0.0426 mmol, 54%). $[\alpha]_D^{26}$ = +6.8 (*c* 0.2, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$) δ 7.38–7.14 (m, 10H), 6.08 (ddd, *J* = 7.91, 10.3, 17.5 Hz, 1H), 5.16 (d, *J* = 17.2 Hz, 1H), 4.99 (d, *J* = 9.9 Hz, 1H), 4.65 (dd, *J* = 5.1, 7.9 Hz, 1H), 4.28 (ddd, *J* = 4.8, 5.1, 7.4 Hz, 1H), 4.09 (d, *J* = 14.1 Hz, 2H), 3.84 (d, *J* = 13.8, 2H), 2.96 (dd, *J* = 4.8, 5.1 Hz, 1H), 2.76 (dd, *J* = 2.7, 7.2, 16.8 Hz, 1H), 2.34 (ddd, *J* = 2.7, 5.1, 16.8 Hz, 1H), 1.77 (t, *J* = 2.7 Hz, 1H), 0.90 (s, 9H), 0.87 (s, 9H), 0.085 (s, 3H), 0.060 (s, 3H), 0.044 (s, 3H), 0.040 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 141.9, 140.9, 129.1, 128.1, 126.6, 115.2, 76.4, 73.1, 70.8, 69.8, 64.7, 56.0, 53.5, 29.8, 26.3, 26.1, 18.3, −3.3, −3.4, −4.2, −4.4; HRMS: (ESI, M + H^+) calcd for $C_{34}H_{54}NO_2Si_2$, 564.3693; found, 564.3648.

2β-*N*-Acetylamine 33. A solution of **31** (127.6 mg, 0.333 mmol) in acetic anhydride (0.3 mL) was stirred for 20 min at room temperature. The reaction mixture was concentrated *in vacuo* to give **33** (140.1 mg, 0.329 mmol, 99%). $[\alpha]_D^{17}$ = +8.90 (*c* 0.6, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$) δ 5.87 (d, *J* = 9.6 Hz, 1H), 5.80 (ddd, *J* = 17.4, 10.1, 7.3 Hz, 1H), 5.19 (d, *J* = 17.4 Hz, 1H), 5.11 (d, *J* = 10.1 Hz, 1H), 4.48 (d, *J* = 7.3 Hz, 1H), 3.97 (ddd, *J* = 9.6, 7.8, 1.8 Hz, 1H), 3.93–3.87 (m, 1H), 2.56 (ddd, *J* = 16.9, 6.4, 2.8 Hz, 1H), 2.36 (ddd, *J* = 16.9, 5.0, 2.8 Hz, 1H), 2.02 (t, *J* = 2.7 Hz, 1H), 2.00 (s, 3H), 0.91 (s, 9H), 0.90 (s, 9H), 0.14 (s, 3H), 0.12 (s, 3H), 0.098 (s, 3H), 0.056 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.9, 138.6, 116.2, 81.8, 72.4, 71.5, 70.3, 58.2, 24.8, 23.4, 18.1, 18.0, −4.4, −4.6; HRMS: (ESI, M + Na^+) calcd for $C_{22}H_{43}NO_3Si_2Na$, 448.2679; found, 448.2667.

2β-*N*-Methanesulfonylamine 34. To a solution of **31** (92.8 mg, 0.242 mmol) in dichloromethane (2 mL) was added triethylamine (81 μL, 0.581 mmol) and methanesulfonyl chloride (22 μL, 0.290 mmol) at room temperature, and the mixture was stirred for 15 min. The resulting mixture was quenched with saturated NH_4Cl and extracted with ethyl acetate. The organic layer was washed with brine, and the extracts were dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified by silica gel chromatography (hexane–ethyl acetate = 30 : 1) to give **34** (94.2 mg, 0.204 mmol, 84%). $[\alpha]_D^{18}$ = +15.2 (*c* 0.9, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$) δ 5.94 (ddd, *J* = 7.2, 10.3, 17.2 Hz, 1H), 5.25 (d, *J* = 17.2 Hz, 1H), 5.22 (d, *J* = 10.3 Hz, 1H), 4.85 (d, *J* = 8.9 Hz, 1H), 4.42 (dd, *J* = 2.4, 7.2 Hz, 1H), 3.91 (dd, *J* = 5.16, 10.6 Hz, 1H), 3.53 (ddd, *J* = 2.7, 5.5, 8.6, Hz, 1H), 3.08 (s, 3H), 2.59 (ddd, *J* = 2.4, 5.1, 17.2 Hz, 1H), 2.47 (ddd, *J* = 2.7, 5.1, 17.2 Hz, 1H), 208–2.02 (m, 1H), 0.91 (s, 9H), 0.89 (s, 9H), 0.15 (s, 3H), 0.12 (s, 3H), 0.088 (s, 3H), 0.059 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.3, 116.9, 81.1, 73.3, 71.3, 62.5, 42.3, 25.8, 25.7, 24.1, 18.0, −3.8, −4.1, −4.5, −4.7; HRMS: (ESI, M + Na^+) calcd for $C_{21}H_{43}NO_4SSi_2Na$, 484.2349; found, 484.2368.

2α-*N*,*N*′-Diethyl-1α-25(OH)$_2$ vitamin D$_3$ (IIb). To a solution of **5** (37 mg, 0.09 mmol) and **14** (45 mg, 0.10 mmol) in toluene and triethylamine (1 : 1, 2 mL) was added $Pd(PPh_3)_4$ (about 50 mg) at room temperature. The resulting mixture was stirred at room temperature for 15 min and 90 °C for 2 h. The reaction mixture was cooled to room temperature, and diluted with ethyl acetate, filtered through a pad of Celite, and concentrated *in vacuo*. The residue was purified by silica gel chromatography (hexane–ethyl acetate = 50 : 1) to give bis silylether. To a solution of in THF (3 mL) was added TBAF (71 mg, 0.27 mmol) at room temperature, and the mixture was stirred at 60 °C for 3 h. To the reaction mixture was added saturated NH_4Cl at room temperature, and the mixture was extracted with ethyl acetate. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified by silica gel chromatography ($CHCl_3$–methanol = 5 : 1) to give **IIb** (8 mg, 0.02 mmol, 22%). $[\alpha]_D^{22}$ = +9.6 (*c* 0.6, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 6.42 (d, *J* = 11.5 Hz, 1H), 5.94 (d, *J* = 11.0 Hz, 1H), 5.32 (s, 1H), 5.00 (s, 1H), 4.45–4.35 (m, 1H), 4.20–4.31 (m, 1H), 3.79–3.36 (m, 5H), 2.84–2.75 (m, 1H), 2.44 (t, *J* = 11.9 Hz, 1H), 2.25–0.70 (m, 37H), 0.50 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 143.8, 143.6, 130.6, 125.5, 117.3, 117.1, 71.5, 70.0, 69.0, 65.3, 56.5, 56.3, 47.8, 45.9, 44.6, 44.4, 40.5, 36.3, 36.1, 29.5, 29.2, 29.1, 27.6, 23.4, 22.1, 20.8, 18.8, 12.1; HRMS: calcd for $C_{31}H_{54}NO_3$ 488.4104; found, 488.4078.

2α-*N*-Substituted vitamin D$_3$ derivatives IId–e, IIIa–c, IVd, Va–d. As described for **IIb**, **IId–e**, **IIIa–c**, **IVd** and **Va–d** were obtained from the corresponding A-ring synthons (**15–24**).

2α-*N*,*N*′-Dibutyl-1α-25(OH)$_2$ vitamin D$_3$ (IId). $[\alpha]_D^{23}$ = +20.7 (*c* 0.5, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 6.49 (d, *J* = 10.8 Hz, 1H), 5.94 (d, *J* = 10.9 Hz, 1H), 5.30 (s, 1H), 5.02 (s, 1H), 4.63 (s, 1H), 4.15–4.29 (m, 1H), 3.35–2.72 (m, 9H), 2.40–2.25 (m, 1H), 2.20–0.70 (m, 42H), 0.51 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.1, 132.1, 130.9, 128.5, 126.1, 116.7, 72.5, 71.1, 68.4, 64.5, 56.5, 56.3, 51.0, 45.9, 44.4, 43.7, 40.5, 36.4, 36.1, 31.9, 29.7, 29.6, 29.4, 29.1, 27.6, 23.5, 22.7, 22.2, 20.9, 20.8, 18.8, 14.1, 14.0, 12.1; HRMS: calcd for $C_{35}H_{62}NO_3$, 544.4730; found, 544.4711.

2α-*N*,*N*′-Dibenzyl-1α-25(OH)$_2$ vitamin D$_3$ (IIe). $[\alpha]_D^{31}$ = +18.3 (*c* 0.5, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.38–7.18 (m, 10H), 6.47 (d, *J* = 11.4 Hz, 1H), 5.94 (d, *J* = 11.4 Hz, 1H), 5.21 (d, *J* = 1.9 Hz, 1H), 4.89 (d, *J* = 1.8 Hz, 1H), 4.66 (d, *J* = 1.8 Hz, 1H), 4.26–4.08 (m, 2H), 3.93 (dd, *J* = 178.4, 13.7 Hz, 4H), 2.82 (d, *J* = 12.4 Hz, 1H), 2.76 (dd, *J* = 13.1, 5.3 Hz, 1H), 2.60 (dd, *J* = 10.1, 2.3 Hz, 1H), 2.18–0.68 (m, 30 H), 0.52 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.0, 139.5, 131.5, 129.1, 127.1, 126.1, 116.7, 114.3, 72.3, 71.1, 66.1, 64.1, 56.5, 56.3, 54.5, 45.9, 44.4, 43.3, 40.4, 36.4, 36.1, 29.4, 29.3, 29.2, 27.6, 23.4, 22.2, 20.8, 18.8, 12.1; HRMS: calcd. for $C_{41}H_{57}NO_3Na$, 634.4236; found, 634.4231.

2α-*N*-Acetyl-1α-25(OH)$_2$ vitamin D$_3$ (IIIa). $[\alpha]_D^{26}$ = +62.8 (*c* 0.6, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 6.46 (d, *J* = 11.4 Hz, 1H), 6.28 (d, *J* = 8.3 Hz, 1H), 5.93 (d, *J* = 11.5 Hz, 1H), 5.35 (d, *J* = 1.8 Hz, 1H), 5.09 (d, *J* = 1.9 Hz, 1H), 4.31 (d, *J* = 3.3 Hz, 1H), 4.03–3.96 (m, 1H), 3.89 (ddd, *J* = 9.7, 9.7, 4.6 Hz, 1H), 2.83 (d, *J* = 12.8 Hz, 1H), 2.70 (dd, *J* = 13.7, 4.6 Hz, 1H), 2.34–2.25 (m, 1H), 2.08 (s, 3H), 2.08–0.80 (m, 30H), 0.53 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.2, 144.5, 144.0, 130.7, 126.1, 117.0, 116.6, 74.7, 71.1, 70.6, 58.1, 56.4, 56.3, 45.9, 44.3, 43.4, 40.4, 36.4, 36.1, 29.5, 29.4, 29.2, 27.6, 23.5, 23.4, 22.2, 20.8, 18.8, 12.1; HRMS: calcd for $C_{29}H_{47}NO_4Na$, 496.3403; found, 496.3399.

2α-*N*-Pivaloyl-1α-25(OH)$_2$ vitamin D$_3$ (IIIb). $[\alpha]_D^{23}$ = +37.5 (*c* 1.8, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.48–6.37 (m, 2H), 5.94 (d, *J* = 11.4 Hz, 1H), 5.35 (s, 1H), 5.08 (s, 1H), 4.33 (d, *J* = 3.2 Hz, 1H), 4.05–3.93 (m, 1H), 3.93–3.84 (m, 1H), 2.82 (d, *J* = 12.3 Hz, 1H), 2.65 (dd, *J* = 13.5, 4.4 Hz, 1H), 2.30 (t, *J* = 11.4 Hz, 1H), 2.05–0.80 (m, 39H), 0.52 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.8, 144.2, 132.1, 128.5, 125.8, 116.7, 116.4, 74.2, 71.1, 70.8, 57.8, 56.4, 56.3, 45.9, 44.3, 43.2, 40.4, 38.9, 36.3, 36.1, 29.5, 29.3, 29.2, 27.7, 27.6, 23.5, 22.2, 20.8, 18.8, 12.0; HRMS: calcd for $C_{32}H_{53}NO_4Na$, 538.3872; found, 538.3834.

2α-*N*-Benzoyl-1α-25(OH)$_2$ vitamin D$_3$ (IIIc). $[\alpha]_D^{23}$ = +26.2 (*c* 1.3, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89–7.31 (m, 5H), 6.98 (d, *J* = 8.0 Hz, 1H), 6.46 (d, *J* = 11.4 Hz, 1H), 5.97 (d, *J* = 11.4 Hz, 1H), 5.38 (s, 1H), 5.12 (s, 1H), 4.45 (d, *J* = 3.4 Hz, 1H), 4.25–4.17 (m, 1H), 4.09–4.00 (m, 1H), 2.83 (t, *J* = 13.1 Hz, 1H), 2.71 (dd, *J* = 13.8, 4.6 Hz, 1H), 2.36 (t, *J* = 11.8 Hz, 1H), 2.23–0.80 (m, 30H), 0.53 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.1, 144.1, 132.5, 132.0, 131.8, 131.0, 128.5, 127.2, 125.8, 116.8, 74.3, 71.1, 70.4, 58.5, 56.4, 56.3, 45.9, 44.3, 43.2, 40.4, 36.3, 36.1, 29.4, 29.3, 29.1, 27.6, 23.5, 22.2, 20.8, 18.8, 12.1; HRMS: calcd for $C_{34}H_{49}NO_4Na$, 558.3559; found, 558.3540.

2α-*N*-*tert*-Butoxycarbonyl-1α-25(OH)$_2$ vitamin D$_3$ (IVd). $[\alpha]_D^{31}$ = +44.7 (*c* 0.8, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.44 (d, *J* = 11.0 Hz, 1H), 5.94 (d, *J* = 11.0 Hz, 1H), 5.35 (s, 1H), 5.07 (s, 1H), 4.35 (d, *J* = 2.7 Hz, 1H), 3.92–3.84 (m, 1H), 3.73–3.64 (m, 1H), 2.82 (d, *J* = 12.8 Hz, 1H), 2.67 (dd, *J* = 13.5, 4.8 Hz, 1H), 2.27 (t, *J* = 11.5 Hz, 1H), 2.06–0.72 (m, 30H), 1.46 (s, 9H), 0.52 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.2, 144.3, 144.1, 131.1, 128.0, 125.8, 116.7, 80.4, 74.7, 71.1, 70.5, 59.1, 56.4, 56.3, 45.9, 44.3, 43.2, 40.4, 36.3, 36.1, 29.7, 29.3, 29.2, 28.3, 27.6, 23.4, 22.2, 20.8, 18.8, 12.1; HRMS: calcd for $C_{32}H_{53}NO_5Na$, 554.3821; found, 554.3814.

2α-*N*-Methanesulfonyl-1α-25(OH)$_2$ vitamin D$_3$ (Va). $[\alpha]_D^{14}$ = +29.4 (*c* 0.5, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.45 (d, *J* = 11.5 Hz, 1H), 5.93 (d, *J* = 11.4 Hz, 1H), 5.39 (s, 1H), 5.12 (s, 1H), 5.01 (d, *J* = 8.3 Hz, 1H), 4.47 (s, 1H), 4.00–3.87 (m, 1H), 3.51–3.38 (m, 1H), 3.01 (s, 3H), 2.82 (d, *J* = 11.9 Hz, 1H), 2.70 (dd, *J* = 13.5, 4.3 Hz, 1H), 2.35–2.20 (m, 1H), 2.10–0.75 (m, 30H), 0.53 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.6, 143.9, 130.3, 126.0, 116.9, 116.5, 74.2, 71.1, 69.3, 61.9, 56.5, 56.3, 46.0, 44.4, 42.1, 41.4, 40.4, 36.3, 36.1, 29.7, 29.4, 29.3, 27.6, 23.5, 22.2, 20.8, 18.8, 12.1; HRMS: calcd for $C_{28}H_{47}NO_5SNa$, 532.3073; found, 532.3076.

2α-*N*-Benzenesulfonyl-1α-25(OH)$_2$ vitamin D$_3$ (Vb). $[\alpha]_D^{17}$ = +29.2 (*c* 0.8, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 (d, *J* = 7.8 Hz, 1H), 7.62 (t, *J* = 7.4 Hz, 1H), 7.55 (t, *J* = 7.6 Hz, 2H), 6.42 (d, *J* = 11.4 Hz, 1H), 5.87 (d, *J* = 11.5 Hz, 1H), 5.31 (d, *J* = 8.3 Hz, 1H), 5.15 (s, 1H), 5.02 (d, *J* = 1.3 Hz, 1H), 3.94 (s, 1H), 3.91–3.81 (m, 1H), 3.23 (ddd, *J* = 8.7, 8.7, 3.2 Hz, 1H), 2.79 (d, *J* = 12.8 Hz, 1H), 2.67 (dd, *J* = 13.6, 4.9 Hz, 1H), 2.32 (s, 1H), 2.20 (t, *J* = 11.7 Hz, 1H), 2.05–0.82 (m, 30H), 0.50 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.6, 143.7, 140.3, 133.0, 130.2, 129.3, 127.1, 126.1, 116.9, 116.5, 73.4, 71.1, 68.7, 62.0, 56.4, 56.3, 45.9, 44.3, 41.6, 40.4, 36.3, 36.0, 29.3, 29.2, 29.1, 27.6, 23.4, 22.1, 20.8, 18.8, 12.1; HRMS: calcd for $C_{33}H_{49}NO_5SNa$, 594.3229; found, 594.3271.

2α-*N*-(4-*tert*-Butyl)-benzenesulfonyl-1α-25(OH)$_2$ vitamin D$_3$ (Vc). $[\alpha]_D^{18}$ = +12.0 (*c* 0.4, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.85 (d, *J* = 8.6 Hz, 2H), 7.55 (t, *J* = 8.6 Hz, 2H), 6.42 (d, *J* = 11.5 Hz, 1H), 5.87 (d, *J* = 10.9 Hz, 1H), 5.24 (d, *J* = 8.6 Hz, 1H), 5.11 (d, *J* = 1.7 Hz, 1H), 5.01 (d, *J* = 1.7 Hz, 1H), 3.90 (d, *J* = 3.5 Hz, 1H), 3.86 (ddd, *J* = 13.7, 9.1, 4.6 Hz, 1H), 3.22 (dt, *J* = 8.6, 3.4 Hz, 1H), 2.79 (d, *J* = 12.6 Hz, 1H), 2.68 (dd, *J* = 13.8, 4.6 Hz, 1H), 2.21 (t, *J* = 11.8 Hz, 1H), 2.10–0.60 (m, 39H), 0.50 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 156.9, 144.6, 143.8, 137.1, 130.2, 127.0, 126.3, 126.2, 116.8, 116.5, 73.4, 71.1, 68.7, 61.9, 56.4, 56.3, 45.9, 44.4, 41.6, 40.4, 36.3, 36.1, 31.6, 31.1, 29.4, 29.2, 29.1, 27.6, 23.5, 22.1, 20.8, 18.8, 12.0; HRMS: calcd for $C_{37}H_{57}NO_5SNa$, 650.3855; found, 650.3858.

2α-*N*-(4-Methoxy)-benzenesulfonyl-1α-25(OH)$_2$ vitamin D$_3$ (Vd). $[\alpha]_D^{20}$ = +12.2 (*c* 0.5, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.86 (d, *J* = 9.1 Hz, 2H), 7.00 (d, *J* = 8.6 Hz, 2H), 6.42 (d, *J* = 11.5 Hz, 1H), 5.87 (d, *J* = 11.5 Hz, 1H), 5.22 (d, *J* = 8.6 Hz, 1H), 5.18 (s, 1H), 5.03 (d, *J* = 1.1 Hz, 1H), 3.99 (d, *J* = 3.5 Hz, 1H), 3.88 (s, 3H), 3.88–3.81 (m, 1H), 3.19 (dt, *J* = 8.6, 3.5 Hz, 1H), 2.79 (d, *J* = 13.2 Hz, 1H), 2.67 (dd, *J* = 13.2, 4.6 Hz, 1H), 2.20 (t, *J* = 11.8 Hz, 1H), 2.10–0.60 (m, 39H), 0.50 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.1, 144.6, 143.8, 131.7, 130.3, 129.3, 126.1, 116.9, 116.5, 114.4, 73.4, 71.1, 68.7, 61.9, 56.4, 56.3, 55.6, 45.9, 44.3, 41.6, 40.4, 36.3, 36.0, 29.3, 29.2, 29.1, 27.6, 23.5, 22.1, 20.8, 18.8, 12.0; HRMS: calcd for $C_{34}H_{51}NO_6SNa$, 624.3335; found, 624.3337.

2β-*N*,*N*′-Dibenzyl-1α-25(OH)$_2$ vitamin D$_3$ (VIIe). To a solution of **5** (40 mg, 0.0346 mmol) and **32** (42 mg, 0.0737 mmol) in toluene and triethylamine (1 : 1, 2 mL) was added $Pd(PPh_3)_4$ (about 50 mg) at room temperature. The resulting mixture was stirred at room temperature for 15 min, then 100 °C for 3 h. The resulting mixture was cooled to room temperature, and diluted with ethyl acetate, filtered through a pad of Celite, and concentrated *in vacuo*. The residue was purified by silica gel chromatography (hexane–ethyl acetate = 20 : 1) to give bis silylether. To a solution of bis silylether in THF (1.0 mL) was added HF–Et_3N (1.0 mL), and the mixture was stirred for 15 h. To the reaction mixture was added saturated $NaHCO_3$, and the mixture was stirred for 30 min. The resulting mixture was extracted with ethyl acetate, and the organic layer was washed with brine. The extracts were dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified by silica gel chromatography ($CHCl_3$–MeOH = 9 : 1) to give **VIIe** (8.7 mg, 0.0142 mmol, 21%). $[\alpha]_D^{18}$ = −35.3 (*c* 0.1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.33–7.24 (m, 10H), 6.34 (d, *J* = 10.8 Hz, 1H), 6.08 (d, *J* = 10.8 Hz, 1H), 5.46 (s, 1H), 4.94 (s, 1H), 4.52 (s, 1H), 4.44 (d, *J* = 10.8 Hz, 1H), 4.16 (d, *J* = 13.7 Hz, 2H), 3.66 (d, *J* = 13.7 Hz, 2H), 2.80 (dd, *J* = 3.4, 12.6 Hz, 1H), 2.56 (d, *J* = 10.8 Hz, 1H), 2.38–0.83 (m, 26H), 0.93 (d, *J* = 5.7 Hz, 3H), 0.53 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 143.7, 139.4, 131.7, 131.0, 128.8, 128.5, 127.1, 126.2, 116.9, 110.4, 71.0, 66.6, 66.0, 65.8, 56.4, 56.3, 54.2, 50.9, 45.9, 44.3, 40.3, 36.3,

36.0, 31.9, 29.6, 29.3, 29.2, 27.6, 23.7, 22.2, 20.7, 18.8, 11.8; HRMS: (ESI, M + H^+) calcd for $C_{41}H_{58}NO_3$, 612.4416; found, 612.4422.

2β-*N*-Substituted vitamin D derivatives VIIIa and Xa. As described for **VIIe**, **VIIIa** and **Xa** were obtained from the corresponding A-ring synthons (**33** and **34**), respectively.

2β-*N*-Acetyl-1α-25(OH)$_2$ vitamin D$_3$ (VIIIa). $[\alpha]_D^{20}$ = −15.6 (*c* 0.3, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.39–6.33 (m, 2H), 6.03 (d, *J* = 11.4 Hz, 1H), 5.59 (s, 1H), 5.09 (s, 1H), 4.13–4.04 (m, 2H), 3.96–3.89 (m, 1H), 2.80 (d, *J* = 10.8 Hz, 1H), 2.61 (d, *J* = 13.1 Hz, 1H), 2.15 (d, *J* = 14.3 Hz, 1H), 2.08 (s, 3H), 2.20–0.84 (m, 24H), 0.93 (d, *J* = 5.1 Hz, 3H), 0.54 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.4, 145.1, 144.1, 131.0, 125.9, 116.7, 112.2, 73.7, 71.0, 69.4, 58.4, 56.5, 56.3, 46.0, 44.3, 42.4, 40.3, 36.3, 36.0, 29.6, 29.3, 29.2, 27.5, 23.7, 23.3, 22.1, 20.7, 18.8, 11.8; HRMS: (ESI, M + Na^+) calcd for $C_{29}H_{47}NO_4Na$, 496.3402; found, 496.3405.

2β-*N*-Methanesulfonyl-1α-25(OH)$_2$ vitamin D$_3$ (Xa). $[\alpha]_D^{21}$ = −41.4 (*c* 0.3, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.35 (d, *J* = 10.9 Hz, 1H), 6.00 (d, *J* = 11.4 Hz, 1H), 5.52 (s, 1H), 5.24 (d, *J* = 8.24 Hz, 1H), 5.12 (s, 1H), 4.23–4.14 (m, 2H), 3.39–3.32 (m, 1H), 3.09 (s, 3H), 2.80 (d, *J* = 10.9 Hz, 1H), 2.57 (d, *J* = 14.6 Hz, 1H), 2.44 (d, *J* = 14.2 Hz, 1H), 2.09–0.84 (m, 24H), 0.93 (d, *J* = 5.9 Hz, 3H), 0.54 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.5, 144.1, 130.9, 125.7, 116.6, 112.3, 71.6, 71.1, 69.8, 62.6, 56.5, 56.3, 46.0, 44.3, 42.0, 41.4, 40.3, 36.3, 36.0, 29.6, 29.3, 29.2, 27.5, 23.7, 22.3, 20.7, 18.8, 11.8; HRMS: (ESI, M + Na^+) calcd for $C_{28}H_{47}NO_5SNa$, 532.3072; found, 532.3089.

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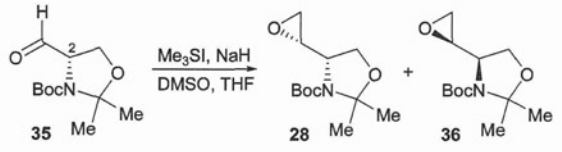
(Scheme 3) afforded **28** as a sole product. $[\alpha]_D^{22} = +12.5$ (*c* 1.1, $CHCl_3$).



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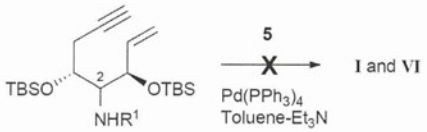

R^1 = Me, Et, *n*-Pr, *n*-Bu, Bn

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Short Communication

Composition of Hemagglutinin and Neuraminidase Affects the Antigen Yield of Influenza A(H1N1)pdm09 Candidate Vaccine Viruses

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SUMMARY: To improve the hemagglutinin (HA) antigen yield of influenza A(H1N1)pdm09 candidate vaccine viruses, we generated 7:1, 6:2, and 5:3 genetic reassortant viruses between wild-type (H1N1)pdm09 (A/California/7/2009) (Cal7) and a high-yielding master virus, A/Puerto Rico/8/34 (PR8). These viruses contained the HA; HA and neuraminidase (NA); and HA, NA, and M genes, respectively, derived from Cal7, on a PR8 backbone. The influence of the amino acid residue at position 223 in Cal7 HA on virus growth and HA antigen yield differed between these reassortant viruses. NIIDRG-7, a 7:1 virus possessing arginine at position 223, exhibited a 10-fold higher 50% egg infectious dose (EID_{50}) (10.0 $\log_{10}EID_{50}$/ml) than the 5:3 and 6:2 viruses. It also had 1.5- to 3-fold higher protein (13.8 μg/ml of allantoic fluids) and HA antigen (4.1 μg/ml of allantoic fluids) yields than the 5:3 and 6:2 viruses, which possessed identical Cal7 HA proteins. However, the HA antigen yield of the other 7:1 virus, which possessed glutamine at position 223 was 60% of that of NIIDRG-7. In addition, a novel 6:2 virus possessing Cal7 HA and the NA of A/Wisconsin/10/98 (a triple reassortant swine-like H1N1 virus), produced 107% of the HA yield of NIIDRG-7. In this study, we showed that the balance between HA and NA in the influenza A(H1N1)pdm09 virus affects its protein and antigen yield.

In April 2009, a novel influenza A(H1N1)pdm09 virus was identified in the United States. Within 2 months, this virus had spread globally, causing the first pandemic of the 21st century (1). The A(H1N1)pdm09 virus is a novel reassortant virus that contains 6 gene segments from the North American triple-reassortant swine-influenza viruses. It also encompasses neuraminidase (NA) and M gene segments from the Eurasian swine influenza viruses (1,2). Due to the origin of the HA gene, the A(H1N1)pdm09 virus is antigenically different from the previous seasonal H1N1 viruses that circulated globally until April 2009 (3). To prevent the spread of the viruses through the population, the World Health Organization (WHO) Global Influenza Surveillance and Response System initiated the development of candidate vaccine viruses (CVVs) (4). Currently, influenza vaccine viruses are propagated in embryonated chicken eggs. However, human influenza isolates, in most cases, grow poorly in chicken eggs; and A(H1N1)pdm09 virus is not an exception. There have been efforts to develop high-growth reassortant viruses between A/California/7/2009 (Cal7) and a high-yielding master virus (A/Puerto Rico/8/34 [PR8] or its derivative) using the classical reassortment method (5,6) or by reverse genetics using plasmid DNAs (7-9). One such reassortant virus, X-179A, exhibited higher growth in eggs (hemagglutination [HA] titer, 2048) than the parental virus, Cal7 (HA titer, 64) (4). Despite the high HA titer of X-179A, the total protein and HA antigen yield was lower than that for the previous seasonal human H1N1 vaccine viruses (4). To overcome this disadvantage, we attempted to develop vaccine viruses with higher growth in eggs and higher protein yield. We used 12 plasmid-based reverse genetics system and the high-growth PR8 virus (UW strain) as the backbone virus (Table 1) (8). For pandemic vaccine viruses, the WHO recommends that the reassortant virus possesses at least the HA and NA genes of the pandemic virus with the remaining genes originating from PR8 (6:2) (10). However, an improvement in virus growth or antigen yield, relative to 6:2 viruses, was observed with H5N1 reassortant viruses possessing the HA (7:1), HA/NA/PB1 (5:3), or HA/NA/M (5:3) from H5 viruses (11). In addition, studies have shown that there are different residues (D/Q, G/Q, or D/R) at the receptor-binding site of Cal7 HA at positions 222/223 (H1HA numbering) (4,12). The residues at these positions reportedly affect the receptor binding specificity of H1N1, H2N2, and H3N2 influenza viruses (13-17). In this study, we examined the effect of gene constellations and amino acid residues at 222/223 in Cal7 HA on virus growth and antigen yield.

We first developed three 6:2 reassortant viruses with D/Q, G/Q, and D/R at positions 222/223 using the appropriate cloned Cal7 HA cDNAs (Table 1). The HA titer of the virus with G/Q was 64. Passaging the viruses 4 times in embryonated chicken eggs did not improve the HA titer. However, the HA titers of the D/Q and D/R viruses increased to 128 and 512, respectively (Table 1). Next, to assess the impact of D/Q and D/R at positions 222/223 and the impact of the gene constellations on virus growth and infectivity, we develped 5:3 (NIIDRG-3, -6) and 7:1 (NIIDRG-7, -8) reassortant viruses, in addition to the 6:2 viruses (NIIDRG-1, -5) (Table 1). We performed 50% egg infective dose (EID_{50}) assays and found that infectivity increased

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Table 1. Differences in the amino acid sequences of HA and the infectivity titers of viruses

Virus[1]	Passage history	Gene constellation	Amino acid at the indicated HA positions[2]					EID_{50} (log_{10}/EID_{50} ml)[3]	
			125	191	209	222	223	Exp. 1	Exp. 2
A/California/7/2009	—	—	N	L	K	D/G	Q/R	n.d.	n.d.
X-179A	E7/E1/E6	5:3	·	·	T	D	R	9.4	9.3
NIIDRG-1	LLC1E2	6:2	·	I	·	D	Q	8.3	8.4
NIIDRG-3	LLC1E2	5:3	·	I	·	D	Q	8.2	8.3
NIIDRG-5	LLC1E4	6:2	·	·	·	D	R	9.1	9.0
NIIDRG-6	LLC1E5	5:3	N/D	·	·	D	R	9.0	8.9
NIIDRG-7	LLC1E2	7:1	·	·	·	D	R	9.9	10.0
NIIDRG-8	LLC1E3	7:1	·	I	·	D	Q	9.3	9.1

[1]: Reassortant viruses were generated by co-transfecting 12 plasmid DNAs into qualified LLCMK2 cells using FuGENE HD Transfection Reagent (Roche) (LLC1) (8). Approximately 18 h post-transfections, the cells were washed, and the medium was replaced with Opti-Pro SFM medium (Invitrogen) containing 1 μg/ml of TrypZean (SIGMA). The supernatant was harvested at 48 h post-transfection and used to inoculate 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs to amplify the rescued viruses. For each reassortant virus, the genetic sequences of HA and/or NA and/or M derived from Cal7 were verified by sequencing the RT-PCR products amplified from viral RNA. All the manipulations were performed in accordance with the Good Manufacturing Practice of NIID.

[2]: H1 numbering; all changes relative to A/California/7/2009.

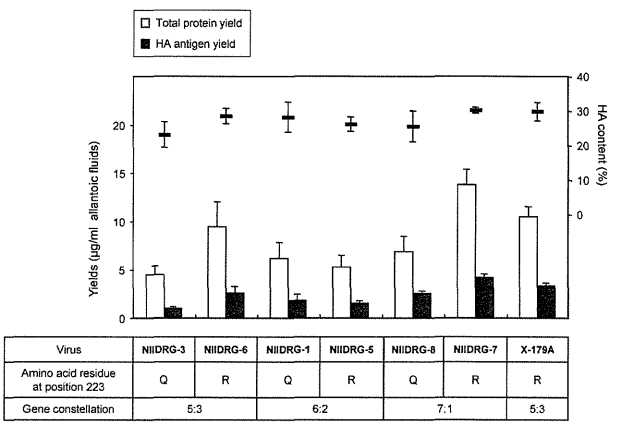
[3]: The EID_{50} was determined as follows: each virus was serially diluted from 10^{-5} to 10^{-11}. Six 10 to 11 day-old embryonated chicken eggs were infected with 0.2 ml of virus for each dilution. An aliquot of the allantoic fluid was harvested from each of the eggs at 48 h post-infection. An HA assay was performed for each allantoic fluid, using 0.5% turkey erythrocytes. The EID_{50} was calculated by using the formula of Reed and Muench. The results are from two separate experiments.

n.d., not done.

among the reassortant viruses (in the order 5:3, 6:2, and 7:1) possessing the identical amino acid residues at positions 222/223. In addition, the influence of 223R on the EID_{50} was greater than that of 223Q, as determined by evaluating viruses with the same gene constellations (Table 1). The HA titers and EID_{50} values were nearly parallel for each virus. Among the reassortant viruses, NIIDRG-7 exhibited the highest growth (HA titer, 1024) and EID_{50} (Table 1). Residue 223 is located on the 220-loop of the receptor-binding site of HA. Avian H1HA has close contact between 223Q and the Gal-2 of α2-3 linked sialylated glycans. However, similar contacts between the A(H1N1)pdm09 HA of A/California/4/2009 and α2-3 linked sialylated glycans are absent (18). The presence of 223R instead of 223Q might overcome the unfavorable interactions between Cal7 HA and α2-3 linked sialylated glycans, which are prevalent in the chorioallantois of chicken eggs. In the case of 223Q viruses (NIIDRG-1, -3, and -8), all rescued viruses possessed an additional substitution, i.e., L191I. The L191I substitution might facilitate viral rescue and amplification in eggs, as was observed with other reassortant viruses derived from A/California/4/2009 (12). However, this substitution did not have a stronger influence on virus growth than that observed with 223R.

The total protein yield (TPY) of the purified NIIDRG-1, -3, and -5 to -8 and X-179A viruses was determined by using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, Ill., USA), according to the manufacturer instructions (Fig. 1). Among the viruses subjected to the assay, NIIDRG-7 exhibited the highest TPY (13.8 μg/ml allantoic fluid [AF]), whereas X-179A had a TPY of 10.4 μg/ml AF. The other viruses exhibited TPYs similar to or lower than that of X-179A (Fig. 1). A correlation between virus proliferation and TPY was observed primarily with the resultant viruses.

The HA yield of each virus was calculated using the TPY and the results of the SDS-PAGE analysis (Fig. 1). The HA content relative to the total protein content of



Virus	NIIDRG-3	NIIDRG-6	NIIDRG-1	NIIDRG-5	NIIDRG-8	NIIDRG-7	X-179A
Amino acid residue at position 223	Q	R	Q	R	Q	R	R
Gene constellation	5:3		6:2		7:1		5:3

Fig. 1. Average yield (μg) of total protein, HA antigen per ml of allantoic fluids and the average HA content (%) of each virus concentrate. Ten-day-old embryonated chicken eggs were inoculated with NIIDRG-1, -3, and -5 to -8 viruses. Forty-eight hours post infection, the allantoic fluid was collected. The viruses were purified by ultracentrifugation using 20% sucrose at 35,000 rpm for 60 min at 4°C. The pellet was re-suspended in a small volume of buffer, which was used as the purified concentrate. The method for preparation of the virus concentrate differed from the method used by others (4,21); therefore, we re-assessed the total protein yield of X-179A and NIIDRG-7. We used viruses concentrated by methods similar to those used by others (4,21), that is, viruses were grown in 50 eggs and purified using 10–40% continuous sucrose gradient with centrifugation at 35,000 rpm for 35 min at 4°C. The total protein yield of X-179A and NIIDRG-7 was estimated at 4.5 mg/50 eggs and 6.25 mg/50 eggs, respectively. The HA content was calculated using the results from the SDS-PAGE analysis, which are shown in Figure 2. The SDS-PAGE results were captured and analyzed using a CS analyzer (ATTO), and the ratio of HA protein to total proteins was calculated. The total HA yields were calculated from the total protein yields and the relative HA contents. Each value is the average of values from 3 analyses.

NIIDRG viruses ranged between 23% (NIIDRG-3) and 30% (NIIDRG-7). NIIDRG-7 had the highest HA yield (4.1 μg/ml AF) whereas X-179A had an HA yield of 3.3

μg/ml AF (Fig. 1). NIIDRG-6 and -8 also exhibited low HA yield (2.5 μg/ml AF). The remaining viruses had yields much lower than that of NIIDRG-7.

The functional balance between HA and NA is known to affect the virus growth (19,20). The novel gene constellation of the A(H1N1)pdm09 virus may not result in the optimal balance between HA and NA activity. The difference in TPY between NIIDRG-5 and NIIDRG-7 is attributable to the imbalance in HA and NA activity. Cal7 HA is genetically close to HA in the triple-reassortant swine-like H1N1 virus, A/Wisconsin/10/98 (Wis98) (3). The similarity of HA1 is 93% between Cal7 and Wis98. To improve the compatibility of HA and NA, we developed another 6:2 reassortant virus, Cal7/Wis98 which harbored Cal7 HA and Wis98 NA with a PR8 backbone. The HA yield (4.4 μg/ml AF) and the TPY (15.2 μg/ml AF) were slightly higher than that of NIIDRG-7. This suggests that the low TPY and HA yield of NIIDRG-5 was a result of the poor compatibility of HA and NA of Cal7.

During the preparation of this manuscript, Harvey et al. reported a new CVV (6:2) with a chimeric sequence derived from PR8 and Cal7 HA (NIBRG-119). This new CVV exhibited improved growth as compared to the current vaccine virus (6:2) (NIBRG-121) (21). Based on their results, we created a new 7:1 reassortant virus by exchanging the 3′- and 5′-non-coding region domains, signal peptide, transmembrane (TM) domain, and cytoplasmic tail of Cal7 HA with those of PR8 (NIIDRG-7.1) (Fig. 2A). NIIDRG-7.1 exhibited an improved HA content (40.1 ± 1.23%) (Fig. 2B) and HA yield (5.2 μg/ml AF). However, it still had a TPY (13.1 μg/ml AF) similar to that of NIIDRG-7. Our result proved the hypothesis proposed by Harvey et al.; they stated that the TM domain of PR8HA plays a role in the efficient incorporation of HA into viral particles, which is caused by the increased association of HA with glycosphingolipid-enriched, detergent-resistant plasma membrane domains. In the case of NIBRG-119, the TPY was higher, but not the HA yield. Although both viruses showed similar HA yields, the mechanisms responsible for improvement might be different.

We performed hemagglutination inhibition (HAI) assays using ferret antisera against the reassortant viruses in order to determine their antigenic characteristics (Table 2). All NIIDRG viruses were antigenically similar to wild-type A(H1N1)pdm09, Cal7, and A/Narita/1/2009, and the vaccine virus X-179A. However, the NIIDRG viruses were distinct from A/Brisbane/59/2007, a past seasonal H1N1 virus (Table 2).

To improve the protein/antigen yields of A(H1N1)pdm09 CVVs, genetic alterations have been introduced into the packaging signal and the oligosaccharide moiety of Cal7 HA (21,22). In this study, we showed that the composition of HA and NA must be controlled to achieve a high protein yield. Although NA immunogenicity of the original pandemic virus is significant, if the antigen yield is extremely low, as observed for the A(H1N1)pdm09 CVV, we can also con-

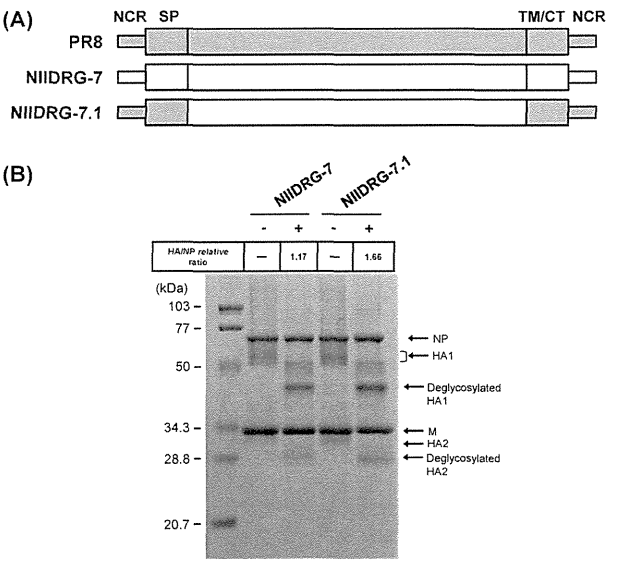


Fig. 2. (A) Schematic diagram of the HA chimera. Domains derived from Cal7 and PR8 are shown in white and black, respectively. NCR, non-coding region; SP, signal peptide; TM/CT, transmembrane and cytoplasmic tail domains of the HA. (B) SDS-PAGE analysis of NIIDRG-7 and NIIDRG-7.1. The purified viruses (6 μg) were treated with (+) or without (−) 10 units of PNGase F according to the manufacturer's instructions (New England Biolabs). Samples were incubated in a total volume of 20 μl at 37°C overnight, and the reducing agent was added before the samples were loaded on 10% SDS-PAGE gels. The relative ratio of HA to NP is shown as the average of the values.

Table 2. Antigenic analysis of viruses

Virus	Titer[1)] of ferret antiserum against indicated virus:			
	A/Brisbane/59/2007	A/California/7/2009	A/Narita/1/2009	NYMC X-179A
A/Brisbane/59/2007	**640**	<10	<10	<10
A/California/7/2009	<10	**2560**	10240	2560
A/Narita/1/2009	<10	2560	**10240**	5120
NYMC X-179A	<10	5120	10240	**5120**
NIIDRG-1	<10	2560	5120	1280
NIIDRG-3	<10	1280	5120	1280
NIIDRG-5	<10	1280	5120	1280
NIIDRG-6	<10	1280	5120	1280
NIIDRG-7	<10	2560	5120	2560
NIIDRG-8	<10	1280	5120	1280

[1)]: HAI assay was performed with 0.5% turkey erythrocytes. HAI titers are presented as the reciprocal value of the highest serum dilution which inhibited hemagglutination ability of the viruses. The homologous titers are boldfaced.

sider the development of a CVV with a different gene constellation without genetic alterations.

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Conflict of interest None to declare.

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Genetics and infectivity of H5N1 highly pathogenic avian influenza viruses isolated from chickens and wild birds in Japan during 2010–11

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ABSTRACT

Outbreaks of H5N1 subtype highly pathogenic avian influenza virus (HPAIV) were recorded in chickens, domesticated birds and wild birds throughout Japan from November 2010 to March 2011. Genetic analysis of the Japanese isolates indicated that all gene segments, except the PA gene, were closely related to Japanese wild bird isolates in 2008 and belonged to clade 2.3.2.1 classified by the WHO/OIE/FAO H5N1 Evolution Working Group. Direct ancestors of the PA gene segment of all Japanese viruses analyzed in this study can be found in wild bird strains of several subtypes other than H5N1 isolated between 2007 and 2009. The PA gene of these wild bird isolates share a common ancestor with H5N1 HPAIVs belonging to clades 2.5, 7 and 9, indicating that wild birds were involved in the emergence of the current reassortant 2.3.2.1 viruses. To determine how viruses were maintained in the wild bird population, two isolates derived from chickens (A/chicken/Shimane/1/2010, Ck10 and A/chicken/Miyazaki/S4/2011, CkS411) and one from a wild bird (A/mandarin duck/Miyazaki/22M-765/2011, MandarinD11) were compared in their ability to infect and be transmitted to chickens. There was a significant difference in the survival of chickens that were infected with 10^6 EID_{50} of CkS411 compared to those with MandarinD11 and the transmission efficiency of CkS411 was greater than the other viruses. The increased titer of CkS411 excreted from infected chickens contributed to the improved transmission rates. It was considered that reduced virus excretion and transmission of MandarinD11 could have been due to adaptation of the virus in wild birds.

1. Introduction

H5N1 subtype highly pathogenic avian influenza viruses (HPAIVs) have spread to many Asian, European and African countries by natural and artificial means since an outbreak in geese in Guangdong province, China, in 1996 (World organization for animal health, Web portal on Avian Influenza: http://www.oie.int/animal-health-in-the-world/web-portal-on-avian-influenza/; Xu et al., 1999). The spread of HPAI infection can be attributed to several factors such as increased infection efficiency of the virus in several hosts through genetic reassortment, movement of poultry and poultry products, and migration of wild birds (Neumann et al., 2010; Vijaykrishna et al., 2008). The spread of HPAI in poultry has caused economic losses in affected countries and increased the threat of the emergence of a pandemic virus (Coker et al., 2011; Pongcharoensuk et al., 2011).

An FAO-OIE-WHO collaboration has been working towards reducing the risks associated with zoonotic infections of influenza viruses through animal, food and human sectors in preparation for a pandemic (The FAO-OIE-WHO Collaboration, http://www.oie.int/fileadmin/Home/eng/Current_Scientific_Issues/docs/pdf/FINAL_CONCEPT_NOTE_Hanoi.pdf#). The collaboration collects and updates information on HPAIVs and viruses are

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classified into twelve clades based on phylogeny of their HA genes (WHO, 2011b). These clades are further classified into several small subclades because HPAIVs have evolved through maintenance in the poultry and wild bird populations, in part, and possibly due to vaccination of domestic birds in particular regions (Cattoli et al., 2011; Li et al., 2010; WHO/OIE/FAO H5N1 Evolution Working Group, 2012).

A virus of clade 2.2 caused mortalities in wild birds at Qinghai Lake in May 2005 and has spread to Europe and Africa (Chen et al., 2005; Liu et al., 2005). Another virus of the same clade was confirmed at Qinghai Lake between 2006 and 2008, but there was no genetic diversity in the isolates at that time. Subsequently, a virus classified as clade 2.3.2 was isolated from dead birds at the lake between May and June of 2009. The emergence of this virus indicated a possible adaptation to wild birds and the virus spread widely, as the clade 2.2 virus had previously (Li et al., 2011). Intensive surveillance of wild birds in Hong Kong has been performed following outbreaks in wild birds and poultry in 2002–03. Surveillance of dead wild birds revealed the two antigenically distinct groups of viruses that belong to either clade 2.3.4, which was established in Asian poultry, or clade 2.3.2, which is a group of viruses that have been circulating in wild birds and poultry since 2008. Clade 2.3.2 viruses have been isolated from poultry and wild birds since 2007 (Smith et al., 2009). A human case in 2010 in Hong Kong was also caused by a clade 2.3.2 virus (WHO, 2011a). HPAIVs of clade 2.3.2 were isolated from wild birds in Mongolia in 2008 and 2009. The HPAIVs in 2009 were distinguished from those in 2008 by the origin of the PA gene segment (Kang et al., 2011).

During the winter of 2010–11 in Japan, there were 24 HPAI outbreaks in chickens, 60 cases in wild birds and 3 cases in domesticated birds (Sakoda et al., 2012). Before these outbreaks in October 2010, an HPAIV belonging to clade 2.3.2.1 was first isolated from wild duck feces in Hokkaido in the northern part of Japan. The incidence of massive viral infection in birds all over Japan suggested that waterfowl, a natural reservoir of type A influenza virus, could play a role as virus disseminators after maintaining HPAIVs during nesting (Kajihara et al., 2011).

The virulence of an HPAIV derived from wild birds decreased in chickens and the mortality of chickens experimentally infected with HPAIV isolate from tree sparrows in Thailand in 2005 was 70% (Hayashi et al., 2011). These results suggest that the maintenance of HPAIVs within wild birds could affect the pathogenicity of a virus in chickens. In this study, characteristics such as genetics, antigenicity, infectivity and transmissibility to chickens of HPAIVs isolated from outbreaks in either wild birds or chickens between 2010 and 2011 in Japan were examined to understand the possible implications of their maintenance in wild birds. When the infectivity and transmissibility of these HPAIVs were compared in chickens, it appeared that a wild bird isolate used in this study had become attenuated. This study will contribute to the available information on HPAI infection and transmission from wild birds to domestic chickens.

2. Materials and methods

2.1. Virus isolation and characterization

Tracheal swabs, cloacal swabs or internal organ homogenates from chickens suspected of HPAIV infection or dead chickens, domesticated or wild birds were used for virus isolation. Specimens were inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs and incubated at 37 °C for about 30 h at each municipal animal health laboratory. After the allantoic fluid was collected, the specimens were sent to the National Institute of Animal Health for subtype identification by the hemagglutination inhibition (HI) test and the neuraminidase inhibition (NI) test with a panel of antisera (Table 1).

Two chicken viruses, A/chicken/Shimane/1/2010 (Ck10), an index strain from the 2010–11 poultry outbreaks and A/chicken/Miyazaki/S4/2011 (CkS411) and a wild bird strain, A/mandarin duck/Miyazaki/22M-765/2011 (MandarinD11) were used in animal experiments.

2.2. Genetic and phylogenetic characterization of HPAIVs from 2010–11

The DNA sequences of the HA genes of A/chicken/Miyazaki/M6/2011, A/chicken/Aichi/T1/2011 and A/chicken/Mie/1/2011 have been published previously (GenBank: AB675739–AB675741) (Sakoda et al., 2012). In this study, the HA gene of the remaining 24 strains (21 chicken, 2 domesticated bird and 1 wild bird), the NA gene of all strains (24 chicken, 2 domesticated and 1 wild bird) and the internal genes of 22 strains (19 chicken, 2 domesticated and 1 wild bird) were sequenced (GenBank: AB684081–AB684263) as described previously (Uchida et al., 2008). Phylogenetic analysis of the amino acid sequences obtained in this study was carried out with BioEdit software (Hall, 1999) and MEGA 4.0 (Tamura et al., 2007) using the neighbor-joining method. Further phylogenetic analysis with other algorithms, such as minimum evolution method, unweighted pair-group method with an arithmetic mean and the maximum parsimony method were performed on the PA gene segment. Bootstrap value was calculated at 1000 replicates.

Putative amino acid substitutions found in Ck10, CkS411 and MandarinD11 relative to A/common magpie/HK/5052/2007 (CmHK07) were illustrated on a three-dimensional structure of the HA protein (Protein Data Bank ID: 2IBX) by PyMOLver. 0.99 (PyMOL, http://pymol.sourceforge.net/).

2.3. Antigenic analysis

The antigenic characteristics of viruses isolated in 2010–11 in Japan were analyzed by the HI test described in the WHO manual on animal influenza diagnosis and surveillance using antiserum against HPAIVs. Antisera against Ck10, A/whooper swan/Akita/1/2008 (WsAkita08) and A/chicken/Yamaguchi/7/2004 (Ck04) were produced by immunizing chickens with formalin-inactivated virus, and ferret antiserum against CmHK07 was kindly provided by Dr. R. Webby, St. Jude Children's Research Hospital, Memphis, TN, USA.

2.4. Animal experiments

Four-week-old specific pathogen-free (SPF) white leghorn chickens, L-M-6 strain, were obtained from Nisseiken Co., Ltd. (Tokyo, Japan). All animal experiments were carried out in biosafety level 3 facilities at the National Institute of Animal Health, Japan and were approved by the Ethics Committee of the institute.

To investigate the infectivity and pathogenicity of each virus, 10^6, 10^4 or 10^2 EID_{50}/100 μl of each virus diluted in PBS were inoculated intranasally into five chickens. Animals were observed for 10 days post inoculation and survival rate and period of infection were recorded for the survival analysis. Tracheal and cloacal swabs taken at 3, 5, 7 and 10 days post inoculation (DPI) or at death were collected and dipped into MEM containing 0.5% BSA, 25 μg/ml of Fungizone, 1000 units/ml of penicillin and 1000 μg/ml of streptomycin, 0.01 M HEPES and 8.8 mg/ml of $NaHCO_3$. Swabs were removed from the MEM and stored at −80 °C until titration. Frozen samples were thawed and centrifuged at 3000 rpm for 5 min

Table 1
List of H5N1 subtype HPAIVs isolated from poultry, domesticated bird and wild bird in Japan during November 2010 to March 2011.

Strain name	Place of isolation	Virus isolated host	Date of specimen collection (mm/dd/yy)
Ck/Shimane/1/2010	Shimane	Layer	11/29/2010
Mute swan/Toyama/1/2010	Toyama	Domesticated bird	12/16/2010
Ck/Miyazaki/M6/2011	Miyazaki	Breeding chicken	1/21/2011
Ck/Miyazaki/S4/2011	Miyazaki	Layer	1/23/2011
Ck/Kagoshima/I-2/2011	Kagoshima	Layer	1/25/2011
Ck/Aichi/T1/11	Aichi	Layer	1/26/2011
Ck/Miyazaki/T10/2011	Miyazaki	Broilar	1/27/2011
Ck/Miyazaki/K3/2011	Miyazaki	Broilar	1/28/2011
Ck/Miyazaki/N7/2011	Miyazaki	Broilar	1/28/2011
Ck/Miyazaki/TA3/2011	Miyazaki	Broilar	1/30/2011
Ck/Miyazaki/MT2/2011	Miyazaki	Broilar	2/1/2011
Ck/Oita/1/2011	Oita	Layer	2/2/2011
Ck/Miyazaki/8/2011	Miyazaki	Broilar	2/4/2011
Ck/Miyazaki/9/2011	Miyazaki	Broilar	2/5/2011
Ck/Miyazaki/10/2011	Miyazaki	Broilar	2/5/2011
Ck/Miyazaki/11/2011	Miyazaki	Broilar	2/6/2011
Black swan/Yamaguchi/1/2011	Yamaguchi	Domesticated bird	2/9/2011
Ck/Aichi/2/2011	Aichi	Broilar	2/14/2011
Ck/Wakayama/1/2011	Wakayama	Layer	2/15/2011
Ck/Mie/1/2011	Mie	Broilar	2/15/2011
Ck/Miyazaki/12/2011	Miyazaki	Broilar	2/16/2011[a]
Mandarin duck/Miyazaki/22M-765/2011	Miyazaki	Wild bird	2/17/2011
Ck/Mie/2/2011	Mie	Layer	2/26/2011
Ck/Nara/1/2011	Nara	Layer	2/28/2011
Ck/Miyazaki/13/2011	Miyazaki	Broilar	3/5/2011
Ck/Chiba/1/2011	Chiba	Layer	3/12/2011
Ck/Chiba/2/2011	Chiba	Broilar	3/14/2011

[a] Date of virus isolation (mm/dd/yy).

at 4 °C. The supernatant was subjected to viral titration by EID_{50}. To confirm viral infection in the surviving chickens, blood samples taken at the end of the observation period were used to detect antibodies against the influenza A virus by FLOCKTYPE recAIV Screening ELISA (VERITAS, Tokyo, Japan).

Viral transmission was assessed by intranasal inoculation of one chicken with 10^6 EID_{50}/100 μl of each virus housed together with four naïve chickens in the same isolator. The observation period was 10 days from the date of the first inoculation and feed and water were shared between the inoculated and naïve chickens. The survival period and rate were recorded to draw a survival curve. Tracheal and cloacal swabs were collected and virus titers were determined as described above.

2.5. Survival analysis

Survival analysis was carried out using the results of survivability in the 10^6, 10^4 and 10^2 EID_{50} inoculation groups and the naïve chickens in the transmission test. A Kaplan–Meier survival curve (Kaplan and Meier, 1958) was constructed using the survival rate and infection period for chickens in each titer group. Differences in the Kaplan–Meier survival curves were analyzed by log-rank test under Bonferroni correction.

3. Results

3.1. Genetic characterization of the Japanese isolates

Twenty four HPAIVs isolated in Japan from chickens from individual outbreaks during 2010–11 and viruses from domesticated birds (2 strains) and a dead wild bird (1 strain) were sequenced and the HA and NA gene segments were phylogenetically analyzed. The deduced amino acid sequence at the HA cleavage site was PQRERRRKR for all isolates except A/chicken/Miyazaki/T10/2011, for which the sequence was PQREKRRKR. All of the Japanese isolates from this period formed a cluster (Fig. 1A cluster A) within clade 2.3.2.1 (Sakoda et al., 2012) at 87% bootstrap values in the HA gene phylogenetic tree and were clearly distinguishable from clade 2.3.2.1 strains isolated in Japan during 2008 from wild birds (Fig. 1A cluster B). Cluster A also included a Mongolian virus isolated in 2010 (A/whooper swan/Mongolia/21/2010) and wild bird isolates from both Japan and South Korea isolated during 2010–11. An isolate, A/hooded crane/Kagoshima/4612J008/2010, from an outbreak in cranes which occurred within 5 km of the farm where A/chicken/Kagoshima/I-2/2011 was isolated, formed a branch with it at 85% bootstrap values. A similar topology was observed for the NA and internal genes except the PA gene of cluster A strains.

The genetic origin of the cluster A strain PA genes was scrutinized by phylogenetic analysis (Fig. 1B). The direct ancestors of the PA gene segment of cluster A strains were found in wild bird strains of several subtypes isolated between 2007 and 2009. H3N8, H4N6, H4N9, H5N2, H5N5, H6N2, H6N8, H10N8 and H11N2 avian influenza viruses formed a cluster in the PA tree as the genetic origin of the PA gene of cluster A strains. These avian influenza viruses and the HPAIVs of clade 2.3.2.1 isolated in 2010–11 shared a common ancestor to HPAIVs of clades 2.5, 7 and 9.

3.2. Antigenic analysis of the Japanese isolates

Inter-clade and intra-clade antigenic variation was demonstrated by HI tests using Ck10, A/chicken/Miyazaki/11/2011 (Ck1111), CmHK07, WsAkita08 isolated in 2008 and Ck04 (clade 5) as antigens (Table 2). Although anti-Ck10 serum reacted well with Ck10, Ck1111 and WsAkita08, the reaction with CmHK07 was 16 fold lower compared to Ck10, although both viruses belong to clade 2.3.2.1. In addition, anti-CmHK07 showed a titer that was 8 fold lower than Ck10 compared to CmHK07. One of the pandemic vaccine candidates, SJRG-166615, from clade 2.3.2.1 was derived from CmHK07 and the HI data indicates that the 2010–2011 Japanese HPAIVs of clade 2.3.2.1 were antigenically distinguishable from this.

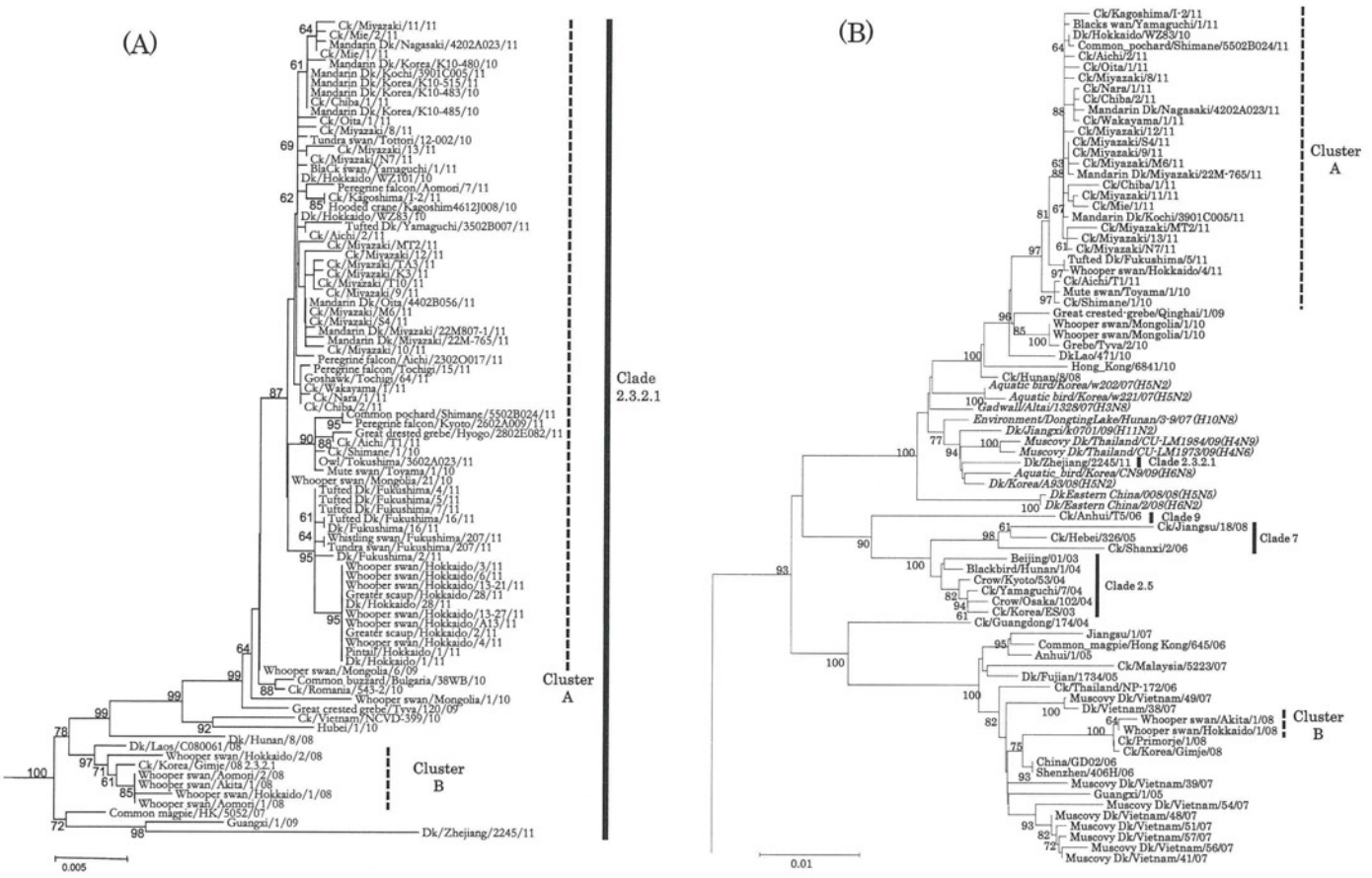

Fig. 1. Phylogenetic tree of HA and PA gene. (A) *Phylogenetic tree of the HA gene*: Phylogenetic tree of the HA gene of the 2010–11 Japanese isolates was constructed by the neighbor-joining method with the sequences published in GenBank. The nucleotide length of the HA gene used for the analysis was 952 base pairs. The strain used to root this tree was A/goose/Guangdong/1/1996. Bootstrap values of more than 60 are shown at each branch. The length of branches represents the distance between sequences and the bar and numbers at the bottom of the tree indicate the number of substitutions. Isolated host and year in the strain names were abbreviated as follows: chicken; Ck, duck; Dk, quail; Qa, 2011; 11. (B) *Phylogenetic tree of PA gene*: Phylogenetic tree of PA gene was constructed by the neighbor-joining methods. PA gene sequences of the H5N1 subtype HPAIVs and other subtype strains published in GenBank are shown as a representative of phylogenetic analysis of the PA gene. Topology of the tree was similar when other calculating methods, such as the minimum evolution method, the unweighted pair-group method with arithmetic mean and the maximum parsimony method, were applied. The nucleotide length of the PA gene used for the analysis was 1975 base pairs. Root strain, drawn bootstrap values and host and year of abbreviation of the isolated strain in the tree were the same as the HA tree. Viruses written in italics mean other subtypes of avian influenza viruses except H5N1 subtype HPAIVs.

3.3. Comparison of infectivity and pathogenicity of isolates derived from chickens and a wild bird

CkS411, a chicken isolate from 2010/11 outbreaks, and a wild bird isolate, MandarinD11, differed in their lethality to chickens when five chickens of each group were inoculated with 10^6 EID_{50} viruses (Fig. 2A). Survival analysis showed a significant difference between the viruses. The mean death time (MDT) was 51.6 hpi for CkS411 and 75.6 hpi for MandarinD11 infected chickens, although all of the infected chickens eventually died. All of the chickens inoculated with 10^4 EID_{50} of Ck10 or MandarinD11 died (Fig. 2B), while 60% of those infected with CkS411 survived and were negative for anti-influenza A virus antibodies by ELISA (data not shown). The MDTs of this infectious dose of CkS411, Ck10 and MandarinD11 were 170.4, 144 and 148.8 hpi, respectively. These values were more than twice the MDTs obtained with 10^6 EID_{50} of each virus. All five chickens infected with 10^2 EID_{50}/100 μl of Ck10 or MandarinD11 virus survived (data not shown). One of five chickens infected with 10^2 EID_{50}/100 μl CkS411 virus died, although no virus was detected in the tracheal or cloacal swabs collected at the time of death. Since the cause of death did not appear to be due to the viral infection, the data for this chicken was excluded from

Table 2
Antigenic characterization of viruses isolated in Japan in 2010–11 by the HI test using hyper immune antisera.

	Hyper immune antisera			
	Chicken		Ferret	Chicken
	Clade 2.3.2.1 Ck/Shimane/1/2010	Clade 2.3.2.1 Ws/Akita/1/2008	Clade 2.3.2.1 Common magpie/HK/5052/2007	Clade 2.5 Ck/Yamaguchi/7/2004
A/chicken/Shimane/1/2010	**1280**	640	20	160
A/chicken/Miyazaki/11/2011	640	640	20	160
A/whooper swan/Akita/1/2008	640	**1280**	40	160
A/common magpie/HK/5052/2007	80	2560	**160**	160
A/chicken/Yamaguchi/7/2004	160	1280	20	**1280**

Boldface shows HI titer used with homogenous antigen and antisera.

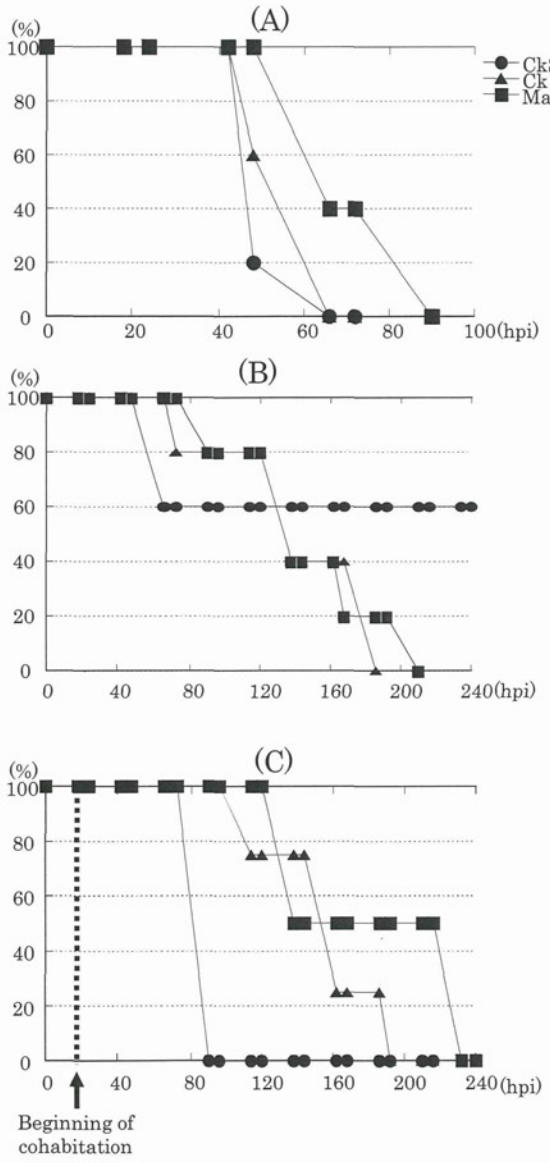

Fig. 2. *The survival curves of the 10^6 (A) and 10^4 (B) viral infection and transmission test (C).* In each figure, circle (●), triangle (▲) and square (■) represent a data set obtained following infection with CkS411, Ck10 and MandarinD11, respectively. The *x*-axis is hours post inoculation (hpi) and the *y*-axis is the survival rate (%). (C) The survival curves of the chickens cohabiting with the chicken infected with either CkS411 (●), Ck10 (▲) or MandarinD11 (■), respectively, are shown by broken lines. Cohabitation with the infected chicken started at 18 hpi.

all of the analyses in this study. There were no significant differences in the survival rates observed for either 10^4 or 10^2 EID_{50} infectious doses. Fifty percent chicken lethal doses (CLD_{50}/ml) were calculated as $10^{4.3}$ EID_{50} for CkS411 and 10^3 EID_{50} for the others.

Viral titers in the tracheal and cloacal swabs from chickens infected with 10^6 EID_{50} of MandarinD11 were lower than in chickens infected with chicken isolates. Significant differences were observed in the viral titers in the tracheal swabs from chickens infected with 10^6 EID_{50} Ck10 compared to MandarinD11 ($p = 0.0098$) (Fig. 3A). Viral load in cloacal swabs from birds infected with CkS411 and Ck10 was significantly higher ($p = 0.0013$ and 0.019) than in MandarinD11 swabs (Fig. 3B). Viral titers in chickens infected with CkS411 and Ck10 reached $>10^{5.1}$ EID_{50}/ml in the tracheal swabs and $10^{3.1}$ EID_{50}/ml in the cloacal swabs shortly before death. The maximum viral titers of chickens infected with MandarinD11 were $10^{4.05}$ EID_{50}/ml and $10^{1.13}$ EID_{50}/ml in the trachea and cloaca, respectively.

There was no difference in the maximum viral titers in the tracheal or cloacal swabs collected on 3, 5, 7 and 10 dpi or at death in the chickens infected with 10^4 EID_{50}. Viral excretion into the trachea and cloaca occurred later in these groups compared to that observed in the 10^6 EID_{50} inoculation groups (Fig. 3C and D).

No virus was isolated from tracheal or cloacal swabs from any of the chickens inoculated with 10^2 of any of the three viruses during the observation period. Anti-influenza A virus antibody was not detected by ELISA in any of the surviving chickens indicating that they were not infected with the influenza virus (data not shown).

3.4. Transmission of virus isolates to chickens

The transmission study demonstrated that CkS411 was more readily transmitted to uninfected chickens compared to the other two isolates. Chickens infected with 10^6 EID_{50} of CkS411 or Ck10 died at 48 hpi whilst the chicken infected with the same dose of MandarinD11 died at 66 hpi. The MDT of the naïve chickens was 72 h after cohabitation (hac) for CkS411, 139.5 hac for Ck10 and 168 hac for MandarinD11 (Fig. 2C). Survival analysis of the groups of cohabiting chickens revealed significant differences between CkS411 compared to Ck10 and MandarinD11 groups. CkS411 replicated well in both inoculated and naïve chickens and was transmitted with greater efficiency (Fig. 4A and B). In the inoculated chicken in the CkS411 group, tracheal and cloacal titers were $10^{6.53}$ and $10^{5.02}$ EID_{50}/ml, and mean max titers of the cohabiting chickens were $10^{6.24}$ and $10^{4.73}$ EID_{50}/ml, respectively. These values in the MandarinD11 infected chickens were low. The efficiency of viral transmission is correlated with the titer of virus excreted from an inoculated chicken. The mean time of the first viral isolation in the trachea or cloaca of cohabiting chickens was 48, 108 and 108 hac in CkS411, Ck10 and MandarinD11 groups, respectively. The time of viral isolation in CkS411 group was significantly shorter than that of Ck10 and MandarinD11.

3.5. Comparison of amino acid substitutions between the isolates

Animal experiments indicated that the replication and transmission characteristics of Ck10 and MandarinD11 viruses were different. Deduced amino acid sequences were compared to explore whether any signature substitutions relating to such phenotypical differences could be found. Only three amino acid substitutions in the HA amino acid sequences were identified between the three viruses. When compared with the sequence of MandarinD11, one substitution at 83 aa (87 aa in H3 numbering) in CkS411 and two substitutions at 84 and 259 aa (88 and 263 aa in H3 numbering) in Ck10 were found. As a result, the amino acid sequences in Ck10 tended to be different from those of the other two viruses. Several amino acid substitutions that were seen in other segments of the viruses did not coincide with amino acids changes that have previously been reported to correlate with pathogenicity (Table 3). Amino acid substitutions in the HA gene of the three viruses compared to CmHK07 are shown in the HA three-dimensional structures (Fig. 5). Five amino acid substitutions from CmHK07were commonly found among the three viruses at antigenic sites A, B, D and E. They were at 66 aa (75 aa in H3 numbering) for site E, 133 and 136 aa (137 and 140 aa in H3 numbering) for site A, 154 aa (158 aa in H3 numbering) for site B and 240 aa (244 aa in H3 numbering) for site D. These substitutions appeared to correlate with antigenic differences between Ck10 and CmHK07. No amino acid substitutions were found within the antigenic sites of CkS411, Ck10 or MandarinD11.

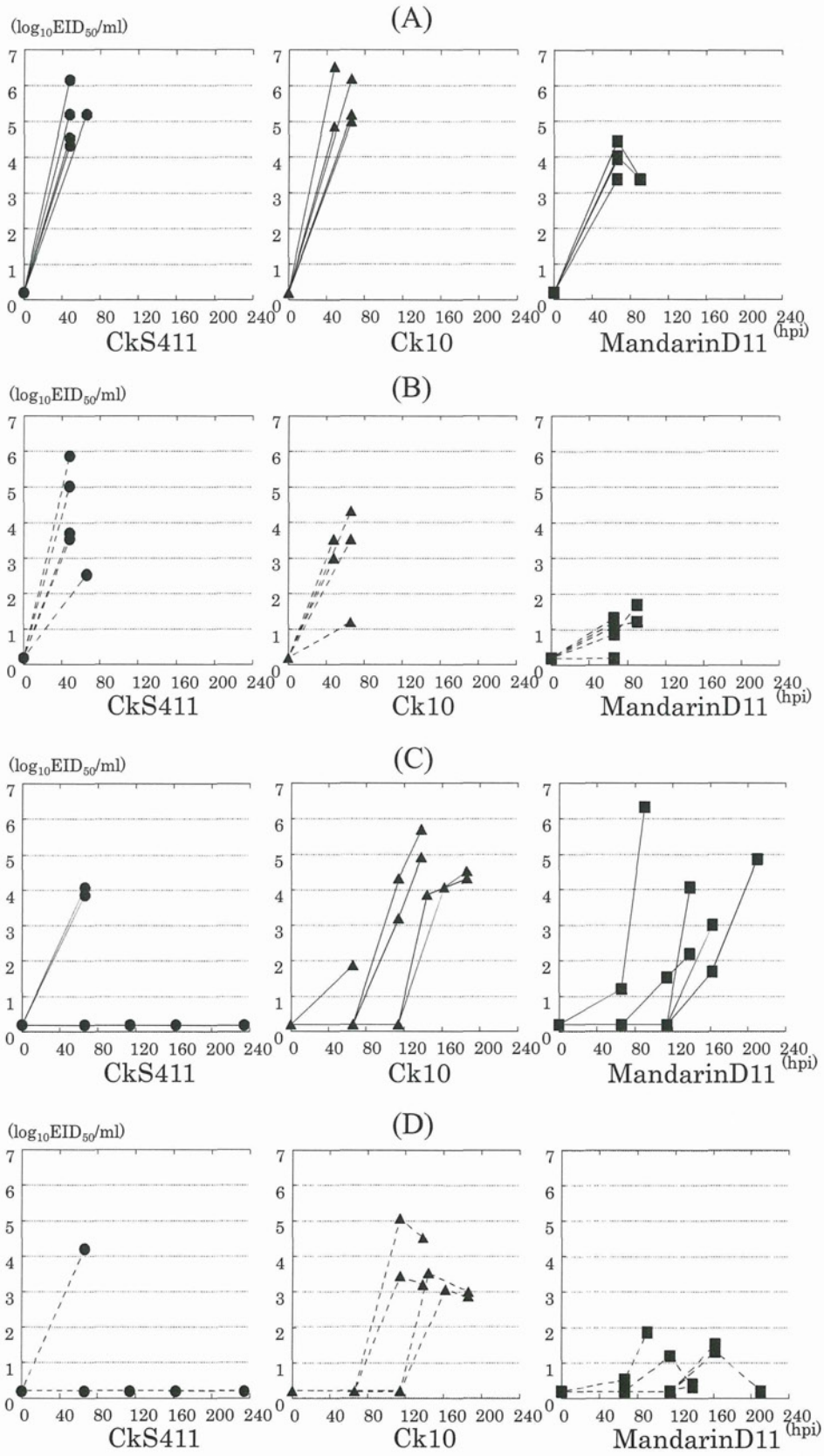

Fig. 3. *The kinetics of viral multiplication in the trachea (A, C) and cloaca (B, D) of infected chickens.* Circle (●), triangle (▲) and square (■) represent a data set obtained during the animal experiment with CkS411, Ck10 and MandarinD11, respectively. The solid or broken lines indicate the results in the trachea or cloaca. The *x*-axis is hours post inoculation (hpi) and the *y*-axis is 50% egg infective dose (log_{10} EID_{50}/ml). Data obtained following infection with 10^6 EID_{50} (A, B) and 10^4 EID_{50} (C, D) are shown.

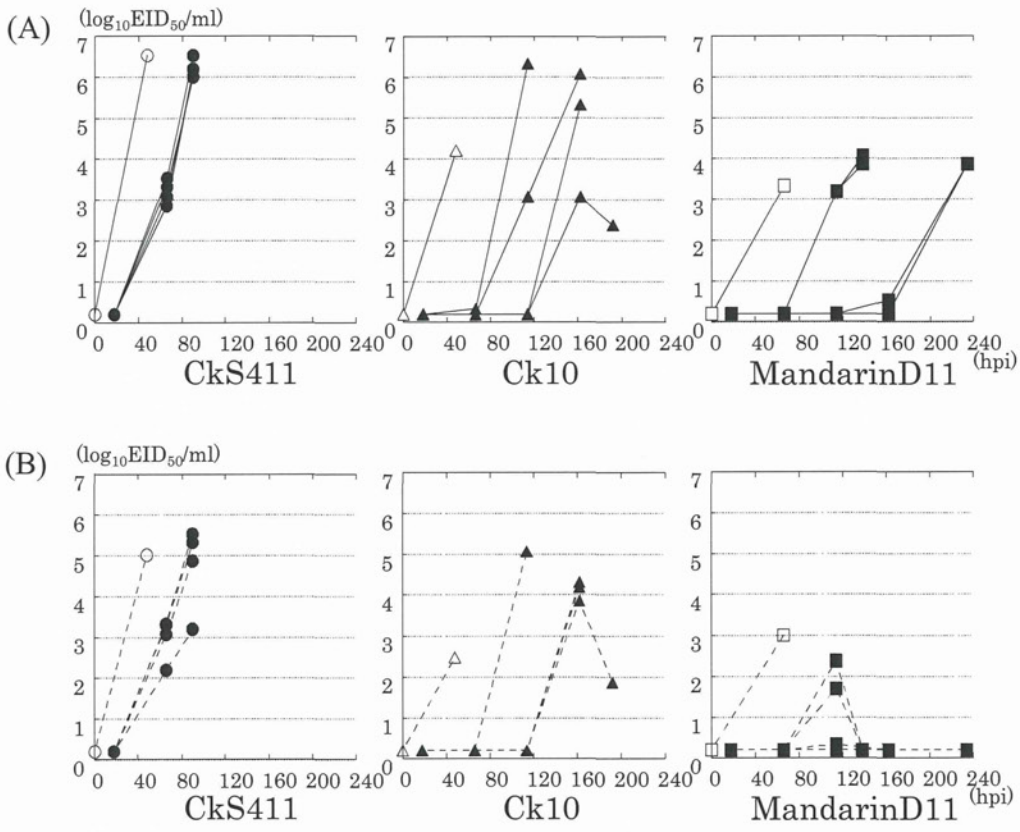

Fig. 4. *The kinetics of viral multiplication in the trachea (A) and cloaca (B) of chickens in the transmission test.* Circle (●), triangle (▲) and square (■) represent a data set obtained in the animal experiments with CkS411, Ck10 and MandarinD11, respectively. The white and black shapes indicate the results of the inoculated and cohabiting chickens, respectively. The *x*-axis is hours post inoculation (hpi) and the *y*-axis is 50% egg infective dose ($\log_{10}$ EID_{50}/ml).

Table 3
Comparison of deduced amino acid sequences among three viruses used for the animal experiment.

	PB2	PB1				PB1-F2	PA					HA[b]			NA							NS1		NS2
	491	175	566	621	746	88	344	347	400	552	578	83	84	259	48	58	60	175	189	346	414	3	79	3
MandarinD11	T	E	T	Q	T	W	K	D	P	A	G	T	K	K	P	N	V	V	K	S	G	S	M	S
CkS411	T	E	T	R	T	W	E	D	P	A	G	A	K	K	P	N	A	V	K	S	G	S	L	S
Ck10	I	D	A	Q	I	[a]	E	N	S	T	S	A	N	R	S	S	V	I	N	N	S	L	M	L

[a] There is a termination codon at 88 aa in PB1-F2 of Ck10, and it is shorter than others.
[b] Numbering starts from the beginning of HA1.

4. Discussion

The PA gene of recent Japanese HPAI isolates form a phylogenetic cluster (A) which also includes avian influenza viruses with low pathogenicity of various subtypes other than H5N1, isolated from wild birds as well as Ck/Hunan/8/2008 (H5N1). The non-H5N1 isolates in this cluster originated from wild birds, poultry and the environment in Russia, Korea, Thailand and China. There are several possible explanations for the gene constellation of cluster A viruses which has occurred. Firstly, cluster A viruses could have arisen from the reassortment between cluster B viruses and wild bird strains with PA gene segments originating from clades 2.5, 7 and 9. A descendant virus from a common ancestor of clades 2.5, 7 and 9 may have entered the wild bird population prior to 2007 and reassortment events may have occurred in the wild birds to generate viruses such as Gadwall/Altai/1328/2007 (H3N8) and Aquatic bird/Korea/w202/2007 (H5N2). Then, after the introduction of an HPAIV related to cluster B viruses into the wild bird population prior to 2008, a wild bird strain with a PA gene derived from the HPAIV reassorted with it to generate an ancestral strain of cluster A viruses. Another possibility is that cluster A viruses could have arisen due to reassortment in poultry between a virus related to cluster B and a descendant HPAIV from a common ancestor of clades 2.5, 7 and 9. The non-H5N1 isolates with PA gene segments originating from clades 2.5, 7 and 9 in the cluster resulted from reassortment between cluster A viruses and low pathogenic avian influenza viruses. However, the host in which the reassortant viruses were generated remains unknown. Which of these pathways actually caused the generation of cluster A viruses remains to be solved. However, there is little doubt that genetic material from HPAIVs of H5N1 subtype have mixed with those of avian influenza viruses in wild birds.

Using molecular epidemiology, Uchida et al., demonstrated that Thai HPAIVs isolated from wild birds could have been maintained in the wild bird population for a certain period of time (Uchida et al., 2008). It is hypothesized that the viruses were brought to Japan after being perpetuated in the wild bird population during the summer nesting season. MandarinD11 replicated exclusively in wild birds for a time while CkS411entered and replicated in chickens before the outbreak was recognized. Passage in wild birds

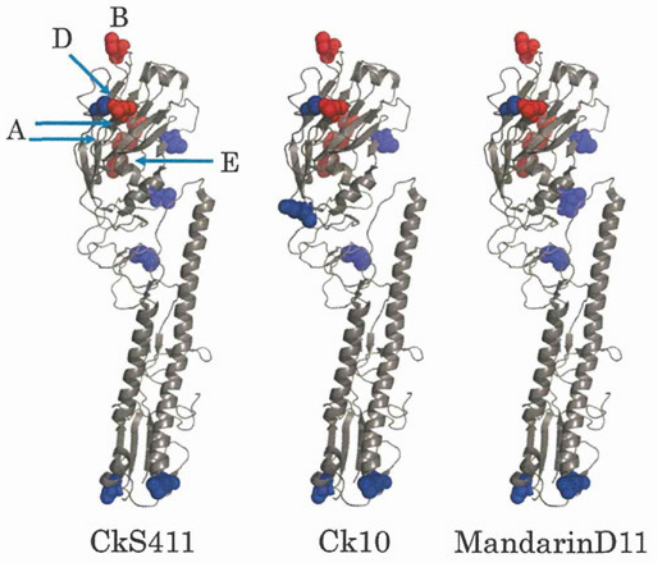

Fig. 5. Illustration of putative amino acid substitutions found in the HA of Ck10, CkS411 and MandarinD11 compared to CmHK07. Substitutions located within the predicted antigenic sites are shown in red, whereas those found elsewhere are colored in blue.

might have resulted in attenuation of MandarinD11 in chickens. An HPAIV isolated from a tree sparrow in Thailand in 2005 (TsThai) killed 70% of chickens infected with 10^6 EID_{50} and the MDT of chickens infected with it was longer than that of chickens infected with a pigeon isolate (Hayashi et al., 2011). It could be considered that passage of HPAIV in different bird species resulted in attenuated virus pathogenicity in chickens. However, in the same study, a pigeon isolate did not appear to be attenuated in chickens, suggesting that passage in wild birds does not necessarily lead to attenuation in chickens. Although we were unable to determine the amino acid substitution(s) responsible for attenuation of MandarinD11 in chickens, there were only four amino acid differences between MandarinD11 and CkS411. The generation of mutants of CkS411 with such substitutions and examination of their pathogenicity in chickens would elucidate the possible changes that may lead to attenuation of HPAIV during passage in wild birds.

It was enigmatic to note that CLD_{50} of CkS411 was higher than those of Ck10 and MandarinD11, although it appeared to be the most pathogenic isolate in chickens in terms of MDT, virus shedding and transmission. CLD_{50} might not be a factor that determines pathogenicity *in vivo* when sufficient amounts of virus are given in an experimental setting. Hayashi et al. (2011) demonstrated that when two HPAIVs with differing LD_{50} in mice, 0.8 log EID_{50} and 2.2 log EID_{50}, respectively, were inoculated at the same dose of 5 log EID_{50} into mice, lethality and death time did not significantly differ. In another study (Spekreijse et al., 2011), it was demonstrated that when a chicken was given $\geq$1 CLD_{50}, the rate of successful transmission did not differ significantly between 3.0 and 4.0 log EID_{50}. The reason for the dissociation between CLD_{50} and other pathogenic parameters remains to be determined.

In the virus transmission study, CkS411 was found to have the highest transmission speed followed by Ck10 and MandarinD11, determined by the timing of virus isolation from cohabiting chickens. It appeared that greater levels of excreted virus from infected chickens correlated with a higher transmission speed. Suzuki et al., reported that transmissibility of a virus to chickens correlated with the viral titer in oropharyngeal and cloacal swabs and the time taken for the virus to replicate to titers corresponding to the 50% chicken infectious dose (Suzuki et al., 2010). Infection of chickens with 10^6 EID_{50} MandarinD11 resulted in significantly lower viral titers from the tracheal and cloacal swabs compared to those of CkS411, coinciding with the slower transmission speed of this strain observed in the transmission study. When the kinetics of viral replication in chickens cohabiting with the chicken inoculated with MandarinD11 were looked at more closely, it was possible to identify two phases of transmission. Viral replication was detected in two chickens in both tracheal and cloacal swabs at 120 hpi, whereas the rest did not show evidence of viral replication in tracheal swabs until 160 hpi or later and not at all in cloacal swabs. It could be that the first two cohabiting chickens were infected from the inoculated chicken and that the virus was subsequently transmitted from these two, not from the inoculated chicken. If this is the case, lower virus excretion could affect the mode of transmission of the virus, although it is possible that lower exposure to the virus resulted in slower replication in affected chickens.

A wild bird isolate in this study, MandarinD11, appeared to become attenuated in chickens which could have been caused by passage and adaptation to wild birds. Under natural conditions, when a wild bird adapted virus first enters a farm, it is likely that a longer time lag would exist before the virus invasion could be detected. Early virus detection is an essential factor for successful eradication programs. It is therefore important to elucidate the molecular basis of the attenuation of virus in chickens following passage in wild birds.

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