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Eur J Pediatr (2010) 169:1221–1225
DOI 10.1007/s00431-010-1208-5

ORIGINAL PAPER

A comparison of conventional and molecular microbiology in detecting differences in pneumococcal colonization in healthy children and children with upper respiratory illness

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Received: 16 March 2010 / Accepted: 20 April 2010 / Published online: 4 May 2010

Abstract Conventional microbiology (CM) and real-time polymerase chain reaction (PCR) were used to determine rate and serotype of pneumococcal nasopharyngeal colonization in healthy children and children with upper respiratory illnesses (URI). One hundred and thirty-six healthy children and 79 children with URI were evaluated. Pneumococcal colonization was detected more often by real-time PCR than CM in healthy children (50% vs. 24%, $p \leq 0.001$), while detection rates were comparable by CM and real-time PCR in children with URI (61% vs. 65%, NS). Pneumococcal serotypes were identified 2.3 times more often in healthy children by real-time PCR than CM, $p \leq 0.001$ and 1.5 times more often in children with URI by PCR than CM, $p=0.01$. Real-time PCR was also more sensitive in detecting multiple strains rather than CM in both healthy ($p=0.001$) and children with URI ($p \leq 0.001$). Overall real-time PCR proved superior to CM in detection and serotyping of *Streptococcus pneumoniae*. Future studies should incorporate real-time PCR technology along with CM to fully understand the epidemiology of colonization in health and illness.

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Keywords *Streptococcus pneumoniae* · Real-time polymerase chain reaction · Nasopharyngeal colonization · Serotype distribution

Introduction

Nasopharyngeal bacterial colonization evolves rapidly during the first year of life [10, 11, 15]. *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* colonize the nasopharynx early in life and are responsible for the vast majority of acute otitis media (AOM) and acute rhinosinusitis (ARS) [5, 10, 11, 15]. *S. pneumoniae* is the most important of the three pathogens because it is ubiquitous and highly pathogenic [4, 7, 8, 10, 13, 16, 22, 29].

S. pneumoniae colonizes as many as 54% of children by 1 year of age [10]. A single strain may persist in the nasopharynx for weeks or months and is often replaced by another strain; multiple serotypes within a single individual are reportedly uncommon [12, 15, 27].

The relationship between colonization and disease has been extensively studied [5, 10, 12, 13, 15, 16, 18, 21, 24, 29]. Children who are colonized early and repeatedly in life are prone to recurrent episodes of otitis media [13]. Current pneumococcal vaccines have been shown to reduce colonization with vaccine strains and thereby reduce otitis media due to the same strains [6, 9, 14]. Unfortunately, non-vaccine strains have been shown to replace the vaccine strains in the nasopharynx [23]. Most studies on the epidemiology of colonization after the introduction of the conjugated pneu-

mococcal vaccines utilized conventional microbiology (CM). The present study was designed to compare real-time polymerase chain reaction (PCR) technology with CM in determining pneumococcal colonization rates and serotypes in healthy and ill children.

Materials and methods

Population

Healthy children and children with upper respiratory illnesses were enrolled. Informed consent was obtained from parents or guardians. This study was approved by the ethical committee of Wakayama Medical University. The healthy children consisted of children who were scheduled to receive immunizations according to the Japanese Preventive Vaccination Law. They were confirmed as healthy by the lack of symptoms and abnormal physical findings. None of children in this study received 7-valent conjugated pneumococcal vaccine, because the conjugate vaccine was licensed in February 2010 in Japan. The children with URI included children with AOM or ARS. AOM was diagnosed on the basis of clinical symptoms such as fever, irritability, otalgia, and tympanic membrane findings of redness, bulging, and obliteration of landmarks [2]. ARS was diagnosed on the basis of clinical symptoms of persistent rhinorrhea, nasal congestion, and physical findings of post-nasal drainage and purulent rhinorrhea [1]. The nasal discharge has been present within 28 days.

Bacterial identification

Specimens were collected from the nasopharynx with a swab. The swabs were cultured on 5% sheep blood agar and chocolate agar (Nippon Becton Dickinson Company Ltd., Tokyo, Japan) within 8 h of collection. The plates were incubated for 48 h at 37°C in humidified atmosphere supplemented with 5% CO_2.

S. pneumoniae was identified by colonial morphology, alpha-hemolysis, Gram's stain characteristics, optochin disk sensitivity, and bile solubility. The density of *S. pneumoniae* colonization was determined semi-quantitatively by placing the swab in broth, vortexing the preparation, and making serial dilutions before plating the dilutions on sheep blood agar impregnated with gentamicin to suppress the growth of other bacteria. The colonies were counted after 24–48 h of incubation.

Serotypes were determined by conventional and real-time PCR methods. For the conventional method, a bacterial suspension was mixed with group-specific or type-specific antisera (The Statens Serum Institute, Copenhagen, Denmark) [26]. The quelling and agglutination was assessed under the phase-contrast microscopy. For the study of the possible existence of multiple serotypes, five pneumococcal colonies on the plate were examined.

Real-time PCR

Pneumococcal DNA genome was determined by real-time PCR using primers, designed primarily to identify pneumococcal serotypes contained in the 7-valent conjugated pneumococcal vaccine, with the TaqMan probes (Table 1) [19, 28]. Briefly, total genomic DNA was extracted from nasopharyngeal swabs, by the phenol-chloroform method, precipitated by ethanol and suspended in 50 μl TE buffer. Real-time PCR assay was carried out in a final 25 μl reaction volume including 12.5 μl TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, USA) and 2.0 μl of DNA template in 96-well plates (Applied Biosystems). DNA was amplified with the Applied Biosystems 7500 Real-time PCR System (Applied Biosystems). After denaturation at 50°C for 2 min and 95°C for 10 min, the reaction was followed by 40 cycles of amplification at 95°C for 15 s and at 60°C for 1 min. Amplification data were analyzed by instrumental software (SDS, Applied Biosystems).

Controls included RNase free water as a negative control and DNA from various pneumococcal serotypes as positive controls and they were serially diluted from 10^1 to 10^7CFU/ml to derive standard curves in every run. The standard curves depended on the cycle threshold (Ct) values of the positive controls. The CFUs of pneumococci in the samples were calculated based on the standard curve. The sample with Ct value over 40 was defined as negative for real-time PCR. The sensitivity of the assay ranged between 0.022 and 0.085 pg/ml.

Statistical analysis

Statistical analyses used Prism 5 (GraphPad Software, Inc., Ja Jolla, CA, USA). The Chi-square test with the Fisher's exact test was used for categorical variables between groups. Spearman's rank correlation was used to assess changes in pneumococcal serotypes over time. A p value of <0.05 was considered statistically significant.

Results

Two hundred and fifteen children were enrolled in the study. There were 136 healthy children. They ranged in age from 1 month to 13 years with a median age of 6 years. There were 85 males and 51 females. There were 79 children with URI, 41 with AOM and 38 with ARS. The children ranged in age from 1 month to 10 years with a

Table 1 Oligonucleotide primers and TaqMan minor groove binder (MGB) probes for each group of serotypes

Serotype/serogroup	Target gene	Forwad/reverse primer sequences (5'-3')	TaqMan MGB probe	References
All	wzg (cpsA)	GCTCCTAAGACGTCTAAGAATCAGTCT/CGACACCGAACTAATAGGACCAT	Pneumo FAM-TCTATGTTAGTGGAATTGAC	[28]
3	cap3A, 3B, 3C	CAGGAGCTGGTAAAGATCGTCAT/ACACTGACACCTCGAGAAATCGT	St3 VIC-ATAGTGGAGCTCATCTGA	Newly designed
6A and 6B	wciP (cpsS)	GCTAGAGATGGTTCCTTCAGTTGAT/CATACTCTAGTGCAAACTTTGCAAAAT	St6A FAM-CTGGCTCATGATAGTT	[28]
			St6B VIC-ACTGTCTCATGATAATT	[28]
14	cps14	TGGCGCAGGTGTCAGAATT/CTTCCTAAAACTCCTGTCCTATAAATAAAAC	St14 VIC-CCTCTACAGTAGATATATTATTG	Newly designed
19F	wchO	AATKCKGTRTTTATGGGRGTTGG/AGAGACGTTTAGGCTCATTWGC	St19F VIC-ATGCAAAAGTCAAATTTAGA	[28]
23F	wchV	CTGGGCCAAGATATTTAAAAGAGAGT/AATTYCGCATCAGAGTATGCAA	St23F FAM-TTGCTCTTCGAAAAATGT	[28]

median age of 1 year. There were 34 males and 45 females. The children with AOM and ARS were similar in age and gender distribution. The URI group was significantly younger, $p<0.001$, and had a greater proportion of females, $p<0.01$, than the healthy group. For these reasons, colonization rates between healthy children and children with URI were not compared.

Conventional microbiology detected *S. pneumoniae* in 23.5% of the healthy group and 60.8% of the URI group. In contrast, real-time PCR detected *S. pneumoniae* in 50.0% of the healthy group and 64.6% of the URI group. The increase in the detection rate of *S. pneumoniae* by PCR compared to CM was statistically significant in the healthy groups alone, 50.0% versus 23.5%, respectively, $p\leq0.001$ (Fig. 1). Colonization rates in children with AOM and ARS were similar and will be considered together in the URI group in all subsequent analyses.

The median concentration of *S. pneumoniae* in the healthy group was 6.1×10^3 CFUs/ml and in the URI group was 6.7×10^5 CFUs/ml ($p\leq0.0001$ by age-matched analysis). There was no significant difference between pneumococcal concentrations in the AOM and ARS groups. The concentration of pneumococci in the healthy group varied with the age of the children. As seen in Fig. 2, the median concentration of *S. pneumonae* significantly related inversely to the age ($r=-0.6978$; $p=0.008$).

The ability to detect various serotypes differed between CM and real-time PCR (Table 2). Among healthy children the distribution of serotypes was comparable for CM and real-time PCR; however, detection was increased for most serotypes by real-time PCR. Among children in the URI group detection and distribution of serotypes were comparable for CM and real-time PCR methods, but in healthy children serotype 6A was significantly more readily detected by real-time PCR, and serotype 23F had the same tendency. Serotypes 6A, 6B and 23F were very common in

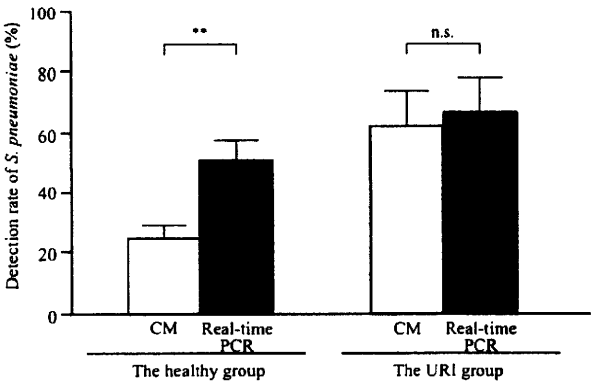

Fig. 1 Differences in nasopharyngeal colonization of pneumococci between conventional microbiology (CM) and Real-time PCR in the healthy group and the URI group. $^{**}p<0.01$ by Fisher's exact test. *n.s.* not significant

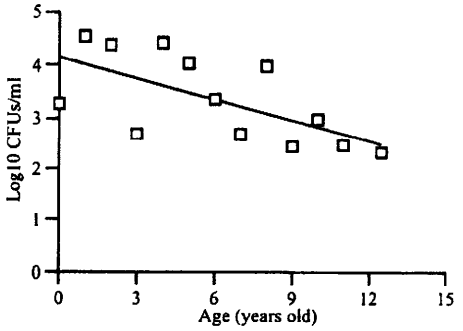

Fig. 2 The changes of nasopharyngeal pneumococcal concentration according to the age in the healthy group. The open square showed the median concentration of *S. pneumoniae*. The median concentration of *S. pneumonae* significantly related to the age (r=−0.6978; p=0.008). The linear line shows the changes of median nasopharyngeal pneumococcal concentration according to the ages

both URI and healthy groups (Table 2). Serotype 19F occurred more frequently in the URI group than the healthy group. The distribution of serotypes within the AOM and ARS groups was similar. More than 70% of these serotypes were detected in the first 5 years of life. Approximately 55% of the children were colonized with *S. pneumoniae*, and 24% were colonized with a single serotype and 10% were colonized with more than one serotype evaluated by real-time PCR. The rates of colonization with multiple serotypes in healthy and URI children were 7.4% and 13.9%, respectively. In contrast, we could not identify multiple serotypes in both healthy and URI children by CM.

Discussion

The present study demonstrates that real-time PCR has an important role to play in assessing the epidemiology of colonization. Real-time PCR was able to detect the presence of *S. pneumoniae* more often than CM. The difference was greater among specimens from healthy children than among ill children. It is possible that the lower concentration of organisms in specimens from healthy children, as demonstrated in this study, was undetectable by the lower sensitivity of CM compared with real-time PCR. Real-time PCR also proved to be superior to CM in identifying a greater number of pneumococcal serotypes in specimens from both healthy and ill children. The ability of real-time PCR to identify multiple serotypes within individuals affected the distribution of serotypes in a positive manner. The number of serotypes identified was limited by the number of serotypes tested in the present report.

Simultaneous carriage of more than one genetically distinct isolate of *S. pneumoniae* has been reported but is difficult to detect by CM [3, 17, 25]. More than 90 serotypes of *S. pneumoniae* have been identified on the basis of capsular polysaccharide differences. Some children were colonized with up to four different serotypes during the first year of life but not necessarily at the same time [15]. Over 95% of children have been colonized with up to six different serotypes by the age of two [18]. There is relatively little data on colonization with multiple serotypes but existing estimates range from 7.5% up to 29.5% [17, 20]. Seven percents of healthy children and 14% of ill children were colonized with multiple serotypes in the present study. These types of data help us understand which pneumococcal serotypes may be important for vaccine development.

Incorporation of real-time PCR technology in vaccine research will enhance the ability to better evaluate the impact of future generations of conjugated pneumococcal vaccines on colonization. Post-marketing studies on the original 7-valent conjugated pneumococcal vaccine demonstrated that the vaccine actually reduced both disease and colonization with vaccine strains; however, it additionally

Table 2 The detection of serotypes in the URI group and the healthy group by conventional microbiology (CM) and real-time polymerase chain reaction (PCR)

Serotypes	The URI group (n=79)			The healthy group (n=136)		
	CM n (%)	PCR n (%)	p Value	CM n (%)	PCR n (%)	p Value
Serotype 3	1 (1.3)	1 (1.3)	n.s.	3 (2.2)	3 (2.2)	n.s.
Serotype 6A	7 (8.9)	14 (17.7)	n.s.	4 (2.9)	13 (9.6)	0.04
Serotype 6B	3 (3.8)	6 (7.6)	n.s.	6 (4.4)	14 (10.3)	n.s.
Serotype 14	3 (3.8)	4 (5.1)	n.s.	1 (0.7)	4 (2.9)	n.s.
Serotype 19F	9 (11.4)	13 (16.5)	n.s.	2 (1.5)	2 (1.5)	n.s.
Serotype 23F	10 (12.7)	11 (13.9)	n.s.	5 (3.7)	13 (9.6)	0.08
Others (without target serotypes)	15 (19.0)	14 (17.7)	n.s.	11 (8.1)	32 (23.5)	<0.01

n.s. not significant

demonstrated that non-vaccine strains filled the void created when the vaccine strains disappeared. Recently, a new 13-valent conjugated pneumococcal vaccine has been approved for use. The use of real-time PCR technology will enhance the evaluation of the new vaccines impact on colonization. It is reasonable to assume that real-time PCR will offer the same advantages over CM for studies on pathogens other than *S. pneumoniae*.

Acknowledgements Our deepest appreciation goes to Prof. Howard Faden (Department of Pediatrics, The State University of New York at Buffalo, Women and Children's Hospital of Buffalo, NY, USA) who gives us insightful comments and suggestions. We greatly thank Miss Yuki Tatsumi (Department of Otolaryngology-Head and Neck Surgery, Wakayama Medical University, Wakayama, Japan) for her technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research (no. 21592165) from the Japan Society for the Promotion of Science (to NY).

Conflict of interest None of the authors have any conflicts of interest associated with this study.

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Vaccine 28 (2010) 7063–7069

Contents lists available at ScienceDirect

Vaccine

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Effectiveness of pneumococcal polysaccharide vaccine against pneumonia and cost analysis for the elderly who receive seasonal influenza vaccine in Japan

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ARTICLE INFO

Article history:
Received 22 April 2010
Received in revised form 29 July 2010
Accepted 2 August 2010
Available online 17 August 2010

Keywords:
Pneumococcal polysaccharide vaccine
Influenza vaccine
Pneumonia
Cost analysis

ABSTRACT

To determine the clinical efficacy and cost-saving effect of pneumococcal polysaccharide vaccine (PPV) against community-acquired pneumonia (CAP), an open-label, randomized clinical trial was conducted involving 786 Japanese subjects older than 65 years of age receiving a routine influenza vaccine during the 2-year period. Study subjects were randomly assigned to either a PPV group ($n = 394$) or to a non-PPV group ($n = 392$). The incidence, admission and the medical cost for all-cause pneumonia were compared between these two groups. PPV vaccination significantly reduced the incidence of admission for all-cause pneumonia for subjects older than 75 years of age (41.5%, $P = 0.039$) and for those who had difficulty walking (62.7%, $P = 0.005$), but not for all study subjects older than 65 years of age ($P = 0.183$), for the 2-year period. The Kaplan–Meier survival curves for subjects who had difficulty walking free from all-cause pneumonia demonstrated a significant difference ($P = 0.0146$) between the two groups. PPV vaccination significantly reduced medical costs for all study subjects during the first year period ($P = 0.027$). Our present data demonstrated that PPV was effective for all-cause pneumonia for study subjects older than 75 years of age, although the effect was not significant for all study subjects older than 65 years of age.

1. Introduction

Streptococcus pneumoniae (*S. pneumoniae*) is a leading human pathogen causing a variety of diseases in children and adults such as otitis media, and invasive pneumococcal diseases (IPD), including sepsis, meningitis and bacteremic pneumonia. The rates of IPD are highest among children under 5 years of age, and among adults who are older than 65 years of age [1,2]. *S. pneumoniae* is the most common cause of community-acquired pneumonia (CAP) in adults [3,4]. The incidence of pneumococcal CAP is also high among the elderly [4–6].

The 23-valent pneumococcal polysaccharide vaccine (PPV) has proven to be protective against IPD in immunocompetent adults. However, information is lacking about its efficacy in adults with chronic illnesses [7–10]. Although PPV vaccination reportedly reduces the mortality and ICU admissions for pneumonia [11,12], the evidence of its efficacy against all-cause pneumonia remains inconclusive [7,9,13,14].

The importance of the cost-effectiveness of vaccines is becoming an increasingly important aspect of decision-making with regard to vaccine policy. Recently, it was reported that PPV is a cost-effective measure for prevention of invasive pneumococcal diseases (IPD) (less than $50,000 per life-year gained [LYG] or per quality-adjusted life-year gained [QALY]) [15–18]. These studies evaluated the cost-effectiveness of PPV only in the base-case analysis for overall IPD. Furthermore, Ament et al. have reported that PPV vaccination is assumed to be cost-effective for pneumococcal pneumonia as well as IPD [19]. However, these findings may not persuade policy makers in Japan because of the differences in the organizations and costs for health care when comparing U.S. or European countries with Japan.

As a result of insufficient evidence supporting the clinical efficacy and cost-effectiveness of PPV against all-cause pneumonia, PPV has not been included in the national immunization program in Japan. This results in a low coverage of PPV (approximately 7%) among Japanese older than 65 years of age. To address these issues, we designed an open-labeled, randomized clinical trial to determine whether PPV vaccination reduces the incidence rate and number of admissions for all-cause pneumonia among individuals older than 65 years of age in Japan who receive routine immunization with seasonal-influenza virus vaccine (IV). Additionally, we

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doi:10.1016/j.vaccine.2010.08.010

compared the medical costs for all-cause pneumonia for the study participants who received PPV with those of participants who did not receive PPV.

2. Materials and methods

2.1. Study design

During the 2-month period of October and November 2005, the present study enrolled 786 adults older than 65 years of age and who had received routine immunization against seasonal influenza. The subjects were randomly assigned to either the PPV group or the non-PPV group by choosing a sealed envelope that contained a card indicating one of the two groups, because we were unable to mobilize the staff needed for a blinded randomization of the participants during the 2-month period of enrollment. All participants received a trivalent, split-virion influenza vaccine (IV). The 2005/2006 vaccine contained A/New Caledonia/20/99H1N1, A/New York/55/04H3N2, and B/Shanghai/361/2002 (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) and the 2006/2007 vaccine contained A/New Caledonia/20/99H1N1, A/Hiroshima/52/05H3N2, and B/Malaysia/2506/04 (The Chemo-Sero-Therapeutic Research Institute). The participants in the PPV group were immunized with 0.5 ml of 23-valent PPV (Pneumovax, Banyu, Japan) with a 1-month interval after receipt of their seasonal influenza vaccine in 2005. The participants in the non-PPV group received only seasonal influenza vaccine in 2005. The participants in both the PPV and the non-PPV groups received only seasonal influenza vaccine in 2006. Demographic data were obtained from each participant at the time of enrollment. The present study was approved by the institutional review boards for the Nagasaki Kawatana Medical Center (NKMC) and the Faculty of Medicine, Nagasaki University, Japan.

2.2. Study population

The study population consisted of individuals routinely followed by pulmonary physicians (KK, RK, TY, KK, and YH) at the Department of Respiratory Medicine of NKMC and by general physicians at twelve private clinics in Kawatana, Hasami and Higashisonogi townships, Nagasaki Prefecture. Individuals who were bed-ridden or had immunocompromised conditions such as active malignant diseases, or anatomical or functional asplenia and who had previously received PPV were excluded from the study.

After written informed consent, 786 individuals older than 65 years of age who received routine immunization by IV between October and November 2005, were assigned to either the PPV ($n = 394$) or to the non-PPV group ($n = 392$) (Fig. 1). Lost to follow-up during the 2-year period were 3 and 5 subjects from the PPV and non-PPV groups, respectively. As a result, 391 subjects in the PPV group and 387 subjects in the non-PPV group completed the entire study. These subjects were further classified into six subgroups according to their underlying diseases, age or physical conditions (Table 1). A subgroup of chronic lung diseases involved subjects with bronchial asthma, chronic obstructive pulmonary diseases (COPD), sequelae of pulmonary tuberculosis, or pneumoconiosis. A subgroup for subjects who experienced a prior episode of pneumonia was defined as subjects who experienced pneumonia during the last 5 years. There was a subgroup for subjects who had difficulty walking and had to use a stick or a wheel-chair. Of the 128 subjects in this subgroup, 105 (82.0%) were older than 75 years of age, and had comorbid illnesses, such as cerebrovascular diseases, congestive heart failure, COPD, and dementia.

Since research of the literature indicated the substantial probability of a false positive finding when multiple subgroup analyses

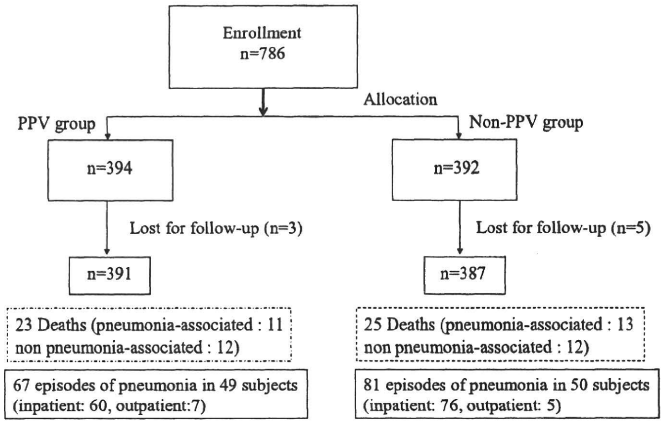

Fig. 1. Flow diagram of study subjects.

are performed [20], we carefully planned, reported and interpreted these results according to the guidelines for reporting subgroup analyses [21].

2.3. Outcome measures

All participants were examined for their underlying diseases either at a private clinic or at the NKMC once or twice per month by the study investigators. Patients were also asked to visit either their clinic or the NKMC for examination by a study investigator, if they developed a fever or respiratory symptoms during the 2-year follow-up period. The primary endpoints were all-cause pneumonia and admissions due to all-cause pneumonia. The secondary endpoint was the medical cost of all-cause pneumonia. The time to the first episode of all-cause pneumonia after the enrollment in this study was recorded. A pneumonia diagnosis was based on clinical symptoms (cough, sputum or fever), increased white blood cell counts or serum C-reactive protein, and the appearance of an infiltration on a chest radiograph or chest computed tomography at the hospital or the clinics of the study investigator [4].

The medical cost for each study participant of the PPV group was defined as the cost for both PPV and IV vaccinations plus cost for medical care of all-cause pneumonia during the study period. The medical cost for each participant of the non-PPV group was defined as the IV vaccination fee plus medical care for all-cause pneumonia during the study period. An indirect cost from loss of patient productivity, which mostly consisted of missed work was disregarded because the study population was older than 65 years of age.

2.4. Data collection

After enrollment, the study participants were followed by the study coordinators (TK, MM) by means of telephone interview on a monthly basis for recent episode of pneumonia. When the study coordinators identified an episode of pneumonia-like illness for the study participant, they confirmed with the responsible physician if the study participant had received a radiological diagnosis of pneumonia, and had received medical care for pneumonia at the private clinics or NKMC. After confirmation of a pneumonia episode for the study participant, the study coordinator collected the receipt for medical care of this pneumonia episode at the inpatient or outpatient clinic from the study participant, and calculated the medical cost for a single episode of pneumonia according to the detailed invoice and the reimbursement system for each individual.

Table 1
Comparison of demographic features of enrolled subjects by vaccine group.

	PPV group (n = 391)	Non-PPV group (n = 387)	P-value
Age; mean (SD)	78.5 (7.3)	77.7 (7.2)	0.133
Male sex (n = 274)	149 (38.1)	125 (32.3)	0.133
Categories (n)	No. of subjects (%)		
Older than 75 years (n = 511)	264 (67.5)	247 (63.8)	0.291
Chronic heart diseases and hypertension (n = 503)	261 (66.8)	242 (62.5)	0.767
Chronic lung diseases (n = 130)	73 (18.5)	57 (14.5)	0.150
Chronic renal diseases (n = 102)	59 (15.1)	43 (9.1)	0.164
Prior episode of pneumonia (n = 50)	25 (6.4)	25 (6.3)	0.464
Difficulty walking (n = 128)	63 (16.1)	65 (16.8)	0.822

2.5. Statistical analysis

An interim target sample size of 658 was chosen to ensure that there would be at least an 80% chance to detect a difference of 0.05 (0.06 vs. 0.1) episodes per person per year, with a one-sided alpha level of 0.05, in the incidence of all-cause pneumonia between the PPV group and the non-PPV group. The incidence of all-cause pneumonia and admission due to all-cause pneumonia, and the total medical costs including vaccine for all-cause pneumonia for several prespecified subgroups were compared using a chi-square test. A Kaplan–Meier estimator was used to calculate the survival curve for subjects who did not acquire all-cause pneumonia during a 2-year period. A Cox's proportional hazard model for adjusting age and gender was also used to determine the effect of PPV on the incidence rate of all-cause pneumonia in the subgroups during the first year period as well as for the entire 2-year period. The effect of PPV on medical cost was estimated using a linear regression model, whereby the medical cost regressed on PPV, age and gender [22–25]. Namely, the estimation equation was as follows; $(\text{medical cost})_i = \alpha + \beta(\text{PPV})_i + \gamma(\text{age})_i + \delta(\text{gender})_i + \varepsilon_i$; where i indicates a study participant. $(\text{PPV})_i$ indicates 0 for the non-PPV group and 1 for the PPV group. The effect of PPV on medical cost was estimated as the estimator of β. All costs were calculated in US dollars (USD) and adjusted for inflation using the 2007 consumer price index and exchange rates (1 USD = 115 yen). The costs for a single dose of PPV and two doses of IV were 61 USD and 73 USD, respectively. Data was considered to be statistically significant, when the P values were less than 0.05.

3. Results

The mean age ± SD for the PPV group was 78.5 ± 7.3 and 38% were male. For the non-PPV group, the mean age ± SD was 77.7 ± 7.2 and 32.3% were male. The numbers of subjects within each subgroup is summarized for the two groups in Table 1. No significant differences were found between the two groups with respect to the number of subjects older than 75 years of age, with chronic heart diseases and hypertension, with chronic lung diseases, with chronic renal diseases, with a prior episode of pneumonia, or with difficulty walking. No significant difference was found between the two groups with respect to the number of pneumonia-associated deaths (11 for the PPV group and 13 for the non-PPV group; P = 0.660) nor for non pneumonia-associated deaths (12 for the PPV group and 12 for the non-PPV group; P = 0.852) during the 2-year period (Fig. 1). The PPV group recorded 67 episodes of pneumonia, and 81 were identified in the non-PPV group. Treatment for pneumonia was performed at the inpatient clinic for 60 episodes and at the outpatient clinic for 7 episodes for the PPV group, while the non-PPV group sought treatment for pneumonia at the inpatient clinic for 76 episodes and at the outpatient clinic for 5 episodes.

No significant difference was found in the incidence of all-cause pneumonia between all subjects in the PPV group (0.086 per person year) and the non-PPV group (0.105 per person year) during the 2-year study period (P = 0.221, Table 2). By contrast, the incidence of all-cause pneumonia among subjects older than 75 years of age for the PPV group (0.068 per person year) was significantly lower than it was for the non-PPV group (0.134 per person year) during the first year of the study (P = 0.017), but not for the entire 2-year period (P = 0.072). This also was true for subjects with chronic lung diseases, who demonstrated a significant reduction in the incidence for the PPV group (0.096 per person year), compared with the non-PPV group during the first year (P = 0.035), but not for the entire 2-year period (P = 0.233). Furthermore, for subjects who had difficulty walking, the incidence of all-cause pneumonia for the PPV group (0.135 per person year) was significantly lower (P = 0.0006) than it was for the non-PPV group (0.331 per person year) for the 2-year period while no significant differences were found between the two groups among the subjects with chronic heart diseases and hypertension, chronic renal diseases, and with a prior episode of pneumonia. While no significant differences were found in the incidence of admissions for all-cause pneumonia between the two groups (Table 3), significant differences were found between the two groups for subjects older than 75 years of age (P = 0.039 for the 2-year period) and for the subjects who had difficulty walking (P = 0.005 for the 2-year period).

The mean cost of 12 subjects for medical care of pneumonia at outpatient clinics was 310 USD per episode. By contrast, the mean cost of 137 subjects for medical care of pneumonia at the inpatient clinics was higher (9195 USD per episode). For all subjects, a significant reduction in the medical cost was found in the PPV group, compared with the non-PPV group during the first year (P = 0.027, Table 4), but not for the 2-year period (P = 0.111). Significant reductions in the medical cost were also found for subjects older than 75 years of age (P = 0.018) and for subjects with chronic lung diseases (P = 0.017) during the first year. The reductions in medical cost averaged 1079 USD for subjects older than 75 years of age and 2672 USD for subjects with chronic lung diseases. It is noteworthy that a significant reduction in medical cost was found for subjects who had difficulty walking (P = 0.004), and this reduction averaged 2467 USD for the 2-year period.

There was a significant association recorded between the receipt of PPV and a low probability of pneumonia was found for the subjects who had difficulty walking for both the first year (P = 0.037) and the entire 2-year period (P = 0.006) (Table 5). However, none of the multivariate analyses demonstrated a significant association between any variables for all study subjects, and the probability of all-cause pneumonia in either the first year or the 2-year period. Similarly, the Kaplan–Meier survival curves for the subjects with difficulty walking who were free from all-cause pneumonia demonstrated a significant difference between the two groups (P = 0.0146) (Fig. 2), while no significant difference was found in the Kaplan–Meier survival curves for study subjects older than either 65 (P = 0.750) or 75 years of age (P = 0.199) who were free

Table 2
Incidences of all-cause pneumonia by vaccine group during a period of 2 years after enrollment among subjects with different categories (age and gender adjusted).

Categories (*n*)	Period	Incidence of all-cause pneumonia (per person years)		% Reduction in incidence of all-cause pneumonia (95% CI)	*P*-value
		PPV group	Non-PP V group		
All subjects (*n* = 778)		*n* = 391	*n* = 387		
	First year	25 (0.064)	37 (0.096)	41.70 (−6.9–69.2)	0.082
	Two years	67 (0.086)	81 (0.105)	24.75 (−18.5–52.8)	0.221
Older than 75 years old (*n* = 511)		*n* = 264	*n* = 247		
	First year	18 (0.068)	33 (0.134)	59.08 (14.05–82.40)	0.017
	Two years	51 (0.097)	69 (0.140)	36.58 (−4.09–61.97)	0.072
Chronic heart diseases and hypertension (*n* = 503)		*n* = 261	*n* = 242		
	First year	18 (0.069)	12 (0.050)	−38.26 (−230.88–43.24)	0.472
	Two years	41 (0.079)	34 (0.069)	−8.45 (−107.30–44.77)	0.810
Chronic lung diseases (*n* = 130)		*n* = 73	*n* = 57		
	First year	7 (0.096)	15 (0.263)	60.60 (6.32–82.63)	0.035
	Two years	25 (0.171)	33 (0.289)	31.59 (−28.26–62.23)	0.233
Chronic renal diseases (*n* = 102)		*n* = 59	*n* = 43		
	First year	4 (0.068)	5 (0.116)	55.33 (−108.74–97.13)	0.325
	Two years	14 (0.119)	10 (0.116)	17.64 (−281.91–95.97)	0.822
Prior episode of pneumonia (*n* = 50)		*n* = 25	*n* = 25		
	First year	7 (0.280)	11 (0.440)	26.33 (−94.17–72.93)	0.539
	Two years	21 (0.420)	21 (0.420)	−20.142 (−136.03–39.12)	0.596
Difficulty walking (*n* = 128)		*n* = 63	*n* = 65		
	First year	8 (0.127)	24 (0.369)	67.05 (17.30–88.37)	0.017
	Two years	17 (0.135)	43 (0.331)	60.91 (23.46–80.78)	0.006

from all-cause pneumonia between the two groups (data not shown).

4. Discussion

In the present study, PPV vaccination, in addition to routine IV vaccination, did not significantly reduce the incidence of all-cause pneumonia for all study subjects older than 65 years of age. In contrast, PPV vaccination significantly reduced the incidence of all-cause pneumonia compared with routine IV vaccination alone, by 59.1% for subjects older than 75 years, by 60.6% for subjects with chronic lung diseases, and by 60.9% for subjects who had difficulty walking for the first year of the study period. Furthermore, for the 2-year period, PPV vaccination in addition to routine IV vaccination resulted in significant reductions in admissions for all-cause pneumonia: by 41.5% for subjects older than 75 years of age and by 62.7% for subjects who had difficulty walking.

A higher reduction in the incidence of all-cause pneumonia brought about by PPV vaccination for subjects older than 75 years of age compared with all study subjects could be explained, in part, by a higher incidence of pneumococcal pneumonia for these subjects [4,26]. The highest reduction in the incidence of all-cause pneumonia brought about by PPV vaccination in subjects who had difficulty walking could be explained by the fact that more than 80% of these subjects were older than 75 years of age and these subjects had comorbid illnesses, such as cerebrovascular diseases and COPD, which are known risk factors for pneumococcal infection [27–29]. Previous studies also demonstrated the role of silent aspiration in the development of CAP in the elderly [30], and a high incidence of pneumonia has been found in the elderly with basal ganglia infarction [31]. In the present study, two different analy-

Table 3
Incidences of admission due to all-cause pneumonia by vaccine group during 2 years after enrollment among subjects with different categories (age and gender adjusted).

Categories (*n*)	Period	Incidence of admission due to all-cause pneumonia (per person years)		% Reduction in incidence of admission due to all-cause pneumonia (95% CI)	*P*-value
		PP V group	Non-PP V group		
All subjects (*n* = 778)		*n* = 391	*n* = 387		
	First year	25 (0.064)	35 (0.090)	38.17 (−13.12–67.29)	0.120
	Two years	60 (0.077)	76 (0.098)	27.33 (−16.32–55.79)	0.183
Older than 75 years old (*n* = 511)		*n* = 264	*n* = 247		
	First year	18 (0.068)	32 (0.130)	57.25 (11.49–81.12)	0.021
	Two years	46 (0.087)	67 (0.136)	41.52 (2.66–65.48)	0.039
Chronic heart diseases and hypertension (*n* = 503)		*n* = 261	*n* = 242		
	First year	18 (0.069)	11 (0.045)	−49.98 (−256.91–38.15)	0.366
	Two years	36 (0.069)	32 (0.066)	0.67 (−95.08–51.03)	0.985
Chronic lung diseases (*n* = 130)		*n* = 73	*n* = 57		
	First year	7 (0.096)	14 (0.246)	58.52 (−0.59–82.24)	0.052
	Two years	22 (0.151)	31 (0.272)	33.53 (−27.14–63.55)	0.180
Chronic renal diseases (*n* = 102)		*n* = 59	*n* = 43		
	First year	4 (0.068)	5 (0.116)	55.33 (−108.74–97.13)	0.325
	Two years	14 (0.119)	10 (0.116)	17.64 (−281.91–95.79)	0.822
Prior episode of pneumonia (*n* = 50)		*n* = 25	*n* = 25		
	First year	7 (0.280)	11 (0.440)	26.32 (−94.17–72.93)	0.539
	Two years	15 (0.300)	21 (0.420)	17.28 (−89.19–64.59)	0.570
Difficulty walking (*n* = 128)		*n* = 63	*n* = 65		
	First year	8 (0.127)	23 (0.354)	65.09 (14.15–87.17)	0.021
	Two years	16 (0.127)	42 (0.323)	62.74 (25.71–82.14)	0.005

Table 4
The total medical costs including vaccine for all-cause pneumonia by vaccine group during 2 years after enrollment among subjects with different categories (age and gender adjusted).

Categories (*n*)	Period	Total medical cost (mean, US$)		Reduction of medical cost due to all-cause pneumonia (US$) (95% CI)	*P*-value
		PPV group	Non-PPV group		
All subjects (*n* = 778)		*n* = 391	*n* = 387		
	First year	499	1225	661 (17–1304)	0.027
	Two years	1273	1978	320 (−113–753)	0.111
Older than 75 years old (*n* = 511)		*n* = 264	*n* = 247		
	First year	597	1741	1079 (136–2022)	0.018
	Two years	1667	2824	546 (−90–1182)	0.075
Chronic heart diseases and hypertension (*n* = 503)		*n* = 261	*n* = 242		
	First year	542	383	−224 (−818–370)	0.600
	Two years	1070	1094	−20 (−482–441)	0.959
Chronic lung diseases (*n* = 130)		*n* = 73	*n* = 57		
	First year	784	3522	2672 (461–4884)	0.017
	Two years	2690	5024	1135 (−316–2586)	0.117
Chronic renal diseases (*n* = 102)		*n* = 59	*n* = 43		
	First year	584	1292	640 (−806–2087)	0.341
	Two years	2100	1957	−104 (−1319–1111)	0.908
Prior episode of pneumonia (*n* = 50)		*n* = 25	*n* = 25		
	First year	1448	5996	4483 (−539–9506)	0.083
	Two years	2936	7745	2372 (−166–4910)	0.070
Difficulty walking (*n* = 128)		*n* = 63	*n* = 65		
	First year	1290	5387	4032 (849–7215)	0.013
	Two years	2193	7193	2467 (798–4137)	0.004

Table 5
Association of the receipt of PPV and the probability of all-cause pneumonia during the first one and two years (age and gender adjusted).

Period	Categories	All-cause pneumonia	
		Hazard ratio (95% CI)	*P*-value
First year	All subjects	0.73 (0.44–1.23)	0.251
	Age older than 75	0.55 (0.30–1.01)	0.053
	Chronic heart diseases and hypertension	1.58 (0.73–3.43)	0.247
	Chronic lung diseases	0.51 (0.21–1.19)	0.12
	Chronic renal diseases	0.56 (0.137–2.28)	0.417
	Prior episode of pneumonia	1.64 (0.63–4.21)	0.303
	Difficulty walking	0.41 (0.17–0.95)	0.037
Two years	All subjects	0.82 (0.55–1.23)	0.348
	Age older than 75	0.67 (0.42–1.06)	0.085
	Chronic heart diseases and hypertension	1.15 (0.64–2.05)	0.644
	Chronic lung diseases	0.79 (0.41–1.54)	0.501
	Chronic renal diseases	0.93 (0.29–2.88)	0.895
	Prior episode of pneumonia	1.51 (0.68–3.30)	0.303
	Difficulty walking	0.38 (0.19–0.76)	0.006

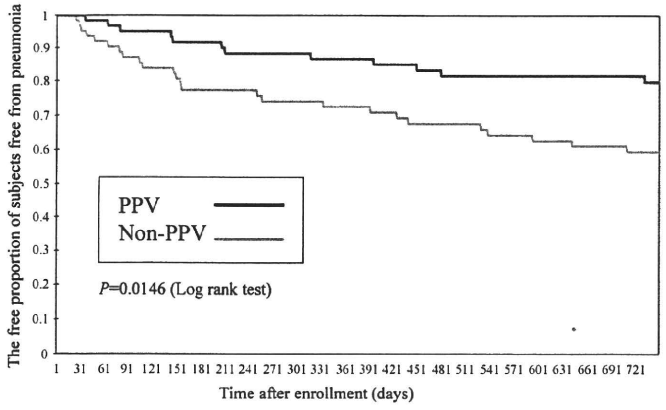

Fig. 2. Kaplan–Meier survival curve for study subjects who had difficulty walking showing the proportion of study subjects free from all-cause pneumonia between the PPV group (black line) and the non-PPV group (gray line) during the follow-up period. A significant difference in the proportion of study subjects who were free-from all-cause pneumonia was found between the two groups ($P = 0.0146$).

ses using the estimated results of Cox's proportional hazard model and the Kaplan–Meier survival curve similarly supported the significant protective effect of PPV vaccination for all-cause pneumonia in subjects who had difficulty walking.

On the other hand, the reason the effects of PPV vaccination were not found in other subgroups, such as chronic heart diseases and hypertension, chronic renal diseases and prior episode of pneumonia, may be explained, in part, by a low incidence in all-cause pneumonia for the subjects affected by such conditions in our patient sample.

The effect of PPV vaccination in the prevention of all-cause pneumonia during the first year period, but not for the 2-year period, for subjects older than 75 years of age and for those with chronic lung disease, may be explained by the kinetics of serotype-specific IgG determined by the third generation ELISA in the sera of subjects who received PPV vaccination [32]. The serum levels of serotype-specific IgG peaked at 2 months after PPV vaccination and they declined at 6 months after vaccination for most serotypes. Our previous study also showed that PPV had an effect on infectious acute exacerbation was found during the first year after vaccination, and was associated with a serotype-specific immune response

in the sera of patients with chronic lung diseases who received PPV vaccination [33].

Significant reductions in admissions for all-cause pneumonia were found following PPV vaccination, in addition to routine IV vaccination in subjects older than 75 years of age and in subjects who had difficulty walking during the 2-year period. Our data on the effects of PPV vaccination for hospital admissions for all-cause pneumonia are in agreement with the previous reports from a large cohort study of subjects older than 65 years of age in Sweden [34], and in a prospective study of the elderly in Japan [35]. A double- blind, randomized, controlled study has recently reported the efficacy of PPV in preventing pneumococcal pneumonia and reducing the mortality for pneumococcal pneumonia in nursing home residents in Japan [36]. Approximately 90% of the participants in this study were found to be older than 75 years of age, and this study also demonstrated a significant reduction in the incidence of all-cause pneumonia by PPV among the study subjects.

Although a previous study reported the cost-effectiveness of PPV for preventing pneumococcal pneumonia in five European countries, their results were based on the estimated incidence of pneumococcal pneumonia and on the estimated medical costs for vaccination and for treatment after admissions in each country [19]. In this regard, it is noteworthy that a significant reduction in direct medical costs (approximately 660 USD) for pneumonia was found in study subjects older than 65 years of age who received routine influenza vaccination during the first year following the PPV vaccinations in the present study. To our knowledge, this is the first report of the cost-saving effect of PPV for all-cause pneumonia among subjects in a randomized-controlled study. Significant reductions in higher medical costs for all-cause pneumonia among study subjects older than 75 years of age, those who had difficulty walking, and study subjects with chronic pulmonary diseases, were associated with a reduced incidence of hospital admissions for all-cause pneumonia, since the cost for one episode of hospital admission for all-cause pneumonia in the present study was more than 9000 USD. A high-risk population for all-cause pneumonia, such as subjects who had difficulty walking and subjects with chronic pulmonary diseases, are likely to have a higher reduction in medical costs for pneumonia.

Since no attempt was made to blind the clinical assessors to the vaccine allocation in the present study, the possibility of bias in the clinical assessments obtained by the investigators cannot be dismissed, and this would be one limitation. Another limitation is that the preventive effects of PPV for pneumococcal pneumonia were not evaluated in the present study, because the microbiological examinations were not routinely performed for all of pneumonia cases particularly in the private clinics.

In conclusion, during the 2-year period of the present study in Japan, PPV vaccination was effective in reducing the incidence of admission for all-cause pneumonia for subjects who were older than 75 years of age, although no significant effect was found for all-cause pneumonia in all study subjects older than 65 years of age. A cost-saving effect of PPV was also found for all-cause pneumonia in all study subjects during the first year of the present study. Our present data suggest a recommendation of PPV in combination with influenza vaccine for the people who are older than 75 years of age in Japan.

Acknowledgments

We are grateful to Dr. F. Konings, International Vaccine Institute, Seoul, Korea, for his critical review of this manuscript and M. Maekawa and K. Terumoto for their study coordination and collecting the information concerning the information medical costs of pneumonia. We are also grateful to the members of Medical Association of Higashisonogi district, Nagasaki Prefecture; Dr. T. Beppu, Dr. H. Kanazawa, Dr. H. Kohno, Dr. J. Matsuo, Dr. M. Motokawa, Dr. N. Nonaka, Dr. N. Okabe, Dr. F. Tamagawa, Dr. S. Tabuchi, Dr. N. Yamamoto and Dr. T. Yamazumi. This study was supported by a research grant "Studies on preventable vaccines for varicella, mumps, and pneumococcal pneumonia and other diseases" from the Ministry of Health, Labor and Welfare, Japan.

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PLoS one

Characterization of Quasispecies of Pandemic 2009 Influenza A Virus (A/H1N1/2009) by *De Novo* Sequencing Using a Next-Generation DNA Sequencer

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Abstract

Pandemic 2009 influenza A virus (A/H1N1/2009) has emerged globally. In this study, we performed a comprehensive detection of potential pathogens by *de novo* sequencing using a next-generation DNA sequencer on total RNAs extracted from an autopsy lung of a patient who died of viral pneumonia with A/H1N1/2009. Among a total of 9.4×10^6 40-mer short reads, more than 98% appeared to be human, while 0.85% were identified as A/H1N1/2009 (A/Nagano/RC1-L/2009(H1N1)). Suspected bacterial reads such as *Streptococcus pneumoniae* and other oral bacteria flora were very low at 0.005%, and a significant bacterial infection was not histologically observed. *De novo* assembly and read mapping analysis of A/Nagano/RC1-L/2009(H1N1) showed more than ×200 coverage on average, and revealed nucleotide heterogeneity on hemagglutinin as quasispecies, specifically at two amino acids ($Gly_{172}Glu$ and $Gly_{239}Asn$ of HA) located on the Sa and Ca2 antigenic sites, respectively. Gly239 and Asn239 on antigenic site Ca2 appeared to be minor amino acids compared with the highly distributed Asp239 in H1N1 HAs. This study demonstrated that *de novo* sequencing can comprehensively detect pathogens, and such in-depth investigation facilitates the identification of influenza A viral heterogeneity. To better characterize the A/H1N1/2009 virus, unbiased comprehensive techniques will be indispensable for the primary investigations of emerging infectious diseases.

Citation: Kuroda M, Katano H, Nakajima N, Tobiume M, Ainai A, et al. (2010) Characterization of Quasispecies of Pandemic 2009 Influenza A Virus (A/H1N1/2009) by *De Novo* Sequencing Using a Next-Generation DNA Sequencer. PLoS ONE 5(4): e10256. doi:10.1371/journal.pone.0010256

Editor: Steven Jacobson, National Institutes of Health, United States of America

Received February 19, 2010; **Accepted** March 27, 2010; **Published** April 23, 2010

Funding: This work was supported by grants for Research on Emerging and Re-emerging Infectious Diseases (H20 and 21 Shinko-Ippan-6), from the Ministry of Health, Labour, and Welfare, Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

In April 2009, an H1N1 triple-reassortant swine influenza virus (A/H1N1/2009) was detected in humans with febrile respiratory illness in North America [1], and the virus has rapidly spread worldwide by human-to-human transmission. According to the disease outbreak news from the World Health Organization, at least 14,711 people died from A/H1N1/2009 between April 2009 and January 2010 (http://www.who.int/csr/don/en/). Fatal cases from A/H1N1/2009 viral infection were summarized in a report by Gill *et al.* [2].

The genome of influenza A virus (family Orthomyxoviridae) consists of 8 single-stranded negative sense RNA molecules spanning approximately 13.5 kb. The segments range in length from 890 to 2341 nucleotides (nt) and encode a total of 11 proteins [3]. Genetic diversity in influenza virus results from a high mutation rate associated with replication using a low-fidelity RNA polymerase and the reshuffling (reassortment) of segments among coinfecting strains. Multiple-reassortant influenza viruses from avian, human, and swine origins emerged as major pandemic influenza viruses (i.e., 1918 H1N1, 1957 H2N2, and 1968 H3N2) causing significant mortality in humans in the 20th century [4]. Such an "antigenic shift" by multiple reassortant drives the emergence of pandemic influenza viruses, with their severity and clinical outcome always unpredictable [5].

Influenza A virus can evade antibodies specific to its attachment protein, hemagglutinin (HA), by the accumulation of amino acid substitutions in HA epitopes [6]. This "antigenic drift" in HA epitopes [7] affects recognition by antibodies that neutralize viral infectivity by blocking the interaction of HA with sialic acid residues on host-cell membranes. The H1 subtype HA has four antigenic sites recognized by monoclonal antibodies with high neutralizing activity, designated Sa, Sb, Ca, and Cb [8]. In addition, 8 continuous B cell/antibody epitopes for human H1N1 HA proteins have been experimentally defined by the Immune Epitope Database and Analysis Resource (IEDB: http://www.immuneepitope.org/) [9]. Immune epitope analysis of HA epitopes in A/H1N1/2009 is also summarized in the Influenza Research Database (http://www.fludb.org/brc/homeExtraPage.do?decorator=influenza&extraPage=separate) [10].

To better predict a future pandemic of influenza A virus, the characterization of possible antigenic drift will be indispensable. Igarashi *et al.* and Shen *et al.* reported that a structural comparison of HAs could predict probable future antigenic changes during the evolution of A/H1N1/2009 in the human population [11,12].

In addition to this prediction, extensive investigations on viral quasispecies will be required to reveal the actual appearance of those antigenic changes. Nakamura *et al.* demonstrated the direct detection of potential pathogens, including influenza virus, using *de novo* pyrosequencing [13], but the detection appeared to have insufficient redundant sequencing reads to reveal the genetic variation of the viruses. Ramakrishnan *et al.* demonstrated the discrimination of quasispecies in mixed HA subtype infections of influenza A virus using the same pyrosequencing approach [14]. However, it was shown that the influenza viral RNA sample should be enriched through sequence-specific oligonucleotide capturing prior to pyrosequencing, indicating that such enrichment might represent a biased result.

Here, we performed *de novo* sequencing using total RNAs extracted from an autopsy lung of a patient infected with A/H1N1/2009, and detected potential pathogens such as bacteria in addition to A/H1N1/2009. Extensive DNA sequencing using the Illumina Genome Analyzer II (GA II) revealed that quasispecies for the HA sequence were generated in single patient. Such heterogeneity demonstrated the antigenic drift of HA, implying the existence of a mechanism to escape pre-existing human immunity to the virus.

Results

Summary of sequencing reads and detection of potential pathogens

To determine the influenza A virus sequence and other potential pathogens, we performed *de novo* sequencing of double-stranded cDNA from total RNA extracted from the autopsy lung of a patient infected with the A/H1N1/2009 virus (A/Nagano/RC1/2009(H1N1)) in August 2009 in Japan. The patient was found to be positive for A/H1N1/2009 by real-time reverse transcriptase-polymerase chain reaction (RT-PCR); histopathological findings were also reported [15]. GA II produced 9.4×10^6 40-mer reads from the cDNA library (Fig. 1B). To exclude the human-derived read sequences, we performed an analysis pipeline as follows (Fig. 1A). Initially, all 9,475,890 reads were aligned to a reference sequence of human genomic DNA, followed by quality trimming to remove low-quality reads and excluding reads with similarities to ambiguous human sequences, resulting in 9,309,538 reads (98.24%) with a possible human source (Fig. 1B). The remaining 166,352 reads (1.75%) were further analyzed using a BLAST search against non-redundant databases, revealing 80,827 (0.85%), 469 (0.005%), and 85,056 (0.90%) reads as influenza A virus, bacteria, and no hits, respectively (Fig. 1B).

Regarding the bacterial hits, species classification was determined based on the results of a BLASTN search against the nt database (Fig. 1C). The most abundant bacterium was *Propionibacterium acnes*, but our other sequencing trials for clinical specimens suggest that this species is always detected (data not shown). Therefore, the presence of *P. acnes* could be the result of contamination at some point from the autopsy to the preparation of the cDNA library. In addition to *P. acnes*, *Escherichia coli* and *Acinetobacter baumannii* were frequently detected as possible contaminants. Suspected bacterial pathogens were identified as *Streptococcus pneumoniae* and *Porphyromonas gingivalis*. Specific PCR using 16S-rDNA and the *lytA* gene was performed for further verification of the presence of *S. pneumoniae* (data not shown). Although *S. pneumoniae* was not sufficiently abundant to conclude a coinfection with A/H1N1/2009, the severity of the A/H1N1/2009 infection could be correlated with *S. pneumoniae*, as reported by Palacios *et al* [16]. The other detected bacteria, such as *Streptococcus* sp., generally constitute the normal human oral flora.

de novo assembly of the A/H1N1/2009 virus

Whole 40-mer short reads, including human-derived reads, were assembled using Euler-SR or the Velvet *de novo* assembler. The resultant contigs generated using Euler-SR had longer extended sequences than those generated using Velvet (data not shown); thus, all further analyses were performed using the contigs generated using Euler-SR (Texts S1 and S2). All contigs showed high similarity to the sequences of A/H1N1/2009 (Table 1). Among the 8 segments, almost the whole lengths of segments 2 (2321 nt), 3 (2231 nt), 4 (1765 nt), 5 (1562 nt), 7 (1026 nt), and 8 (892 nt) were correctly assembled as single contigs of 2204, 2198, 1761, 1514, 1019, and 834 nt, respectively (Table 1 and Fig. 2). Segments 1 and 6 were divided into several contigs, but were correctly aligned to the known sequences (Table 1 and Fig. 2).

Read mapping analysis of the A/H1N1/2009 virus

To obtain whole sequences and identify single nucleotide polymorphisms (SNPs) for the 8 segments, the 40-mer short reads were aligned to the sequence of A/Tronto/T0106/2009(H1N1), which was found to be the most similar to the A/H1N1/2009 virus using a BLASTN search. Figure 2 shows dot plot images of the coverage at every nucleotide of the segments. Read coverage was observed at ~×200 on average for all segments, indicating a sufficient redundancy to identify the viral sequences and SNPs. The obtained viral sequences, designated as A/Nagano/RC1-L/2009(H1N1), were consistent with those from A/Nagano/RC1/2009(H1N1) passaged in the Madin-Darby canine kidney (MDCK) cell line, except for 3 possible heterogeneous nucleotides in HA.

The coverage plot curves were not flat throughout the segments. Intriguingly, both ends of segment 1 (encoding PB2), the 3′–end of segment 3 (encoding PA), and approximately 700 nt of segment 8 (encoding NS) showed significant abundant coverage greater than ×1000.

Genetic population analysis of the A/H1N1/2009 virus

To identify heterogeneous populations, alignment results were screened using MapView software (Fig. 3B). Three potential heterogeneous genetic populations were found in segment 4 (encoding HA) at the 515, 715, and 716 nt positions (Fig. 3A), but not in other segments. The read alignments shown in Fig. 3B indicate that either the GGT or AAT sequence appeared at the 715–717 nt position, but not the GAT or AGT sequence. In addition, the read coverage implied that the major (GG; HA-Major) or minor (AA; HA-Minor) nucleotides were detected at the frequencies of 75% and 25%, respectively. To validate these variations, HA-Major- or HA-Minor-specific quantitative RT-PCR (qRT-PCR) was performed for the preparation of specific PCR products between the 434 and 738 nt in the HA coding sequence (Fig. 3C). qRT-PCR demonstrated that the expression of HA was ~40,000-fold greater than that of human β-actin, and the expression ratio of HA-Major/HA-Minor was 4.05, suggesting that it corresponds to the read mapping shown in Fig. 3A. Furthermore, HA-Major and HA-Minor sequences were verified by Sanger DNA sequencing of the specific PCR products (Fig. 3D). Taken together, these results suggest the following amino acid substitutions of HA: one nucleotide alteration causes $Gly_{172}Glu$ and the other two alterations cause $Gly_{239}Asn$ (Fig. 3D).

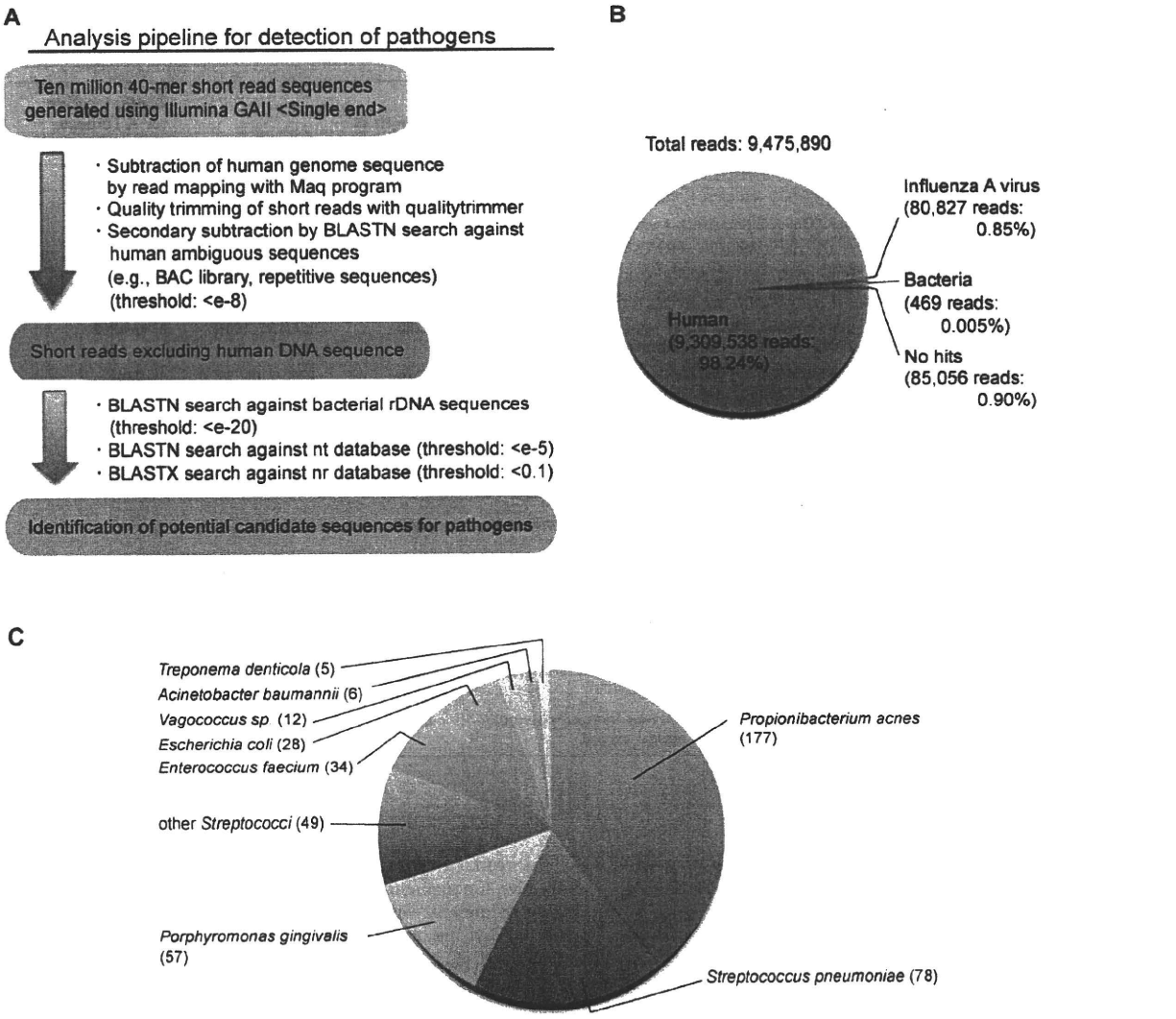

Figure 1. Detection of potential pathogens by comprehensive *de novo* sequencing. (**A**) Schematic representation of the analysis pipeline for the detection of pathogens from comprehensive sequencing of human clinical specimens. After excluding human-derived DNA sequences using Maq software and a BLAST homology search against human genomic DNA and human ambiguous sequences extracted from the nt database, the remaining short reads were subjected to a BLAST search to detect potential pathogens. (**B**) Pie chart of the homology search results for the 40-mer short reads. Read numbers and their percentage to the total reads are shown in parenthesis. (**C**) Pie chart of identified bacterial hits. Number of hit reads is shown in parenthesis. Bacteria with less than 5 hit reads were excluded.
doi:10.1371/journal.pone.0010256.g001

Epitope analysis of heterogeneous HA

To elucidate whether the $Gly_{172}Glu$ and $Gly_{239}Asn$ amino acid substitutions in the HA sequence could be associated with antigenic drift, they were compared to known potential epitopes [8,9]. Representative HA amino acid sequences of the H1N1 influenza A virus were aligned with the heterogeneous HA-Major and HA-Minor sequences. The $Gly_{172}Glu$ substitution (corresponding to Gly158 in the mature HA lacking a signal peptide) was located on the Sa antigenic site (Fig. 4A).

The HA $Gly_{172}Glu$ substitution is likely to be rare thus far because a BLASTP search against the non-redundant nr database revealed only two identical hits, A/Bayern/62/2009(H1N1) in Germany and A/Catalonia/S1207/2009(H1N1) in Spain (data not shown). One intriguing hit was to A/Pennsylvania/14/2009(H1N1) isolated in the US, whose HA sequence has an Xaa amino acid at position 172 due to the presence of the heterogeneous nucleotide R (A or G) (Fig. 5A). This deposited sequence completely coincides with our observation, suggesting that two variants of HAs are likely to coexist in the human lung, further implying that such heterogeneous populations might frequently be generated during infection.

Furthermore, HA $Gly_{239}Asn$ was located on the Ca2 antigenic site that contributes to binding with the host's sialic acid receptor [17]. Asp239 (corresponding to Asp225 in the mature HA lacking a signal peptide) was frequently distributed in H1N1 HAs (Fig. 4B), but Gly239 and Asn239 were found to be minor amino acids among HAs; a BLASTN search found 18 and 5 hit entries on the nt database, respectively. As was observed for Gly172, Xaa239

Table 1. BLASTN search results of *de novo* assembly contigs against database of Influenza virus sequences.

Euler-SR_contigs	Contig length (bp)	Virus segment	Top hit of accession number using BLASTN search against databse of Influenza virus sequences	Length of subject (bp)	Identities	Contig location for A/Toronto/ T0106/2009(H1N1)
>826 183 2835	183	1	gb|GQ328865|INFLUENZA A virus (A/Finland/553/ 2009(H1N1)) segment 1 polymerase PB2 (PB2)	2345	167/168 (99%)	5–168
>324 1558 136	1558	1	gb|GQ365425|INFLUENZA A virus (A/Fukushima/1/ 2009(H1N1)) segment 1 polymerase PB2 (PB2)	2280	1556/1558 (99%)	201–1758
>1194 239 112	239	1	gb|GQ894926|INFLUENZA A virus (A/Delaware/03/ 2009(H1N1)) segment 1 polymerase PB2 (PB2)	2280	214/214 (100%)	1894–2107
>887 174 3294	174	1	gb|GQ894833|INFLUENZA A virus (A/Rhode Island/08/ 2009(H1N1)) segment 1 polymerase PB2 (PB2)	2280	156/156 (100%)	2145–2300
>890 2204 4651	2204	2	gb|GQ894924|INFLUENZA A virus (A/New Mexico/04/ 2009(H1N1)) segment 2 polymerase PB1 (PB1)	2274	2200/2204 (99%)	41–2244
>696 2198 3968	2198	3	gb|GQ866924|INFLUENZA A virus (A/Thailand/CU-H106/ 2009(H1N1)) segment 3 polymerase PA (PA)	2238	2152/2155 (99%)	54–2208
>868 1761 3831	1761	4	gb|CY045503|INFLUENZA A virus (A/Bayern/66/ 2009(H1N1)) segment 4 sequence	1754	1750/1754 (99%)	1–1741
>897 1514 1710	1514	5	gb|GQ502907|INFLUENZA A virus (A/Toronto/R8557/ 2009(H1N1)) segment 5 nucleocapsid protein	1558	1511/1514 (99%)	36–1549
>224 101 9	101	6	gb|GQ502908|INFLUENZA A virus (A/Toronto/R8557/ 2009(H1N1)) segment 6 neuraminidase (NA)	1458	101/101 (100%)	3–103
>1206 1302 2468	1302	6	gb|GQ906584|INFLUENZA A virus (A/Stockholm/49/ 2009(H1N1)) segment 6 neuraminidase (NA)	1447	1299/1300 (99%)	124–1423
>750 1019 1128	1019	7	gb|CY045957|INFLUENZA A virus (A/Toronto/T0106/ 2009(H1N1)) segment 7 sequence	1026	1017/1017 (100%)	9–1025
>809 834 4399	834	8	gb|GQ485660|INFLUENZA A virus (A/Ekaterinburg/01/ 2009(H1N1)) segment 8 nuclear export	877	828/830 (99%)	52–881

Schematic representation of contigs is shown in Fig. 2.
doi:10.1371/journal.pone.0010256.t001

was found in the nt database (Fig. 5B), suggesting that HA heterogeneity of both minor amino acids may affect its binding affinity to the sialic acid receptor.

Discussion

In this study, we demonstrated the detection of potential pathogens using a next-generation DNA sequencer. We speculated that, in addition to influenza A virus, additional potential pathogens such as *S. pneumoniae* could contribute to the severity and fatality of infection with the A/H1N1/2009 virus [2,16]. In this case, the amount of bacteria detected was small (Fig. 1B and 1C), and they were considered to be the result of contamination during the course of the experiment, from autopsy to short read sequencing. The clinical outcome of the patient and histopathological examination suggest that this was a case of influenza viral pneumonia rather than bacterial infection [15], although *S. pneumoniae* coinfection has been reported to play a crucial role in the severity of influenza virus infection in some cases [16,18].

In the present autopsy lung sample, very significant viral copies (~40,000-fold greater than β-actin) were determined using qRT-PCR, but this was not always observed in autopsy samples from other patients (less than β-actin) (data not shown). Such abundant read sequencing enabled us to obtain almost full-coverage contig sequences for the viral segments using *de novo* assembly, suggesting the importance of this result in terms of being able to evaluate uncharacterized emerging infectious agents using an unbiased sequencing technique at the outbreak of a pandemic. Indeed, this study demonstrated that whole contigs can be identified as A/H1N1/2009, but not seasonal H1N1 or other subtypes (Table 1).

The read coverage profile generated by mapping was very indicative for segment 1 encoding PB2 (Fig. 2). Both ends were highly redundant with up to ×3000 coverage. The coverage is reflected by the amounts of vRNA, cRNA, and mRNA of influenza A virus, implying that the coverage bias may detect a more stable region as it is dependent on the expression level or manner of replication.

Contrary to the viral sequences obtained for A/Nagano/RC1/2009(H1N1) isolated from passaging in MDCK cells, *de novo* sequencing revealed the presence of A/Nagano/RC1-L/2009(H1N1) HA quasispecies in the autopsy sample (Fig. 3). Despite the fact that immunity to A/H1N1/2009 viruses is supposed to be limited among the general human population [19], we detected the amino acid substitution $Gly_{172}Glu$ in the HA Sa antigenic site in A/Nagano/RC1-L/2009(H1N1), and this appears to be a very rare event among A/H1N1/2009 viruses to date.

We also observed another substitution of $Gly_{239}Asn$ in the HA Ca2 antigenic site of A/Nagano/RC1-L/2009(H1N1). This antigenic site plays a crucial role in conferring specificity to galactose of the human α2-6 sialylated glycan receptor [20]. Interestingly, Asp239 (corresponding to Asp225 in the mature HA that lacks a signal peptide) is highly prevalent in known H1N1 HAs, indicating that both Gly239 and Asn239 appear to be very minor amino acids among all HA sequences.

Thus far, amino acid substitutions in the HAs of A/H1N1/2009 have been identified compared with seasonal H1N1 HAs. Homology-based structural investigations [17,21] suggest that A/H1N1/2009 HA has the necessary residues to provide optimal contacts for high affinity binding to α2-6 sialylated glycans present

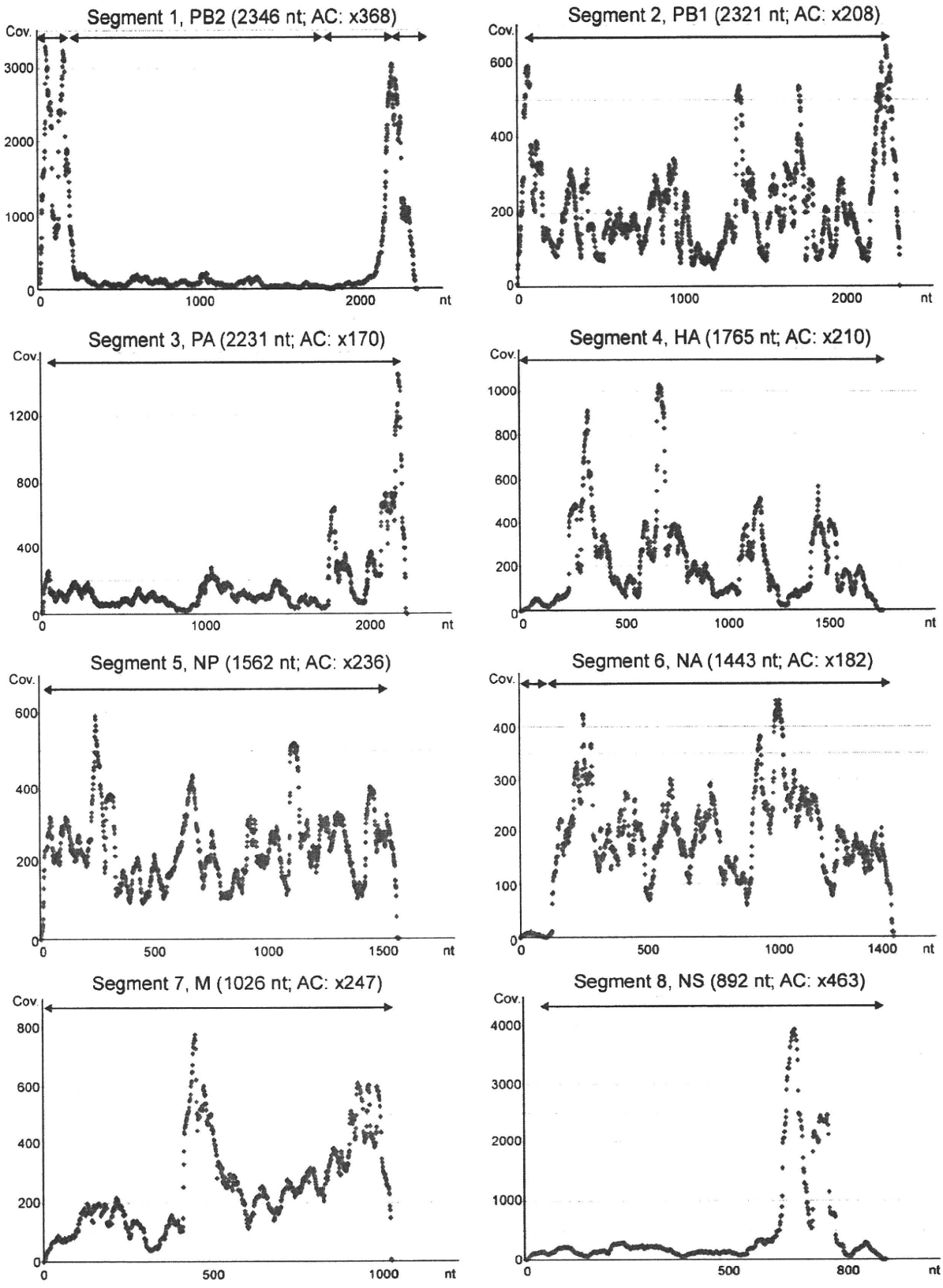
Segment 1, PB2 (2346 nt; AC: x368)
Cov.
3000
2000
1000
0
0
1000
2000
nt
Segment 2, PB1 (2321 nt; AC: x208)
Cov.
600
400
200
0
0
1000
2000
nt
Segment 3, PA (2231 nt; AC: x170)
Cov.
1200
800
400
0
0
1000
2000
nt
Segment 4, HA (1765 nt; AC: x210)
Cov.
1000
800
600
400
200
0
0
500
1000
1500
nt
Segment 5, NP (1562 nt; AC: x236)
Cov.
600
400
200
0
0
500
1000
1500
nt
Segment 6, NA (1443 nt; AC: x182)
Cov.
400
300
200
100
0
0
500
1000
1400
nt
Segment 7, M (1026 nt; AC: x247)
Cov.
800
600
400
200
0
0
500
1000
nt
Segment 8, NS (892 nt; AC: x463)
Cov.
4000
3000
2000
1000
0
0
500
800
nt

Figure 2. Dot plot of short read coverage (Cov.) at every nucleotide for the 8 segments of A/Nagano/RC1-L/2009(H1N1). To obtain the consensus sequences for the respective 8 segments, 40-mer short reads were aligned to the complete segment sequences of A/Tronto/T0106/2009(H1N1) (gb|CY045951.1 – .8). Short read sequencing was performed using total RNA including human RNA, and also vRNA, cRNA, and mRNA from influenza A virus; thus, coverage bias was detected throughout the segments, but the average coverage (AC) is likely to be similar at approximately ×200 or more. The horizontal red arrows show the location of the contigs obtained by *de novo* assembly, as shown in Table 1.
doi:10.1371/journal.pone.0010256.g002

in the human upper airway [22], while it apparently shows minimal contact with α2-3 sialylated glycans present in the human lower respiratory tract [23]. Indeed, the recombinant A/H1N1/2009 HA has been characterized to exhibit lower binding to the alveolar tissue of the lower respiratory tract [17]. However, we previously detected abundant viral nucleoprotein of A/Nagano/

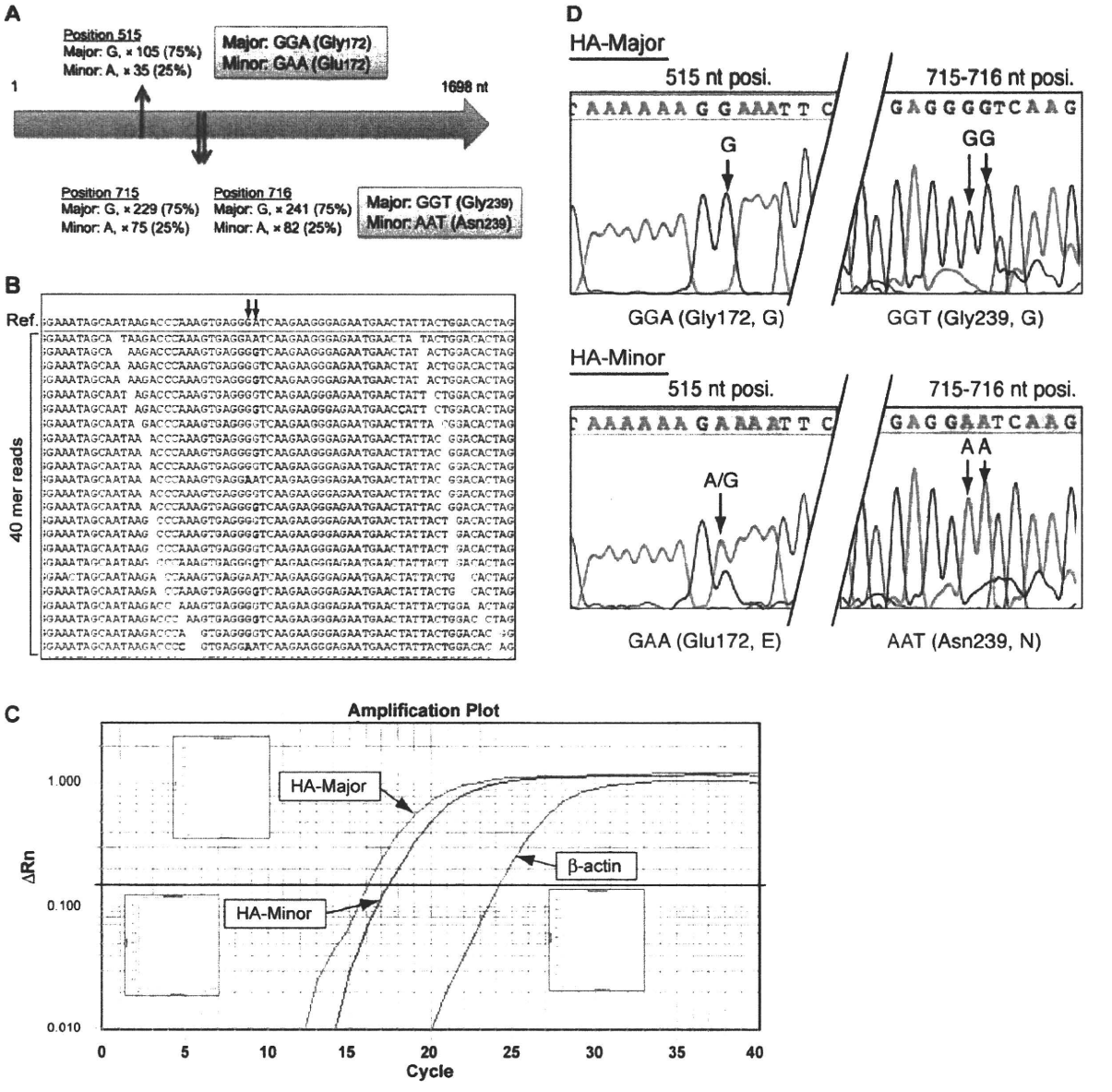

Figure 3. Genetic variations of the HA nucleotide sequence. (**A**) Schematic representation of 3 nucleotide variations (positions 515, 715, and 716 nt) in the HA coding nucleotide sequence. Three variations were classified as Major (75% appearance) or Minor (25% appearance) by read coverage (×), and the coding amino acids are also shown. (**B**) Arrows indicate positions 715 and 716 nt of the HA sequence, and the alignment image of the 40-mer reads. Nucleotides shown in red are the mismatches to the reference sequence of A/Tronto/T0106/2009(H1N1). Every read suggested that either the GGT or AAT sequence was present, but not the GAT or AGT sequence. (**C**) An amplification plot for HA-specific qRT-PCR. (**D**) Validation of genetic variation by Sanger capillary sequencing. HA-Major or HA-Minor PCR products were obtained by qRT-PCR using HA-Major- or HA-Minor-specific PCR primers. HA-Major PCR product shows G nucleotides at positions 515, 715, and 716 nt, while HA-Minor shows A nucleotides.
doi:10.1371/journal.pone.0010256.g003

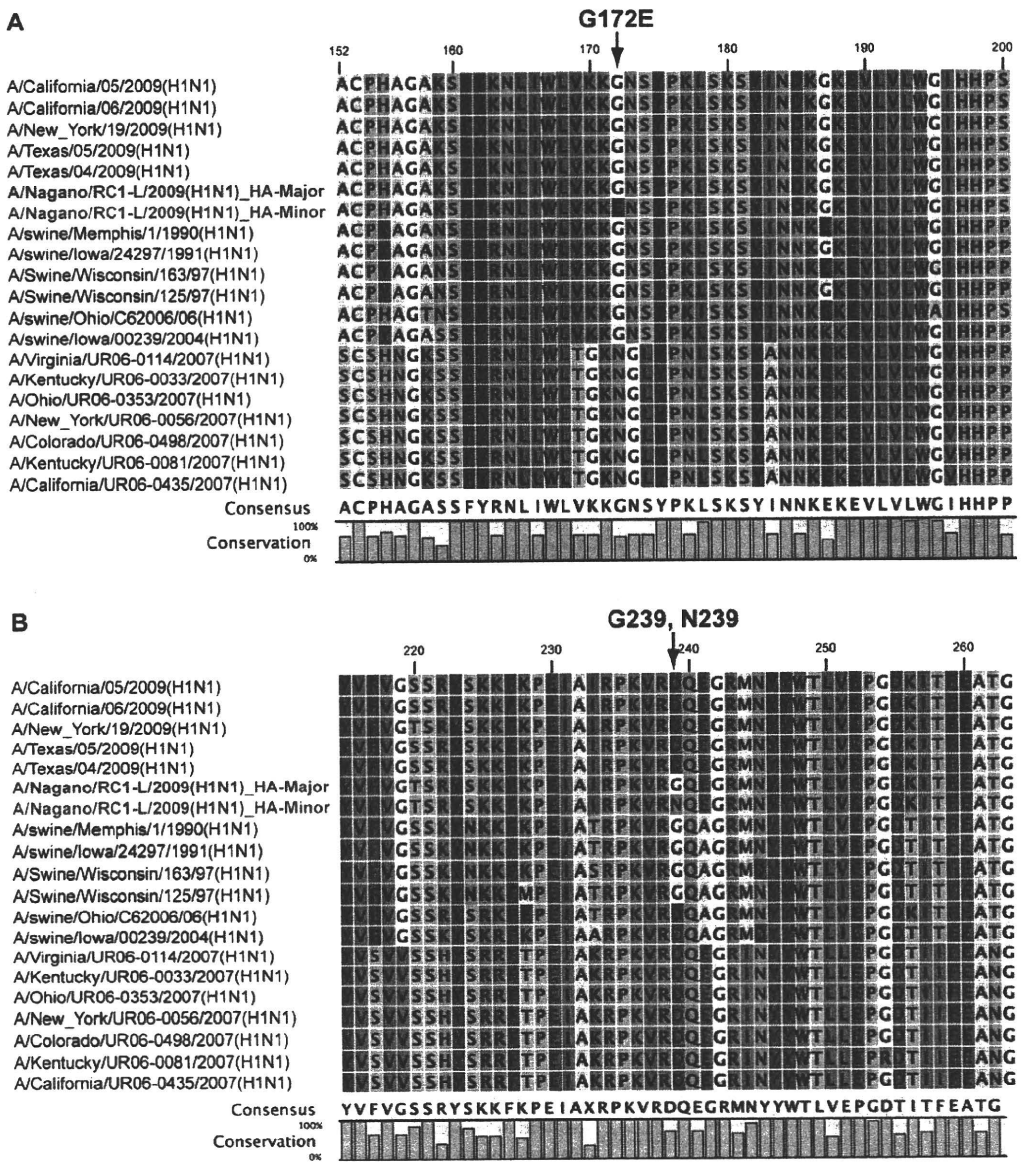

Figure 4. Alignment of HA amino acid sequences in influenza A virus around the identified mutations in A/Nagano/RC1-L/2009(H1N1). (**A**) Genetic variation at position 515 nt causes the amino acid substitution $Gly_{172}Glu$; HA-Major: Gly172, HA-Minor: Glu172. (**B**) Genetic variation at position 715 and 716 nt causes the amino acid substitution $Gly_{239}Asn$; HA-Major: Gly239, HA-Minor: Asn239.
doi:10.1371/journal.pone.0010256.g004

RC1-L/2009(H1N1) in pneumocytes expressing α2-3 sialylated glycans in autopsy lung tissue sections [15], suggesting that the above substitutions could affect the binding affinity to both types of sialylated glycans.

Very suggestive reports predicted the possible future antigenic drift of A/H1N1/2009 viruses from viral sequence and structural comparative analyses [11,12]. Prior to the initiation of the current study (September 2009), Igarashi *et al.* predicted possible substitutions and these included the two amino acid substitutions presented here ($Gly_{172}Glu$ and $Asp_{239}Gly$) [11]. Furthermore, Shen *et al.* suggested that host-driven antigenic drift based on evolutionary trends appeared to favor Asp239 (corresponding to Asp225 in the mature HA) in swine HAs and the 1918 pandemic, while Asp204 (corresponding to Asp190 in the mature HA) was favored in seasonal H1N1 HAs [12]. These predictions are very attractive and our experiments demonstrated one of them *a posteriori*. Furthermore, recent study has shown that receptor-binding avidity can influence antigenic drift [24]. HA antigenic sites Sa is the membrane proximal region, therefore, the identified variations on both Sa and Ca2 might contribute to the alteration of antigenicity and receptor-binding avidity by synergistic effect. The newly identified Asn239 substitution could be a probable candidate for further investigation of the manner of viral binding to sialic acid on the host receptors.

In conclusion, this study demonstrated that *de novo* sequencing can comprehensively detect pathogens, and such in-depth