with rgVac1-P3 were

Please cite this article in press as: Yamamoto, N., et al., Proteins of duck influenza virus responsible for acquisition of pathogenicity in chickens. Virus Res. (2013), http://dx.doi.org/10.1016/j.virusres.2013.02.001

Table 1
Difference of the amino acid sequence in each protein, pathogenicity score, and infectivity rate of each virus in chickens.

H5N1 virus	Virus protein [a]										Pathogenicity score [d]	Infectivity rate (%)
	PB2	PB1	PA	HA	NP	NA	M1	M2	NS1	NS2		
rgVac1-P0			672[b] L	308 H	374 M		89|101 DR	45 R			0.05	50
rgVac1-P3/0PA											0.64	100
rgVac1-P3/0NP											0.25	83
rgVac1-P3/0M1											0.51	100
rgVac1-P3/0M2											0.00	33
rgVac1-P3/0M1,2											0.07	17
rgVac1-P3			672 F	308|338 Q▲[c] R	374 V		89|101 NK	45 H			2.23	100

[a] White box indicates the protein derived from rgVac1-P0 and black box indicates the protein derived from rgVac1-P3.
[b] Methionine encoded by the AUG start codon is defined as position 1.
[c] Triangle indicates that arginine is inserted at position 338.
[d] Score of 3.00 means that all chickens died within 24 h, and score of 0.00 means that no chicken showed any clinical signs during the 14-day observation period.

higher than those with rgVac1-P0 (Table 2). Virus titers of the tissue samples from chickens infected with rgVac1-P3/0PA, rgVac1-P3/0NP, or rgVac1-P3/0M1 were lower than those with rgVac1-P3. The viruses were scarcely recovered from chickens infected with rgVac1-P3/0M2. In addition, no virus was recovered from chickens infected with rgVac1-P3/0M1,2.

3.4. Morphology of recombinant virus particles

rgVac1-P0, rgVac1-P3, rgVac1-P3/0M1, rgVac1-P3/0M2, and rgVac1-P3/0M1,2 were examined by electron microscopy. Most particles of the viruses with the M1 of rgVac1-P0; rgVac1-P0, rgVac1-P3/0M1, and rgVac1-P3/0M1,2 were spherical (Fig. 1). On the other hand, filamentous particles of those with the M1 of rgVac1-P3; rgVac1-P3 and rgVac1-P3/0M2 were observed in 40.3% and 38.4%, respectively. The present results indicate that the M1 is responsible for the morphology of the virus particles.

3.5. Polymerase activity of nucleocapsids of rgVac1-P0 and Vac1-P3 in replicon assay

To examine the activity of polymerase with the PA and NP of rgVac1-P0 or rgVac1-P3, the replicon assay using firefly luciferase gene was carried out in CEF cells. Polymerase activity with the PA

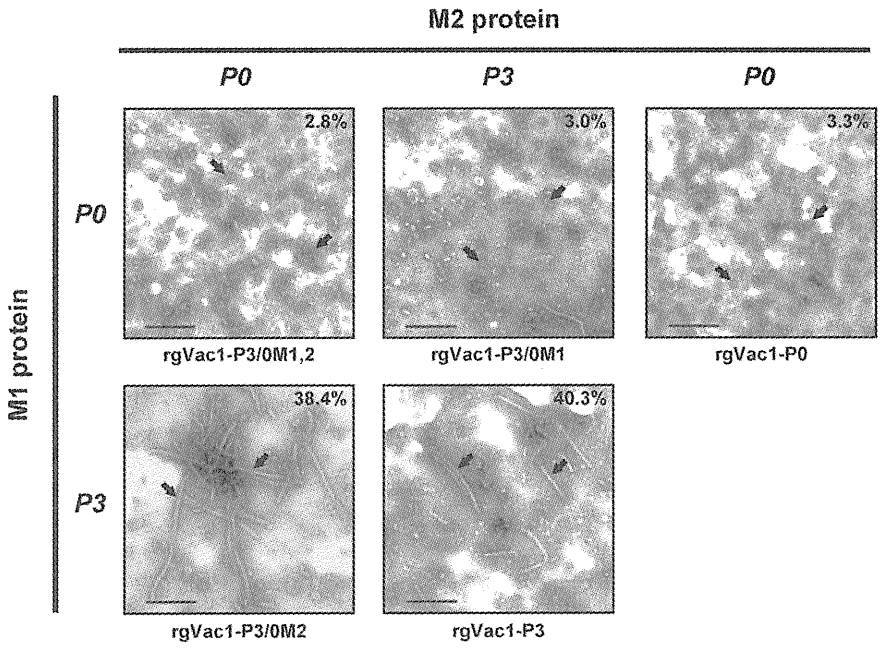

Fig. 1. Morphology of the virus particles. rgVac1-P0, rgVac1-P3, rgVac1-P3/0M1, rgVac1-P3/0M2, and rgVac1-P3/0M1,2 were negatively stained with 2% phosphotungstic acid (pH 5.8). Virions were visualized by transmission electron microscopy. "P0" and "P3" indicate that the M1 and M2 proteins are derived from rgVac1-P0 and rgVac1-P3, respectively. Bar represents 1 μm. Arrows indicate virus particles. Percentage of filamentous particle was shown in each picture.

Please cite this article in press as: Yamamoto, N., et al., Proteins of duck influenza virus responsible for acquisition of pathogenicity in chickens. Virus Res. (2013), http://dx.doi.org/10.1016/j.virusres.2013.02.001

Table 2
Virus recovery from the chickens intranasally inoculated with each virus on 3 days post-infection.

H5N1 virus	Virus recovery (log EID_{50}/g, mean ± standard deviation)			
	Brain	Trachea	Lung	Colon
rgVac1-P0	2.5 ± 0.9 (3.5/1.7/2.3)[a]	2.7 ± 0.9 (3.5/1.7/3.0)	3.1 ± 1.0 (3.8/2.0/3.5)	2.4 ± 0.7 (3.0/1.7/2.5)
rgVac1-P3/0PA	3.4 ± 1.1 (2.3/4.5/3.5)	3.1 ± 1.4 (2.0/4.7/2.7)	5.1 ± 1.3 (4.8/6.5/4.0)	4.0 ± 2.0 (5.5/4.8/1.7)
rgVac1-P3/0NP	4.2 (<[b]/5.3/3.0)	3.2 (</3.8/2.5)	2.3 (</2.3/2.3)	< (</</<)
rgVac1-P3/0M1	3.7 ± 0.3 (3.5/4.0/3.7)	4.5 ± 0.4 (4.0/4.7/4.7)	4.4 ± 1.5 (5.0/5.5/2.7)	4.0 ± 1.3 (2.7/4.0/5.3)
rgVac1-P3/0M2	< (</</<)	2.5 (2.5/</<)	< (</</<)	2.4 (2.5/</2.3)
rgVac1-P3/0M1,2	< (</</<)	< (</</<)	< (</</<)	< (</</<)
rgVac1-P3	5.0 ± 0.5 (5.0/5.5/4.5)	5.2 ± 0.6 (5.5/5.5/4.5)	4.9 ± 1.0 (6.0/4.5/4.2)	4.9 ± 0.5 (4.5/5.5/4.7)

[a] Numbers indicate virus titers in organs of each chicken.
[b] Titer is <1.5 log EID_{50}/g.

and/or NP of rgVac1-P3 was higher than that with both the PA and NP of rgVac1-P0 (Fig. 2).

4. Discussion

It has been proposed that some virus factors are required in addition to the HA which has a pair of dibasic amino acid residues at the cleavage site (Bogs et al., 2010; Stech et al., 2009; Tada et al., 2011; Veits et al., 2012). In the present study, comparison of amino acid sequences of virus proteins and experimental infection of chickens with a low pathogenic avian influenza virus of duck origin demonstrated that each of the structures of the PA, NP, M1, and M2 of Vac1-P3 in addition to the HA were responsible for the acquisition of high pathogenicity in chickens (Table 1).

In the present study, the virus that has the M2 with histidine at position 45 was selected through passaging in chickens. In addition, infectivity rates of the viruses with the M2 of rgVac1-P3 were higher than those of rgVac1-P0 in chickens (Table 1). These findings agree with other research showing that the virus which has the M2 with histidine at this position was selected as a highly pathogenic strain in chickens (Veits et al., 2012). An amino acid residue at position 45 of the M2 of influenza virus is located in the ion channel of the M2 tetramer (Schnell and Chou, 2008), suggesting that the M2 with histidine at position 45 of Vac1-P3 is responsible for efficient membrane fusion at the infection step in chickens.

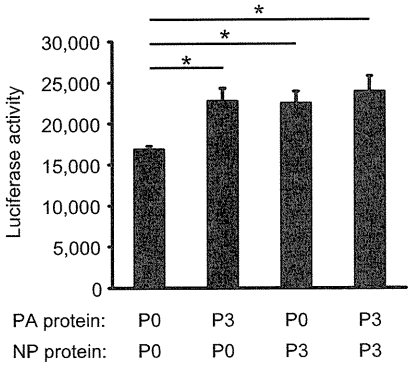

Fig. 2. Polymerase activity in CEF cells. Polymerase activity was evaluated by viral UTR-driven luciferase reporter gene. CEF were transfected with plasmids encoding the PB2 and PB1 of rgVac1-P0 and the PA and NP of rgVac1-P0 or rgVac1-P3 (H5N1). Luciferase activity was normalized by renilla luciferase activity. P0 indicates the gene derived from rgVac1-P0 (H5N1) and P3 indicates the gene derived from rgVac1-P3 (H5N1). The mean value and standard error are shown according to the results of 3 independent experiments. Statistical comparison was performed using the *t*-test. Asterisk indicates significant difference ($p < 0.05$).

Infectious titers of the recovered viruses from the tissue samples of chickens infected with rgVac1-P3/0M1 were lower than those with rgVac1-P3 (Table 2). The M1 of influenza virus plays an essential role in virus assembly (Gomez-Puertas et al., 2000), associating with nucleocapsid and virus RNA (Elster et al., 1997; Ye et al., 1999), and control of nucleocapsid transportation in the cells infected with the virus (Boulo et al., 2007), suggesting that the M1 of Vac1-P3 contributes to efficient replication in chickens. In addition, particles of the viruses with the M1 of Vac-P3 were filamentous (Fig. 1). Influenza virus is known to be polymorphic, spherical or filamentous, and the filamentous form is typical of clinical isolates of influenza virus, whereas virus particles with the spherical form are observed in laboratory viruses that have been passaged in chicken embryos (Choppin et al., 1960). Association between the pathogenicity and virus forms in chickens needs to be clarified in a future study.

The PA and NP are requisite proteins in both the transcription and replication of virus genes (Biswas et al., 1998; Yuan et al., 2009). The C-terminal regions of the PA and NP interact with the PB1 and PB2, respectively, and regulate polymerase activity (Biswas et al., 1998; He et al., 2008; Obayashi et al., 2008). Amino acid residues at positions 627 of the PA and 374 of the NP were located in the C-terminal region of these proteins. Although the synergistic effect of the PA and NP of rgVac1-P3 was not shown in luciferase assay (Fig. 2), virus titers in the tissue samples from chickens infected with rgVac1-P3/0PA and rgVac1-P3/0NP were lower than those with rgVac1-P3 (Table 2), indicating that the PA and NP of Vac1-P3 contribute to efficient virus replication in chickens.

In conclusion, in addition to the HA, the PA, NP, M1, and M2 of Vac1-P3 contribute to the virus that acquires pathogenicity in chickens, indicating that the functions of the proteins synergistically contribute to the acquisition of pathogenicity of rgVac1-P0 in chickens. Molecular basis of the synergy remains to be investigated.

Acknowledgments

We gratefully acknowledge the technical support of M. Endo, Y. Sato, and R. Sawayama. The present work was supported in part by the Global Centers of Excellence Program from Japan Society for the Promotion of Science. This work was also supported by the J-GRID; The Japan Initiative for Global Research Network on Infectious Disease and Japan Science and Technology Agency Basic Research Programs.

Please cite this article in press as: Yamamoto, N., et al., Proteins of duck influenza virus responsible for acquisition of pathogenicity in chickens. Virus Res. (2013), http://dx.doi.org/10.1016/j.virusres.2013.02.001