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Co-incorporation of the PB2 and PA polymerase subunits from human H3N2 influenza virus is a critical determinant of the replication of reassortant ribonucleoprotein complexes

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The influenza virus RNA polymerase, composed of the PB1, PB2 and PA subunits, has a potential role in influencing genetic reassortment. Recent studies on the reassortment of human H3N2 strains suggest that the co-incorporation of PB2 and PA from the same H3N2 strain appears to be important for efficient virus replication; however, the underlying mechanism remains unclear. Here, we reconstituted reassortant ribonucleoprotein (RNP) complexes and demonstrated that the RNP activity was severely impaired when the PA subunit of H3N2 strain A/NT/60/1968 (NT PA) was introduced into H1N1 or H5N1 polymerase. The NT PA did not affect the correct assembly of the polymerase trimeric complex, but it significantly reduced replication-initiation activity when provided with a vRNA promoter and severely impaired the accumulation of RNP, which led to the loss of RNP activity. Mutational analysis demonstrated that PA residues 184N and 383N were the major determinants of the inhibitory effect of NT PA and 184N/383N sequences were unique to human H3N2 strains. Significantly, NT PB2 specifically relieved the inhibitory effect of NT PA, and the PB2 residue 627K played a key role. Our results suggest that PB2 from the same H3N2 strain might be required for overcoming the inhibitory effect of H3N2 PA in the genetic reassortment of influenza virus.

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INTRODUCTION

The genome of influenza A virus is composed of eight segments of negative-sense, ssRNA (Palese & Shaw, 2007). This segmented genome structure facilitates genetic reassortment with other influenza strains that co-infect the same cells, and has played a pivotal role in the emergence of influenza pandemics. The pandemic strains of the past century have incorporated the avian genes expressing the superficial haemagglutinin and neuraminidase (NA) glycoproteins, giving rise to new subtypes with novel surface antigens. Besides the introduction of genes for superficial glycoproteins, at least one internal gene of RNA polymerase from the avian strains has been concurrently incorporated into human strains [Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, 2009; Itoh *et al.*, 2009; Kawaoka *et al.*, 1989]. Recent studies have suggested that the RNA polymerase has a potential role in the generation of reassortant viruses. The introduction of avian PB2 into human strains has often severely reduced polymerase activity and virus replication (Chen *et al.*, 2008; Li *et al.*, 2010), suggesting that the avian PB2 may interfere with human polymerase during genetic reassortment (Nakazono *et al.*, 2012).

Co-incorporation of PB2 and PA from the same virus of origin appears to be important for efficient virus replication (de Wit *et al.*, 2010; Labadie *et al.*, 2007; Treanor *et al.*, 1994), particularly in the reassortment between human H3N2 strains and other influenza strains (Chen *et al.*, 2008; Li *et al.*, 2010). Interestingly, the 1968 H3N2 (Hong Kong flu) strain simultaneously incorporated PB2 and PA genes from the 1957 H2N2 (Asian flu) strain, which was originally derived from the 1918 H1N1 strain (Spanish flu) (Fig. 1a) (Wright *et al.*, 2007). Similarly, natural-reassortant H3N2 viruses isolated from swine possess PB2 and PA genes from the same virus of origin (Fan *et al.*, 2012; Olsen, 2002). Simultaneous incorporation of PB2 and PA was also found in the 2009 H1N1 strain [Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, 2009; Itoh *et al.*, 2009; Peiris *et al.*, 2009]. These observations imply the importance of functional cooperation between PB2 and PA in the generation of viable reassortant viruses. However, the mechanism of this phenomenon remains poorly understood.

In this study, we investigated the molecular basis of the relationship between the PB2 and PA subunits from a human H3N2 strain during genetic reassortment. We

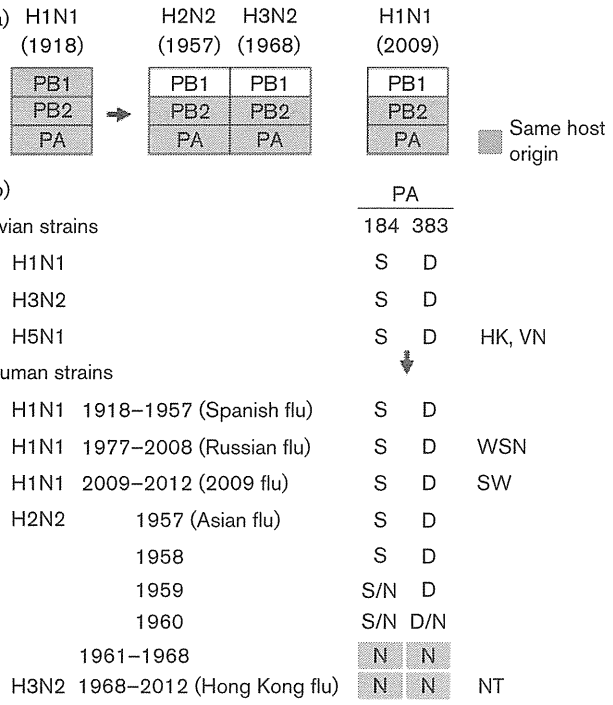

Fig. 1. Specific mutation at positions 184 and 383 in H3N2 PA. (a) Reassortment of the polymerase gene in the past pandemics. The PB2 and PA genes have been concurrently incorporated into the pandemic strains. (b) Amino acid sequence at positions 184 and 383 in PA. The H3N2-specific PA 184N/383N sequence appeared in the 1960s (shaded), which might have played a role in the regulation of reassortment. Influenza strains used in this study are indicated on the right: HK, H5N1 strain A/HongKong/156/1997; VN, H5N1 strain A/Vietnam/1194/2004; WSN, H1N1 strain A/WSN/1933; SW, H1N1 strain A/Kurume/K0910/2009; NT, H3N2 strain A/NT/60/1968.

reassorted the H3N2 polymerase with subunits from other influenza strains and reconstituted the influenza ribonucleoprotein (RNP) in 293T cells. Our data showed that H3N2 PA had a significant inhibitory effect on RNP activity when introduced into the polymerases of other influenza strains. The inhibitory effect was specifically relieved by H3N2 PB2, suggesting that co-incorporation of PB2 and PA subunits from the same H3N2 strain might be a prerequisite for generation of viable reassortant viruses.

RESULTS

H3N2 PA impairs RNP activity when reconstituted with H1N1 or H5N1 polymerase

Previously, we have shown that the PA subunit of H3N2 strain A/NT/60/1968 (NT PA) severely impaired RNP activity when introduced into the human-isolated H5N1 strain A/HongKong/156/1997 (HK) (Fig. 2, lane 20) or the closely related A/Vietnam/1194/2004 (VN) (Nakazono *et al.*, 2012). To generalize this finding to other influenza strains, we introduced NT PA into two human H1N1 strains, A/WSN/1933 (WSN) and A/Kurume/K0910/2009 (SW). The RNP-containing hybrid polymerase was reconstituted in human 293T cells in a background of WSN-derived nucleoprotein (NP) and NA vRNA, and the steady-state levels of NA reporter mRNA, vRNA and cRNA were measured by primer extension. The levels of all three RNA species were reduced significantly, or decreased to essentially inactive levels when NT PA alone was introduced into the WSN or SW polymerase, respectively [20±2.9 % of mRNA in WSN (mean±SD, n=3), 7±2.8 % of mRNA in SW; Fig. 2, lanes 4 and 12]. This result provides further support for the inhibitory effect of NT PA on the reassortment of the polymerase. It is also noteworthy that this impaired RNP activity appears to be restored slightly (24±14.4 % of mRNA in WSN; Fig. 2, compare lane 6 with 4), or significantly (69±23.2 % of mRNA in SW, 118±11.6 % of mRNA in HK; Fig. 2, compare lane 14 with 12, and lane 22 with 20, respectively) when the PB2 was replaced with the NT PB2. This suggests that the co-incorporation of NT PB2 and NT PA is important for the reassortant RNP activity. In addition, the NT PB2 alone appears to enhance the RNP activity when reconstituted with SW (180±26.7 % of mRNA; Fig. 2, compare lane 11 with 9) or HK (148±53.4 % of mRNA; Fig. 2, compare lane 19 with 17) polymerase.

Impaired RNP activity is rescued by mutations at positions 55, 86, 184 and 383 in the NT PA

To identify the amino acid residues of NT PA responsible for the impaired RNP activity, we aligned the PA sequences of four influenza strains and found 15 amino acids that differ only in NT PA (Fig. 3a). We speculated that substitutions of these amino acids in NT PA to the sequences conserved in the other three strains might rescue the impaired RNP activity. In particular, we focused on the N-terminal region of PA (55–404), because several functional domains have been mapped (Dias *et al.*, 2009; Hara *et al.*, 2006; Yuan *et al.*, 2009). We included positions 86, 91, 114, 277 and 388, because these positions are highly conserved in two or three strains. We excluded positions 385 and 268, due to the similar basic (R and K) amino acid or complete loss of RNP activity by mutation, respectively. The single mutations D66G, N142K, F277S and Y321N did not rescue the impaired RNP activity (Fig. 3b, lanes 4–7). However, the single mutation N383D showed a slight increase in the RNP activity (40±5.7 % of mRNA, 13±2.3 % of cRNA and 23±5.6 % of vRNA) when compared with NT PA WT (20±2.9 % of mRNA, 6.7±3.8 % of cRNA and 12±2.3 % of vRNA; Fig. 3b, compare lane 8 with 3). Although the RNP activity was decreased again by the additional mutation E114K (Fig. 3b, compare lane 14 with 12), the RNP activity was gradually increased by the additional mutations N55D (38±7.0 % of mRNA, 7.6±2.0 % of cRNA and 17±8.0 % of vRNA) and M86I (59±4.5 % of mRNA, 24±3.0 % of cRNA and

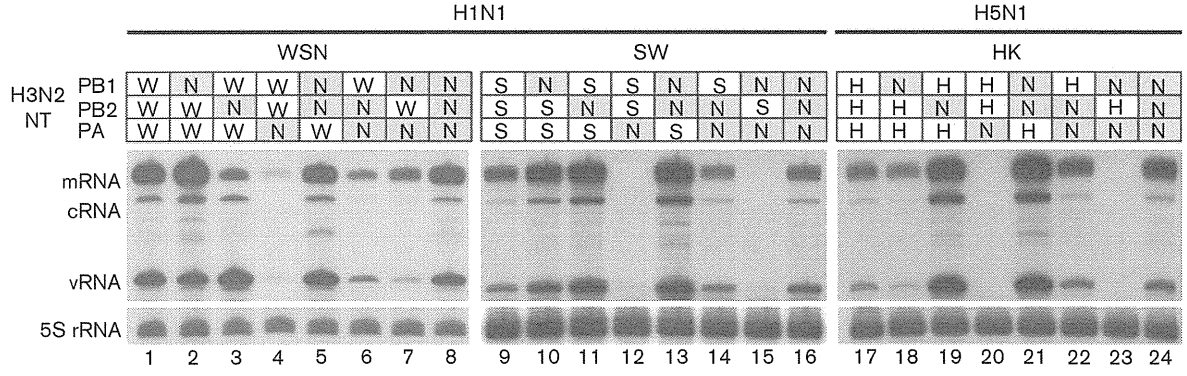

Fig. 2. H3N2 PA impairs RNP activity. Polymerase subunits from A/WSN/1933 (H1N1) (W), A/Kurume/K0910/2009 (H1N1) (S) and A/HongKong/156/1997 (H5N1) (H) were replaced with the corresponding subunits from A/NT/60/1968 (H3N2) (N). The RNP was reconstituted in 293T cells by transfection of plasmids expressing various combinations of polymerase subunits and WSN-derived NP and NA vRNA. The RNP activity was measured by primer extension.

41 ± 10.3 % of vRNA; Fig. 3b, lanes 15 and 16). Mutation V91I did not appear to increase the RNP activity (Fig. 3b, lane 17). The level of mRNA was rescued to approximately 90 ± 7.9 % by the subsequent mutation N184S (sixfold mutant N55D/M86I/V91I/E114K/N184S/N383D), although the level of cRNA (64 ± 9.7 %) and vRNA (70 ± 12.3 %) were still slightly lower than those of WSN PA (Fig. 3b, compare lane 18 with 11). Additional mutation C225S did not increase the RNP activity further (Fig. 3b, compare lane 19 with 18). Overall, we suggest that at least four positions, 55, 86, 184 and 383, in the NT PA are responsible for the impaired RNP activity. This finding was further supported by the observations that the NT PA carrying mutations only at positions 55, 86, 184 and 383 showed a significant RNP activity (87 ± 1.7 % of mRNA, 56 ± 8.2 % of cRNA and 66 ± 3.4 % of vRNA; Fig. 3b, lane 23), comparable to that of sevenfold mutant (N55D/M86I/V91I/E114K/N184S/C225S/N383D) (Fig. 3b, compare lane 23 with 22). In addition, mutation at only two positions, 184 and 383, significantly rescued the impaired RNP activity (50 ± 11.0 % of mRNA, 15 ± 7.3 % of cRNA and 25 ± 5.7 % of vRNA; Fig. 3b, lane 25).

We further asked whether the substitution at four positions, 55, 86, 184 and 383, in WSN PA or HK PA with the NT PA sequence would impair RNP activity. In the case of WSN PA, there was only a slight effect on the RNP activity by a single mutation at positions 86, 184 and 383 (Fig. 3c, compare lanes 3, 4, 5 with 2). However, the RNP activity was severely reduced by a double mutation at positions 184 and 383 (66 ± 7.9 % of mRNA, 32 ± 7.1 % of cRNA and 41 ± 3.0 % of vRNA; Fig. 3c, compare lane 6 with 2), and further decreased by the additional mutation at positions 55 (33 ± 3.2 % of mRNA, 11 ± 1.9 % of cRNA and 13 ± 2.6 % of vRNA; Fig. 3c, compare lane 7 with 6) and 86 (22 ± 5.4 % of mRNA, 5.3 ± 2.1 % of cRNA and 7.8 ± 1.3 % of vRNA; Fig. 3c, compare lane 8 with 7), comparable to that of NT PA (Fig. 3b, lane 3). In the case of HK PA, we did not mutate position 86, because this residue is identical to that of NT PA (Fig. 3a). The RNP activity was markedly reduced to almost inactive levels by double mutation at positions 184 and 383 or by triple mutation at positions 55, 184 and 383 (Fig. 3c, lanes 12 or 13, respectively). Taken together, we suggest that four positions, 55, 86, 184 and 383, in NT PA are involved in the optimal activity of the reassortant polymerase. In particular, two positions, 184 and 383, are the major determinants.

NT PA impairs the replication-initiation activity and accumulation of the RNP

To address the reason for the functional loss of RNP activity by NT PA, we examined: (i) the assembly of the trimeric complex of PB1, PB2 and PA; (ii) the *in vitro* polymerase activities; and (iii) the RNP assembly. We partially purified the reassortant polymerase or RNP using C-terminally tandem-affinity purification (TAP)-tagged PB2. In the NT PA reassortant polymerase, the amount of PB2-TAP and PB1-PA co-purifying with PB2-TAP were comparable to those of the WSN polymerase (Fig. 4a, left panel, compare lane 2 with 1). Similar levels of the three subunits were observed in the NT PA mutant (Fig. 4a, left panel, lane 3) that possessed significant RNP activity (Fig. 3b, lane 18). This indicates that the NT PA does not appear to impair the correct assembly of the trimeric complex. Interestingly, the NT PA WT migrated faster than WSN PA (Fig. 4a, left panel, compare lane 2 with 1), as observed before (Kashiwagi *et al.*, 2009). However, the NT PA mutations caused a decrease in its migration rate (Fig. 4a, left panel, lane 3).

Using partially purified polymerase, we evaluated the promoter-binding activity by a UV cross-linking assay and the replication-initiation activity by the dinucleotide ApG-synthesis assay. In a UV cross-linking analysis, we cannot separate the UV cross-linked PA and PB2-TAP bands in our conditions (Maier *et al.*, 2008) and thus these were assigned as PB2/PA (Fig. 4a, middle panel), even

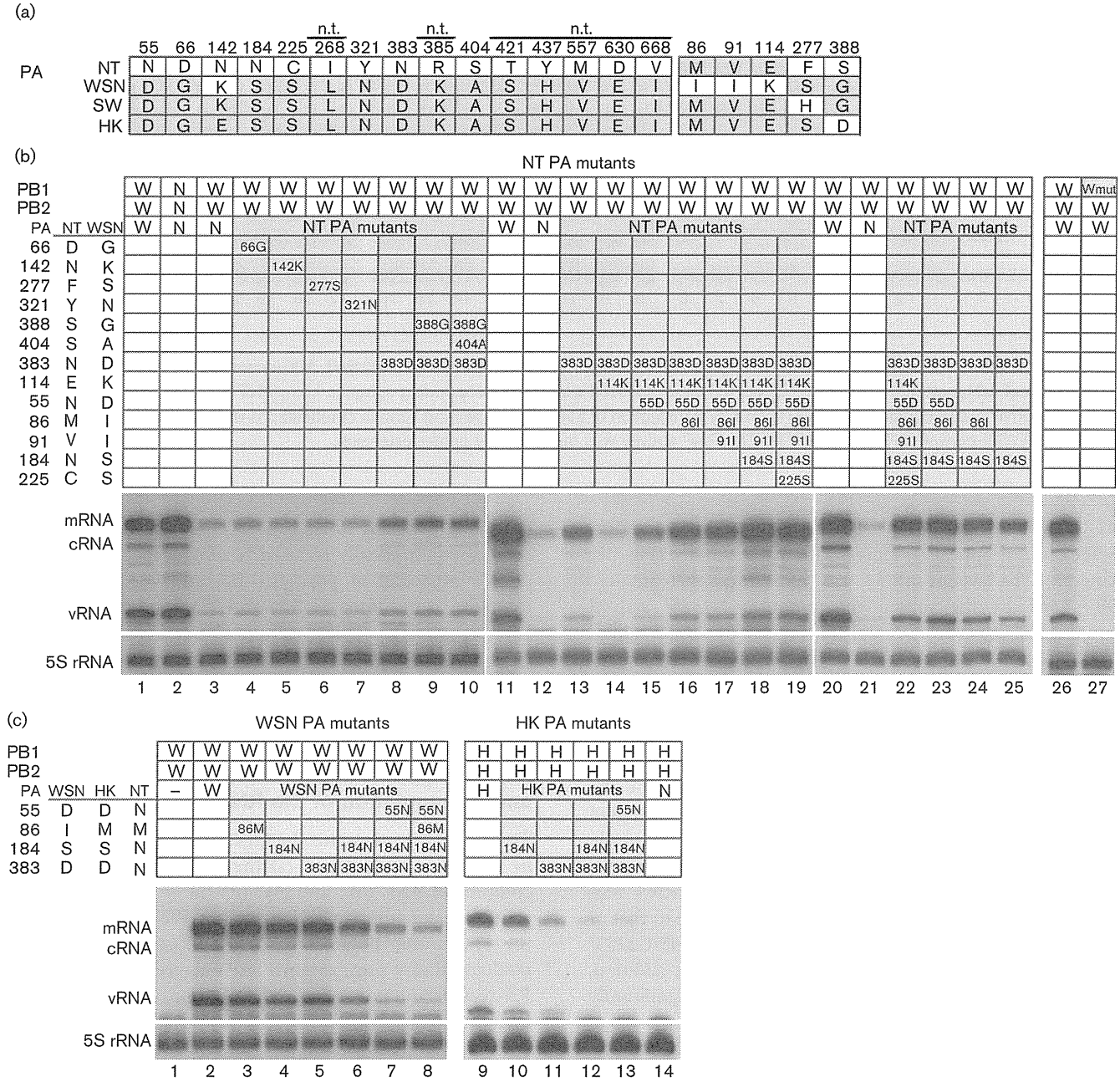

Fig. 3. Amino acids responsible for the inhibitory effect of NT PA. (a) Amino acids in NT PA that completely or partially differ from those of WSN, SW or HK were systemically substituted for the WSN PA sequences. n.t., Not tested. (b) The RNP activity which was reconstituted from WSN (W) hybrid polymerase by replacing PA subunit with NT PA WT (N) (lanes 3, 12 and 21) or NT PA mutants (lanes 4–10, 13–19, 22–25). W mut, WSN PB1with mutation D446Y at the SDD motif of the catalytic site (lane 27). (c) Effects of mutations in WSN PA (lanes 2–8) or HK (H) PA (lanes 10–13) on the RNP activity. The RNP was reconstituted in a background of WSN NP and NA vRNA. The amino acid sequences of WSN PA WT, NT PA WT and HK PA WT are indicated on the left. The numbers indicate the mutated positions in PA.

though the PA subunit migrated slightly faster or slower on the silver-stained gel. Regardless of the defect in RNP activity, the NT PA reassortant polymerase showed enhanced binding to the vRNA promoter (146 % of WSN polymerase), and significant binding to the cRNA promoter comparable to that of WSN polymerase (Fig. 4a, middle panel, lane 2). The binding to the cRNA promoter was significantly reduced to 55 % of WSN polymerase in the NT PA mutant (Fig. 4a, middle panel, lane 3), while significant RNP activity was retained (Fig. 3b, lane 18).

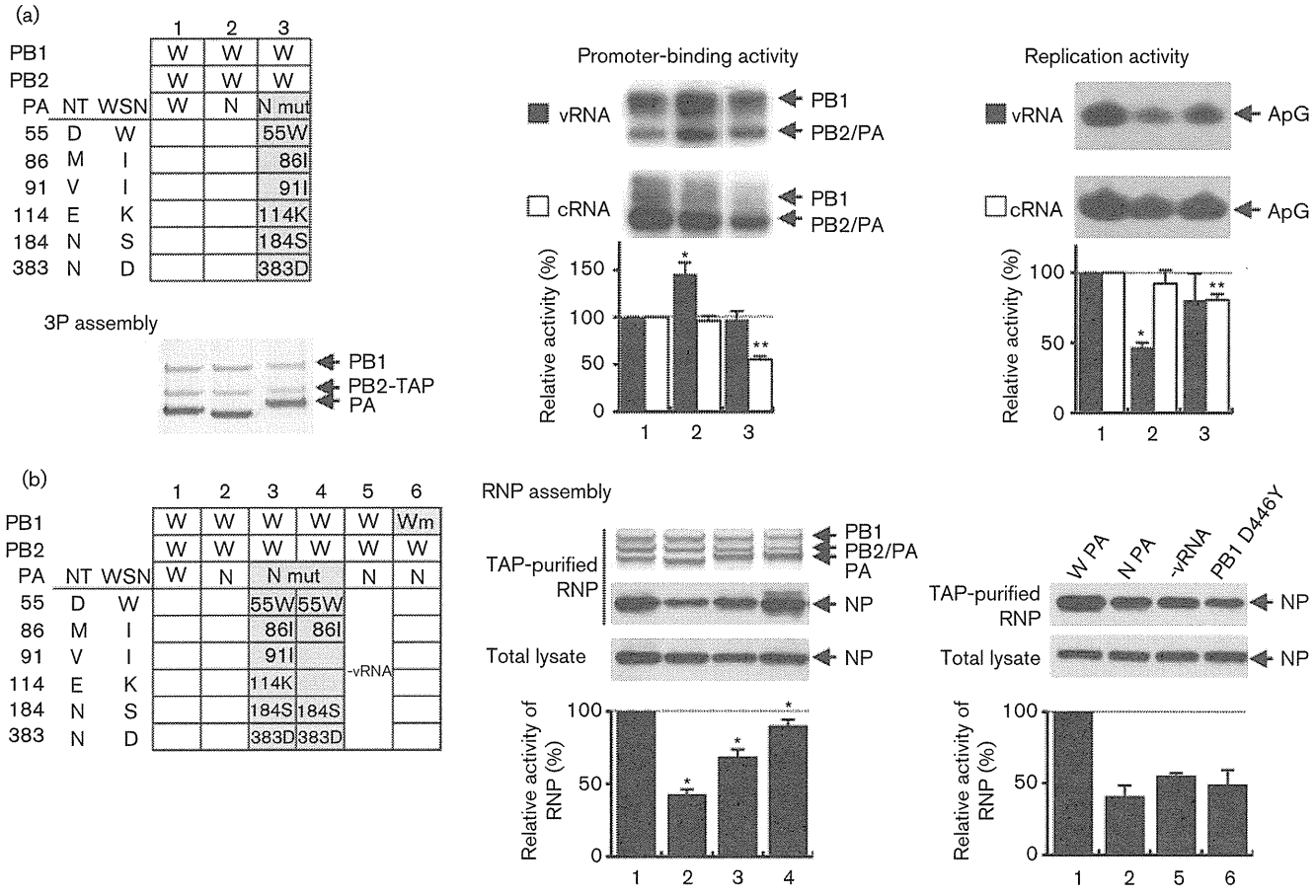

Fig. 4. NT PA reduces replication activity and the RNP accumulation. (a) NT PA reassortant polymerase (lanes 2 and 3) was transiently expressed in 293T cells and partially purified using TAP-tagged PB2. The purified polymerase was analysed by silver-stained 7.5 % SDS-PAGE (left panel), and assayed for the promoter-binding activity (middle panel) and replication-initiation activity (right panel) with a model vRNA (■vRNA) or cRNA promoter (□cRNA). The PA was stained more efficiently than other subunits probably because the silver staining was more sensitive for acidic protein (PA) than for basic protein (PB1 and PB2). * and ** shows statistical significance at $P<0.01$ and <*P* value?>, relative to WSN polymerase in a Student's *t*-test. (b) NT PA reassortant RNP (lanes 2–6) was reconstituted in 293T cells in a background of WSN NP and NA vRNA (lanes 1–4 and 6), or in the absence of NA vRNA (lane 5). The RNP was partially purified using TAP-tagged PB2 and analysed by silver-stained 7.5 % SDS-PAGE (middle top panel) or Western blotting using specific antibodies for NP. Overall expression of NP from total lysate from cells was also analysed by Western blotting with NP antibodies. Quantification of results was obtained by imaging analyser and data were expressed as percentages relative to WSN polymerase (mean ± SD; $n=3$). * shows statistical significance at $P<0.01$, relative to WSN polymerase in a Student's *t*-test. The NP signal in TAP-purified material in lanes 5 and 6 is not significantly different from lane 2 (*P* values: 0.02 in lane 5, 0.15 in lane 6). W, WSN; N, NT PA WT; N mut, NT PA mutant; Wm, WSN PB1 mutant D446Y.

The NT PA did not largely affect the replication-initiation activity on the cRNA template (92 % of WSN PA; Fig. 4a, right panel, lane 2). Notably, however, a remarkable reduction of activity was observed on the vRNA template (47 % of WSN polymerase; Fig. 4a, right panel, lane 2), suggesting that the NT PA specifically reduces vRNA→cRNA synthesis. Nonetheless, the NT PA reassortant polymerase possessed a strong activity to bind the vRNA promoter (146 % of WSN PA). Excessive binding to the promoter might impair the promoter clearance, which leads to the abortive initiation of RNA synthesis as previously suggested (Kashiwagi *et al.*, 2009). The reduced replication-initiation activity on the vRNA template was restored to 81 % of WSN polymerase in the NT PA mutant (Fig. 4a, right panel, lane 3).

Because we have previously shown that the defect in RNP accumulation leads to the loss of RNP activity (Nakazono *et al.*, 2012), we purified reconstituted RNP using TAP-tagged PB2 and quantified the amount of RNP by Western blotting with NP-specific antibodies. Remarkably, the NT PA reduced the NP signal in the TAP-purified material to 43 % of that achieved with WSN RNP (Fig. 4b, lane 2), indicating a significant reduction of RNP accumulation.

Nonetheless, the polymerase trimeric complex was properly formed (Fig. 4b, middle top panel, lane 2) and the overall expression of NP was comparable to that of WSN (Fig. 4b, middle bottom panel, lane 2). The reduced accumulation of RNP was rescued to 69 % and 90 % in the NT PA sixfold mutant and fourfold mutant, respectively (Fig. 4b, middle panel, lanes 3 and 4, respectively). Taken together, these results suggest that the loss of RNP activity was due to a significant reduction of polymerase activity in replication and also to a defect in the accumulation of RNP.

The NP signal was significantly decreased in the NT PA reassortant RNP, but still obtained to some extent (43 %). This suggests the presence of partially formed RNP. It was also possible that the NP signal might represent NP co-purified with polymerase, because NP binds to PB1 and PB2 (Biswas *et al.*, 1998; Poole *et al.*, 2004). To test this, WSN polymerase and NP was expressed in the absence of vRNA (Fig. 4b, lane 5). In addition, we constructed WSN PB1 carrying the D446Y mutation (Biswas & Nayak, 1994; Cauldwell *et al.*, 2013) in the SDD motif that was catalytically inactive (Fig. 3b, lane 27). When the RNP is reconstituted with the PB1 D446Y mutant, the polymerase and NP could be expressed normally, and vRNA could be transcribed by cellular RNA polymerase I from the input pPOLI plasmid and then assembled into RNP, which could not be amplified. TAP-purified material both in the absence of vRNA and in the PB1 D446Y mutant showed the level of NP signal not significantly different from NT PA (55.2 ± 1.8 %, 49.4 ± 9.8 % and 40.9 ± 7.7 %, respectively; Fig. 4b, right top panel, compare lanes 5 and 6 with 2). The overall expression level of NP was not affected in the absence of vRNA or the PB1 D446Y mutant (Fig. 4b, right bottom panel, compare lanes 5 and 6 with 2). These results suggest that the NP signal observed in the NT PA reassortant RNP might primarily represent the NP co-purified with polymerase.

The NT PA inhibitory effect is specifically relieved by NT PB2

We initially observed that the inhibitory effect of the NT PA was relieved by NT PB2 (Fig. 2), suggesting that the co-incorporation of PB2 and PA from the same NT strain was important for polymerase activity. To confirm this, we tested the effect of PB2s from other strains on RNP activity. The impaired RNP activity was not restored by PB2 from WSN or SW (Fig. 5a, lanes 3 and 4), and only marginally restored by VN PB2 in a background of HK polymerase (Fig. 5a, lane 5). In contrast, the RNP activity was significantly restored by NT PB2 (Fig. 5a, lane 6), supporting the requirement for the specific combination of NT PB2 and NT PA for significant RNP activity.

We expected that a significant RNP activity achieved by NT PB2-NT PA would be impaired by mutation in an NT PB2 amino acid which differs from those in other strains. An alignment of PB2 sequences shows that the NT PB2 amino acid sequence differs at positions 41, 34, 35 and 40 from those of HK, VN, WSN and SW, respectively. Among them, we focused on 20 positions (Fig. 5b). First, ten amino acids (44, 67, 82, 338, 344, 358, 382, 394, 463 and 676) that differ only in NT strains were mutated to the sequence conserved in the other four strains. Secondly, five residues (613, 627, 674, 684 and 717) that differ from those in the HK PB2 C-terminal region were mutated to the HK PB2 sequence, since the PB2 C-terminal domain contains important determinants for host adaptation, including residue 627. Thirdly, another five residues (271, 292, 478, 655 and 667) were mutated to the sequence conserved in VN and WSN. The RNP activity was not affected by the serial mutations at positions 44, 67, 82, 338, 344, 358, 382, 394, 463, 676 and 684 (Fig. 5b, lanes 5–9, 11–15 and 17–18). However, the additional mutation K627E markedly reduced the RNP activity to undetectable levels (Fig. 5b, lane 19). This reduced activity was not further affected by additional mutations at positions 674, 717 and 613 (Fig. 5b, lanes 20–22). Importantly, the single mutation K627E dramatically decreased RNP activity (Fig. 6, lane 2), suggesting that the NT PB2 627E is sufficient to impair the RNP activity. The loss of RNP activity was also observed by the K627E mutation in VN PB2 (Fig. 5c, compare lane 7 with 6). Moreover, we tested the effect of mutations except for mutation at position 627. However, in the presence of residue 627K, the serial mutations at positions 44, 67, 82, 338, 344, 358, 382, 394, 463, 676, 684, 674, 717, 613, 271, 292, 478, 655 and 667 did not reduce the RNP activity at all (Fig. 5b, lanes 25–31 and 33–34). Overall, we suggest that PB2 627K plays a pivotal role in overcoming the inhibitory effect of NT PA. This was further supported by the observation that the impaired RNP activity was significantly rescued by E627K mutation in HK PB2 and SW PB2 (Fig. 5c, compare lane 3 with 2 and lane 5 with 4).

The combination of NT PB2 627K and NT PA 184N/383N determines RNP activity

We demonstrated that two positions, 184N and 383N, were the major determinants of the inhibitory effect of NT PA, and the NT PB2 627K was involved in overcoming the inhibitory effect. Amino acid sequences PA 184N and 383N are unique to human H3N2 strains. These findings raise the possibility that the combination of three residues, PA 184N and 383N, and PB2 627K, might be critical for the RNP activity. To test this, we constructed each single mutant NT PB2 K627E, NT PA N184S and N383D, and double mutant NT PA N184S/N383D. The RNP activity was severely impaired by a single 627E mutation in the NT PB2 (Fig. 6, lane 2). However, the impaired RNP activity was partially rescued by a single 184S or 383D mutation in the NT PA (Fig. 6, lanes 3 or 4), and almost fully recovered by a double 184S/383D mutation in the NT PA (95 ± 11.3 % of mRNA, 98 ± 23.4 % of cRNA and 91 ± 17.2 % of vRNA; Fig. 6, lane 5). This suggests that PA 184S/383D are involved in overcoming the impaired RNP activity caused by PB2 627E. On the other hand, PB2 627K showed a significant RNP activity in all NT PA mutants (Fig. 6, lanes 6–8), and the highest activity was observed in the presence of PA 184S/

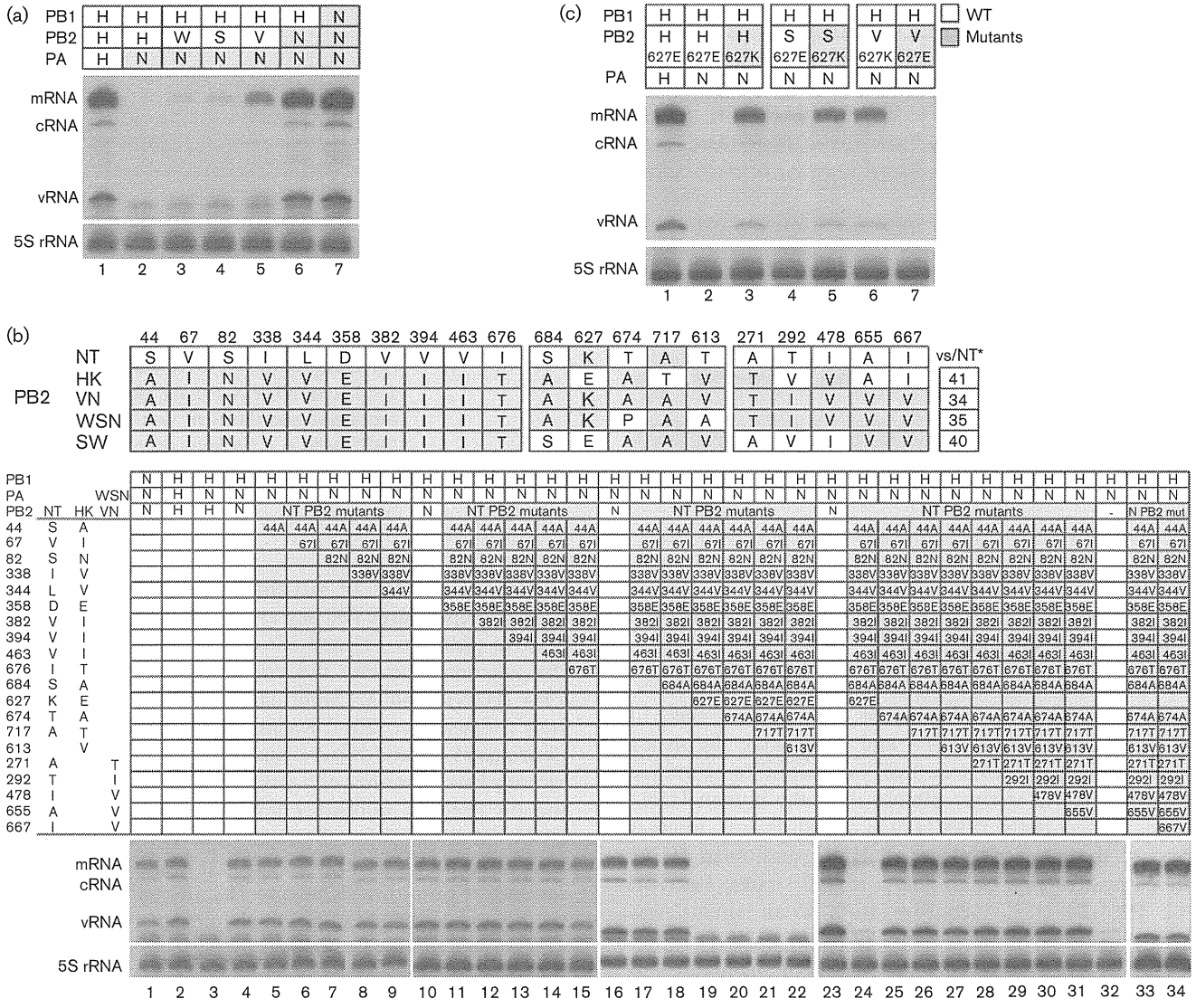

Fig. 5. The inhibitory effect of NT PA is relieved by NT PB2. (a) Effects of PB2 from WSN, SW, HK or VN on the impaired RNP activity. (b) Alignment of amino acid residues selected for mutations of NT PB2 and the effects of mutations in NT PB2 (lanes 5–9, 11–15, 17–22, 24–31, 33–34) on the impaired RNP activity. *, Number of amino acid differences to NT PB2. The PB2 amino acid sequences of NT, HK, WSN and VN are indicated on the left. (c) Effects of mutation at position 627 in HK PB2, SW PB2 and VN PB2 on the RNP activity. The RNP was reconstituted from hybrid polymerase in a background of WSN NP and NA vRNA in 293T cells, and the RNP activity was measured by primer extension. The numbers in the NT PB2 mutants indicate mutated positions. W, WSN; N, NT; H, HK; V, VN; S, SW.

383D (135±33.0 % of mRNA, 292±88.7 % of cRNA and 163±39.2 % of vRNA; Fig. 6, lane 8). These results indicate that the specific combination of PB2 627E and PA 184N/383N results in the loss of RNP activity; however, the defect is recovered by PB2 627K or PA 184S/383D.

DISCUSSION

Recent studies suggest that the co-incorporation of PB2 and PA from the same host origin might be important for the efficient replication of reassortant H3N2 influenza virus. We initially demonstrated that H3N2 PA (A/NT/60/1968) severely impaired the RNP activity when reassorted with H1N1 or H5N1 polymerase. This might be supported by the observation that the introduction of H3N2 PA (A/Yokohama/2017/2003) into H7N7 polymerase (A/equine/Prague/1/1956) resulted in the loss of RNP activity (Li *et al.*, 2008). A similar reduction of RNP activity was described following the introduction of H3N2 PA (A/Aichi/2/1968) into the H1N1 polymerase (A/California/04/2009) (Bussey *et al.*, 2011). We suggest that H3N2 PA plays a key role in the generation of viable reassortant viruses. However, we

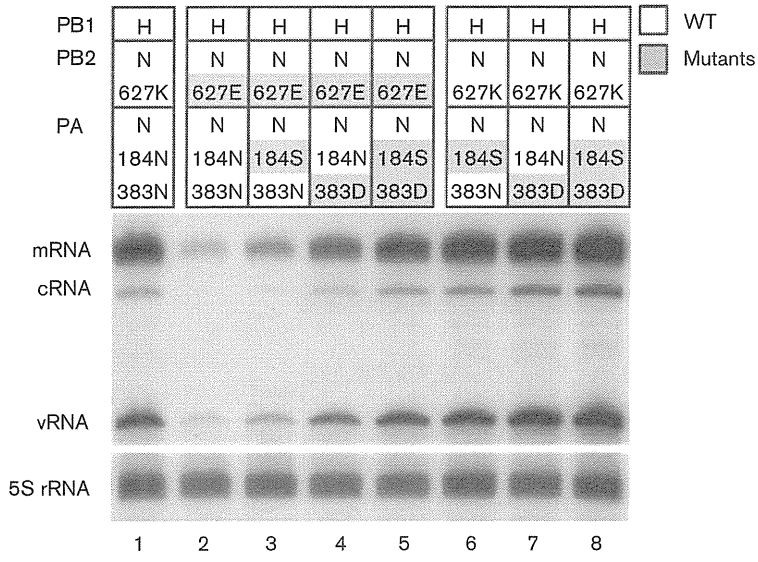

Fig. 6. The effect of the combination of PB2 627K and PA 184N/383N. Hybrid polymerase consisting of HK PB1, NT PB2 mutant (lanes 2–5) and NT PA mutant (lanes 3–8) was expressed and the RNP was reconstituted in 293T cells in a background of WSN NP and NA vRNA. The RNP activity was measured by primer extension.

cannot rule out the possible contribution of NP to the reassortment (Bean, 1984; Li *et al.*, 2010; Scholtissek *et al.*, 1985; Snyder *et al.*, 1987).

We found that four residues, 55N, 86M, 184N and 383N, in the NT PA were responsible for the inhibitory effect of NT PA. Among them, 184N and 383N were the major determinants and their sequences were found to be unique to human H3N2 strains. Almost all of the human H3N2 strains possess 184N/383N (100 % of 100 strains), whereas most of the avian H1N1, avian H3N2 or human H1N1 strains possess 184S/383D (98 % of 410 strains; Fig. 1b). The PA 184S/383D sequence from avian strains was probably incorporated into the 1918 H1N1 strain (Taubenberger *et al.*, 2005) and has been maintained in all human H1N1 strains. The PA 184S/383D sequence was also inherited by the 1957 H2N2 strain. Interestingly, however, the S184N/D383N mutation appeared to occur in the late 1950s in human H2N2 strains and became fixed in the 1960s (97 % of 38 strains). The resulting 184N/383N sequence has been inherited by the 1968 H3N2 strain and maintained over 40 years. While the significance of acquiring PA residues 184N/383N for the adaptation of influenza viruses to humans is not clear, we provide evidence suggesting that PA residues 184N/383N play a key role in the generation of viable H3N2 reassortant viruses.

Asparagine has a structural role in the formation of hydrogen bonds with the peptide backbone near the beginning or end of alpha-helices (Richardson & Richardson, 1989). Acquiring asparagine at positions 184 and 383 in the H3N2 PA may induce structural changes in PA, thereby affecting the assembly of functional RNP. Position 184 is located at the end of helix α6 (Dias *et al.*, 2009; Yuan *et al.*, 2009) and may be linked to the cRNA promoter-binding region: positions 163–178 (Maier *et al.*, 2008). Alternatively, position 184 may be related to position 186, which is involved to some extent in the polymerase activity of A/California/04/2009 (H1N1) at higher temperatures (Bussey *et al.*, 2011). Position 383 is close to positions 349 and 336, which are associated with the polymerase activity and virulence of A/Puerto Rico/8/1934 (H1N1) and A/California/04/2009 (H1N1), respectively (Bussey *et al.*, 2011; Rolling *et al.*, 2009). In addition, positions 55, 86 and 184 are located in the PA-X, which has recently been identified as a second protein, produced from the PA gene by ribosomal frame-shifting (Jagger *et al.*, 2012). PA-X comprises the N-terminal 191 aa of PA fused to 61 aa derived from the +1 ORF. It is unlikely that three residues at positions 55, 86 and 184 affect the function of PA-X, because the putative frame-shift signal of UCC UUU CGU C (PA codons 190–193) is found in all strains used in this study.

We demonstrated that the specific combination of PB2 627E and PA 184N/383N resulted in the loss of RNP activity. However, this defect was overcome by replacement with PB2 627K or PA 184S/383D. This suggests that PB2 627 and PA 184/383 might compensate each other by individually improving the polymerase activity. The compensatory effect of PB2 and PA might be supported by the observations indicating a functional relationship between PB2 and PA (Chen *et al.*, 2008; de Wit *et al.*, 2010; Labadie *et al.*, 2007; Li *et al.*, 2008; Mehle *et al.*, 2012; Treanor *et al.*, 1994). A weak interaction between PB2 and PA has also been proposed recently by using bimolecular fluorescence complementation (BiFC) assay which can detect weak and transient protein–protein interactions in living cells (Hemerka *et al.*, 2009). This compensatory effect might affect the interaction of polymerase with vRNA (Hara *et al.*, 2006; Maier *et al.*, 2008) or host factors (Bradel-Tretheway *et al.*, 2011; Engelhardt *et al.*, 2005; Mayer *et al.*, 2007; Momose *et al.*, 2001; Pérez-González *et al.*, 2006). More recently, PB2 627K was demonstrated to play a role in efficient recruitment of importin-α1 and -α7 to the RNP complex (Hudjetz & Gabriel, 2012). It would

be interesting to analyse whether PB2 627 and PA 184/383 affect the binding of importin-α to the RNP complex.

Our findings also could explain recent studies pointing out that the human H3N2 strain can efficiently reassort with other strains when the polymerase possesses PB2 and PA subunits from the same H3N2 strain. Li *et al.* (2010) showed that the combination of H3N2 PA (A/Tokyo/Ut-Sk-1/2007) and H5N1 PB2 (A/chicken/South Kalimantan/UT6028/2006) significantly impaired RNP activity, but this defect was recovered by replacing H5N1 PB2 with H3N2 PB2. We speculate that the loss of RNP activity could be due to the combination of H3N2 PA 184N/383N and H5N1 PB2 627E. Replacing H5N1 PB2 627E with H3N2 PB2 627K could probably lead to overcoming the inhibitory effect of H3N2 PA 184N/383N. However, we cannot fully explain why the RNP activity was impaired when the NT PA alone was introduced into WSN or VN polymerase, in spite of the presence of 627K in WSN PB2 and VN PB2. The amino acid residues of NT PB2 differ at 35 or 34 positions from those of WSN PB2 or VN PB2, respectively (Fig. 5b), and 20 residues of them were tested in this study. We suppose that in addition to 627K, there might be other determinants in NT PB2 that overcome the inhibitory effect of NT PA.

The NT PB2 appeared to enhance RNP activity when introduced alone into SW (180 % of mRNA) or HK (148 % of mRNA) polymerase (Fig. 2, lanes 11 and 19). In the presence of PA 184S/383D, the E→K replacement at PB2 627 slightly increased the RNP activity (135 % of mRNA; Fig. 6, compare lane 5 with 8). Indeed, such enhanced activity by H3N2 PB2 has been described in reassortants between A/Tokyo/Ut-Sk-1/2007 (H3N2) and A/chicken/South Kalimantan/UT6028/2006 (H5N1), and A/Wyoming/3/2003 (H3N2) and A/Thailand/16/2004 (H5N1) (Chen *et al.*, 2008; Li *et al.*, 2010).

In summary, we suggest that acquiring both PB2 627K and PA 184N/383N might be a prerequisite step for the generation of viable H3N2 reassortant viruses. Although these findings remain to be tested in the virus rescue experiment, these results could help understand why natural-reassortant H3N2 viruses isolated from human and swine possess PB2 and PA subunits from the same H3N2 strain of origin.

METHODS

Strains and plasmids. cDNA clones isolated from the following influenza strains were used: A/HongKong/156/1997 (H5N1; abbreviated as HK or H), A/Vietnam/1194/2004 (H5N1; abbreviated as VN or V), A/WSN/1933 (H1N1; abbreviated as WSN or W), A/Kurume/K0910/2009 (H1N1; abbreviated as SW or S) and A/NT/60/1968 (H3N2; abbreviated as NT or N) (Kashiwagi *et al.*, 2009). The plasmids expressing the PB1, PB2, PA and NP of different influenza strains, and the plasmid expressing vRNA from the WSN NA gene (vNA) have been described previously (Vreede *et al.*, 2004). Mutant PB1, PB2 and PA plasmids were prepared by site-directed mutagenesis and were fully sequenced.

Analysis of RNP activity. The RNP activity was analysed by primer extension as described previously (Fodor *et al.*, 2002; Hara *et al.*, 2006). Human embryonic kidney 293T cells were transfected with each of PA, PB1, PB2, NP and vNA expression vectors from each strain (WSN, NT, HK, VN or SW) and total RNA was isolated 30 h post-transfection. Primer extension was performed using three primers labelled with ^{32}P: one for vRNA, one for mRNA and cRNA, and one for host-cell 5S rRNA as an internal control. All assays were carried out at least three times with independently transfected cells.

Preparation of TAP-tagged polymerase RNP and *in vitro* assays. For preparation of the polymerase, 293T cells were transfected with expression vectors containing PB1, TAP-tagged PB2 and the PA subunit of the polymerase. For preparation of the RNP, NP and vNA expression vectors were also transfected simultaneously. The polymerase or RNP was purified by the TAP method as described before (Deng *et al.*, 2005). Briefly, 293T cells transfected with plasmids were harvested 2 days post-transfection, lysed and incubated with IgG-Sepharose (Amersham). After washing, the polymerase or RNP was released from IgG-Sepharose by cleavage with tobacco etch virus protease (Nakalai). The partially purified polymerase or RNP was analysed by 7.5 % SDS-PAGE with silver staining (Invitrogen), confirmed by Western blotting with specific antibodies against PB1, PB2, PA and NP. The polymerase was adjusted by quantitative measurements of the levels of PA on silver-stained SDS-PAGE gels, because PA or PB1 levels indicate the level of the trimeric complex. The quantitatively adjusted polymerases were used to test the promoter-binding activity by UV cross-linking assay and replication-initiation activity by dinucleotide initiation of replication assay, as reported previously (Fodor *et al.*, 2002; Hara *et al.*, 2006). Data were quantified by an image analyser (GE Healthcare, Image Quant LAS 4000 mini).

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REFERENCES

Bean, W. J. (1984). Correlation of influenza A virus nucleoprotein genes with host species. *Virology* **133**, 438–442.

Biswas, S. K. & Nayak, D. P. (1994). Mutational analysis of the conserved motifs of influenza A virus polymerase basic protein 1. *J Virol* **68**, 1819–1826.

Biswas, S. K., Boutz, P. L. & Nayak, D. P. (1998). Influenza virus nucleoprotein interacts with influenza virus polymerase proteins. *J Virol* **72**, 5493–5501.

Bradel-Tretheway, B. G., Mattiacio, J. L., Krasnoselsky, A., Stevenson, C., Purdy, D., Dewhurst, S. & Katze, M. G. (2011). Comprehensive proteomic analysis of influenza virus polymerase complex reveals a novel association with mitochondrial proteins and RNA polymerase accessory factors. *J Virol* **85**, 8569–8581.

Bussey, K. A., Desmet, E. A., Mattiacio, J. L., Hamilton, A., Bradel-Tretheway, B., Bussey, H. E., Kim, B., Dewhurst, S. & Takimoto, T. (2011). PA residues in the 2009 H1N1 pandemic influenza virus enhance avian influenza virus polymerase activity in mammalian cells. *J Virol* **85**, 7020–7028.

Cauldwell, A. V., Moncorgé, O. & Barclay, W. S. (2013). Unstable polymerase–nucleoprotein interaction is not responsible for avian influenza virus polymerase restriction in human cells. *J Virol* **87**, 1278–1284.

Chen, L. M., Davis, C. T., Zhou, H., Cox, N. J. & Donis, R. O. (2008). Genetic compatibility and virulence of reassortants derived from contemporary avian H5N1 and human H3N2 influenza A viruses. *PLoS Pathog* **4**, e1000072.

de Wit, E., Munster, V. J., van Riel, D., Beyer, W. E., Rimmelzwaan, G. F., Kuiken, T., Osterhaus, A. D. & Fouchier, R. A. (2010). Molecular determinants of adaptation of highly pathogenic avian influenza H7N7 viruses to efficient replication in the human host. *J Virol* **84**, 1597–1606.

Deng, T., Sharps, J., Fodor, E. & Brownlee, G. G. (2005). *In vitro* assembly of PB2 with a PB1-PA dimer supports a new model of assembly of influenza A virus polymerase subunits into a functional trimeric complex. *J Virol* **79**, 8669–8674.

Dias, A., Bouvier, D., Crépin, T., McCarthy, A. A., Hart, D. J., Baudin, F., Cusack, S. & Ruigrok, R. W. (2009). The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. *Nature* **458**, 914–918.

Engelhardt, O. G., Smith, M. & Fodor, E. (2005). Association of the influenza A virus RNA-dependent RNA polymerase with cellular RNA polymerase II. *J Virol* **79**, 5812–5818.

Fan, X., Zhu, H., Zhou, B., Smith, D. K., Chen, X., Lam, T. T., Poon, L. L., Peiris, M. & Guan, Y. (2012). Emergence and dissemination of a swine H3N2 reassortant influenza virus with 2009 pandemic H1N1 genes in pigs in China. *J Virol* **86**, 2375–2378.

Fodor, E., Crow, M., Mingay, L. J., Deng, T., Sharps, J., Fechter, P. & Brownlee, G. G. (2002). A single amino acid mutation in the PA subunit of the influenza virus RNA polymerase inhibits endonucleolytic cleavage of capped RNAs. *J Virol* **76**, 8989–9001.

Hara, K., Schmidt, F. I., Crow, M. & Brownlee, G. G. (2006). Amino acid residues in the N-terminal region of the PA subunit of influenza A virus RNA polymerase play a critical role in protein stability, endonuclease activity, cap binding, and virion RNA promoter binding. *J Virol* **80**, 7789–7798.

Hemerka, J. N., Wang, D., Weng, Y., Lu, W., Kaushik, R. S., Jin, J., Harmon, A. F. & Li, F. (2009). Detection and characterization of influenza A virus PA–PB2 interaction through a bimolecular fluorescence complementation assay. *J Virol* **83**, 3944–3955.

Hudjetz, B. & Gabriel, G. (2012). Human-like PB2 627K influenza virus polymerase activity is regulated by importin-α1 and -α7. *PLoS Pathog* **8**, e1002488.

Itoh, Y., Shinya, K., Kiso, M., Watanabe, T., Sakoda, Y., Hatta, M., Muramoto, Y., Tamura, D., Sakai-Tagawa, Y. & other authors (2009). *In vitro* and *in vivo* characterization of new swine-origin H1N1 influenza viruses. *Nature* **460**, 1021–1025.

Jagger, B. W., Wise, H. M., Kash, J. C., Walters, K. A., Wills, N. M., Xiao, Y. L., Dunfee, R. L., Schwartzman, L. M., Ozinsky, A. & other authors (2012). An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. *Science* **337**, 199–204.

Kashiwagi, T., Leung, B. W., Deng, T., Chen, H. & Brownlee, G. G. (2009). The N-terminal region of the PA subunit of the RNA polymerase of influenza A/HongKong/156/97 (H5N1) influences promoter binding. *PLoS ONE* **4**, e5473.

Kawaoka, Y., Krauss, S. & Webster, R. G. (1989). Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. *J Virol* **63**, 4603–4608.

Labadie, K., Dos Santos Afonso, E., Rameix-Welti, M. A., van der Werf, S. & Naffakh, N. (2007). Host-range determinants on the PB2 protein of influenza A viruses control the interaction between the viral polymerase and nucleoprotein in human cells. *Virology* **362**, 271–282.

Li, C., Hatta, M., Watanabe, S., Neumann, G. & Kawaoka, Y. (2008). Compatibility among polymerase subunit proteins is a restricting factor in reassortment between equine H7N7 and human H3N2 influenza viruses. *J Virol* **82**, 11880–11888.

Li, C., Hatta, M., Nidom, C. A., Muramoto, Y., Watanabe, S., Neumann, G. & Kawaoka, Y. (2010). Reassortment between avian H5N1 and human H3N2 influenza viruses creates hybrid viruses with substantial virulence. *Proc Natl Acad Sci U S A* **107**, 4687–4692.

Maier, H. J., Kashiwagi, T., Hara, K. & Brownlee, G. G. (2008). Differential role of the influenza A virus polymerase PA subunit for vRNA and cRNA promoter binding. *Virology* **370**, 194–204.

Mayer, D., Molawi, K., Martínez-Sobrido, L., Ghanem, A., Thomas, S., Baginsky, S., Grossmann, J., García-Sastre, A. & Schwemmle, M. (2007). Identification of cellular interaction partners of the influenza virus ribonucleoprotein complex and polymerase complex using proteomic-based approaches. *J Proteome Res* **6**, 672–682.

Mehle, A., Dugan, V. G., Taubenberger, J. K. & Doudna, J. A. (2012). Reassortment and mutation of the avian influenza virus polymerase PA subunit overcome species barriers. *J Virol* **86**, 1750–1757.

Momose, F., Basler, C. F., O'Neill, R. E., Iwamatsu, A., Palese, P. & Nagata, K. (2001). Cellular splicing factor RAF-2p48/NPI-5/BAT1/UAP56 interacts with the influenza virus nucleoprotein and enhances viral RNA synthesis. *J Virol* **75**, 1899–1908.

Nakazono, Y., Hara, K., Kashiwagi, T., Hamada, N. & Watanabe, H. (2012). The RNA polymerase PB2 subunit of influenza A/HongKong/156/1997 (H5N1) restricts the replication of reassortant ribonucleoprotein complexes [corrected]. *PLoS ONE* **7**, e32634.

Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, Dawood, F. S., Jain, S., Finelli, L., Shaw, M. W., Lindstrom, S., Garten, R. J., Gubareva, L. V., Xu, X., Bridges, C. B. & Uyeki, T. M. (2009). Emergence of a novel swine-origin influenza A (H1N1) virus in humans. *N Engl J Med* **360**, 2605–2615.

Olsen, C. W. (2002). The emergence of novel swine influenza viruses in North America. *Virus Res* **85**, 199–210.

Palese, P. & Shaw, M. L. (2007). *Orthomyxoviridae*: the viruses and their replication. In *Fields Virology*, 5th edn, pp. 1647–1689. Edited by D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roitman & S. E. Straus. Philadelphia, PA: Lippincott Williams & Wilkins.

Peiris, J. S., Poon, L. L. & Guan, Y. (2009). Emergence of a novel swine-origin influenza A virus (S-OIV) H1N1 virus in humans. *J Clin Virol* **45**, 169–173.

Pérez-González, A., Rodriguez, A., Huarte, M., Salanueva, I. J. & Nieto, A. (2006). hCLE/CGI-99, a human protein that interacts with the influenza virus polymerase, is a mRNA transcription modulator. *J Mol Biol* **362**, 887–900.

Poole, E., Elton, D., Medcalf, L. & Digard, P. (2004). Functional domains of the influenza A virus PB2 protein: identification of NP- and PB1-binding sites. *Virology* **321**, 120–133.

Richardson, J. S. & Richardson, D. C. (1989). Prediction of protein structure and the principles of protein conformation. In *Principles and Patterns of Protein Conformation*, pp. xxx–yyy. Edited by G. D. Fasman. New York: Springer.

Rolling, T., Koerner, I., Zimmermann, P., Holz, K., Haller, O., Staeheli, P. & Kochs, G. (2009). Adaptive mutations resulting in enhanced polymerase activity contribute to high virulence of influenza A virus in mice. *J Virol* **83**, 6673–6680.

Scholtissek, C., Bürger, H., Kistner, O. & Shortridge, K. F. (1985). The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. *Virology* **147**, 287–294.

Snyder, M. H., Buckler-White, A. J., London, W. T., Tierney, E. L. & Murphy, B. R. (1987). The avian influenza virus nucleoprotein gene and a specific constellation of avian and human virus polymerase genes each specify attenuation of avian-human influenza A/Pintail/79 reassortant viruses for monkeys. *J Virol* **61**, 2857–2863.

Taubenberger, J. K., Reid, A. H., Lourens, R. M., Wang, R., Jin, G. & Fanning, T. G. (2005). Characterization of the 1918 influenza virus polymerase genes. *Nature* **437**, 889–893.

Treanor, J., Perkins, M., Battaglia, R. & Murphy, B. R. (1994). Evaluation of the genetic stability of the temperature-sensitive PB2 gene mutation of the influenza A/Ann Arbor/6/60 cold-adapted vaccine virus. *J Virol* **68**, 7684–7688.

Vreede, F. T., Jung, T. E. & Brownlee, G. G. (2004). Model suggesting that replication of influenza virus is regulated by stabilization of replicative intermediates. *J Virol* **78**, 9568–9572.

Wright, P. F., Neumann, G. & Kawaoka, Y. (2007). Orthomyxoviruses. In *Fields Virology*, 5th edn, pp. 1692–1740. Edited by D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roitman & S. E. Straus. Philadelphia, PA: Lippincott Williams & Wilkins.

Yuan, P., Bartlam, M., Lou, Z., Chen, S., Zhou, J., He, X., Lv, Z., Ge, R., Li, X. & other authors (2009). Crystal structure of an avian influenza polymerase PA(N) reveals an endonuclease active site. *Nature* **458**, 909–913.

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Improvement Rate of Acute Otitis Media Caused by Haemophilus influenzae at 1 Week Is Significantly Associated with Time to Recovery

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Improvement Rate of Acute Otitis Media Caused by *Haemophilus influenzae* at 1 Week Is Significantly Associated with Time to Recovery

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Acute otitis media (AOM) is the most common upper respiratory tract infection in childhood. Children with AOM were enrolled at Tohoku Rosai Hospital between July 2006 and June 2011 if their middle ear fluid cultures after tympanocentesis yielded only *Haemophilus influenzae*. The susceptibilities of the isolates to ampicillin were determined, and microtiter biofilm assays and invasion assays using BEAS-2B cells were performed. The association between these bacterial characteristics and clinical relapses of AOM and treatment failures was evaluated. Seventy-four children (39 boys and 35 girls) with a median age of 1 year (interquartile range [IQR], 0.25 to 2 years) were enrolled. Among 74 *H. influenzae* isolates, 37 showed intermediate resistance or resistance to ampicillin (MIC, ≥2 μg/ml). In the microtiter biofilm assay, the median optical density at 600 nm (OD_{600}) was 0.68 (IQR, 0.24 to 1.02), and 70 isolates formed biofilms. The median invasion rate was 15% (IQR, 0 to 10%), and 46 isolates invaded BEAS-2B cells. Relapses and treatment failures occurred in 19 and 6 children, respectively. There was no significant difference in the invasion rates between patients with and those without relapses or treatment failures. Also, there was no significant association between biofilm formation and relapse or treatment failure. The improvements in the severity scores after 1 week were significantly associated with the recovery time ($P < 0.0001$). We did not identify any significant association between relapse or treatment failure and bacterial factors. AOM has a multifactorial etiology, and this may explain why we could not find a significant association. An improvement in the severity score after 1 week of treatment may be a useful predictor of the outcome of AOM.

Acute otitis media (AOM) is the most common disease of the upper respiratory tract in childhood, and treatment of AOM is the most frequent reason that children in the United States take antibiotics (1). In Japan, the incidence of AOM has increased recently, and many children now need hospitalization to receive intravenous antibiotics for the treatment of intractable AOM with persistent purulent otorrhea (2).

We previously reported that *Haemophilus influenzae* can form a biofilm both *in vitro* (3) and *in vivo* (4). Bacterial biofilms are recognized as having an important role in various human infections, and the bacteria in a biofilm are more resistant to antibiotic therapy than are planktonic microorganisms, suggesting that biofilms might play an important role in the pathogenesis and chronicity of otitis media (3). In addition, an investigation of the mechanism of airway epithelium invasion by *H. influenzae* revealed that the bacteria are internalized by the adenoid cells of children (5). We also previously demonstrated that *H. influenzae* isolated from clinical samples can invade and destroy human bronchial epithelial cells (BEAS-2B cells) (6), suggesting that such activity might delay the resolution of AOM. However, the association between biofilm formation or invasion of bronchial epithelial cells and the clinical course and outcome of AOM due to *H. influenzae* has been unclear.

Accordingly, we measured the biofilm formation and invasion of bronchial epithelial cells by *H. influenzae* isolated from children with AOM, and we evaluated the association between these bacterial characteristics and the clinical course and outcome of AOM.

MATERIALS AND METHODS

Patients and study design. Children who attended the Department of Otolaryngology of Tohoku Rosai Hospital between July 2006 and June 2011 were enrolled if they were (i) aged <6 years, (ii) were given a diagnosis of AOM by an otolaryngologist on the basis of symptoms (fever, irritability, and tugging of the ear) and signs (redness and bulging of the tympanic membrane), (iii) had acute illness lasting <7 days, (iv) had no spontaneous perforation and no tympanostomy tubes, and (v) had follow-up until at least day 10 ± 2 of the study (the 3rd visit). In addition, the cultures of middle ear fluid (MEF) specimens after tympanocentesis yielded only *H. influenzae* in all patients. The patients' age, sex, and attendance at a day care center were recorded.

The MEF specimens from tympanocentesis were immediately collected with a sterile cotton swab (Seed swab γ no. 2; Eiken Chemical Co. Ltd., Tokyo, Japan). Each specimen was plated onto chocolate and sheep blood agar plates, which were incubated at 35°C under a 5% CO_2 atmo-

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sphere for 18 to 24 h. The *H. influenzae* strains were identified and confirmed by colony morphology, Gram staining, growth in chocolate agar but not in blood agar, the catalase test, and X and V factor requirements.

The first follow-up visit was scheduled for day 4 or 5 (the day of enrollment was defined as day 1), with additional follow-up visits for clinical evaluation on day 10 ± 2 and day 17 ± 2. All patients were followed until the recovery score was 0 at 7- to 10-day intervals. In addition, unscheduled visits were allowed at any time if the patient's condition deteriorated.

Scores were assigned for temperature (0, <38°C; 1, 38.0 to 38.5°C; 2, 38.6 to 39°C; and 3, >39°C) and for irritability, ear tugging, and redness and bulging of the tympanic membrane (0, absent; 1, mild; 2, moderate; and 3, severe) (7).

The criteria for clinical failure and relapse were as follows. By definition at enrollment, the initial examination of the MEF always detected purulent, mucopurulent, or seropurulent fluid. The persistence of MEF for >2 weeks after the first visit was defined as a treatment failure. A relapse was defined as occurring when an ear that had responded previously developed new MEF at any time during the follow-up period. After examination and tympanocentesis at the first visit, each child was treated with either amoxicillin (60 mg/kg of body weight/day), amoxicillin-clavulanic acid (90/6.4 mg/kg/day), or cefditoren pivoxil (18 mg/kg/day). When the clinical findings for the children treated with amoxicillin or amoxicillin-clavulanic acid did not improve at the first follow-up visit, the children were treated with cefditoren pivoxil.

This study was approved by the human ethics review boards of Tohoku Rosai Hospital (no. Oki-1), Tohoku University Graduate School of Medicine (no. 11518), and Kurume University (no. 07002).

Antimicrobial susceptibility testing, detection of β-lactamase, and serotypes. The MICs of ampicillin (AMP), amoxicillin-clavulanic acid, piperacillin, cefaclor, ceftriaxone, cefditoren, meropenem, and levofloxacin were determined by the broth dilution method, in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (8). *H. influenzae* ATCC 49247 was used as the reference strain. β-Lactamase production was assessed by the nitrocefin test. Among the β-lactamase-nonproducing isolates, strains with MICs of ≥2 μg/ml for AMP were defined as β-lactamase-nonproducing AMP-resistant (BLNAR) *H. influenzae*, while those with MICs of 1 μg/ml were defined as low BLNAR and those with MICs of ≤0.5 μg/ml as β-lactamase-nonproducing AMP-susceptible (BLNAS) *H. influenzae*. Among the β-lactamase-producing isolates (β-lactamase-producing AMP-resistant [BLPAR]), strains that were resistant to amoxicillin-clavulanic acid (MIC, ≥4/2 μg/ml) were classified as β-lactamase-producing amoxicillin-clavulanic acid-resistant (BLPACR) *H. influenzae*. The *H. influenzae* isolates were serotyped by slide agglutination with antisera purchased from Denka Seiken Co., Ltd. (Tokyo, Japan).

Microtiter biofilm assay. Biofilm formation by all *H. influenzae* isolates was assessed using 96-well microplates as described previously (4). The culture medium containing planktonic cells was stained with 1% crystal violet at room temperature. After the biofilm was rinsed three times with water, the dye bound to it was extracted with 230 μl of 95% ethanol for 15 min, and the amounts of dye extracted were quantified by measuring the optical density at 600 nm (OD_{600}) with a microplate reader. The strains were tested in quadruplicate for each experiment, and representative results from three different experiments are reported here.

Invasion assay with BEAS-2B cells. Invasion assays using BEAS-2B cells were done with all isolates of *H. influenzae* from patients enrolled in this study as described previously (6). Bacterial suspensions of *H. influenzae* (about 6×10^6 CFU/ml) were added at 10 μl/well to the cell monolayers (multiplicity of infection [MOI], 0.6) and incubated for 3 h at 37°C under a 5% CO_2 atmosphere, followed by 3 washes with phosphate-buffered saline (PBS) and treatment with gentamicin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 200 μg/ml for 2 h. Then, the monolayers were washed 3 more times with PBS, and viable intracellular bacteria were released by treatment with 0.5 ml of 1% Triton X-100 (Sigma-Aldrich) in PBS for 15 min, after which the samples were harvested and vortexed for 1 min to lyse the cells. The resultant suspensions were plated in serial dilutions on chocolate agar plates (Nissui Pharmaceutical Co., Tokyo, Japan) at 35°C, and the colonies were counted after overnight incubation. *Pseudomonas aeruginosa* PAO1 and *Escherichia coli* RDEC-1 were used as the positive- and negative-control strains for the invasion assays. In the preliminary experiment, we confirmed that 1% Triton X-100 in PBS does not lyse *H. influenzae* isolates. The experiments were repeated at least 3 times with each strain. The bacterial invasion rate was calculated as (bacteria recovered from BEAS-2B cells [CFU/ml]/inoculated bacteria [CFU/ml]) × 100 (%).

Statistical analyses. Statistical analyses were performed as follows. First, we examined differences in the times to recovery (when the severity score declined to 0) among patients with different severity scores at the initial examination by creating box-and-whisker plots and performing the Kruskal-Wallis test. We also examined differences in the relapse rates using Fisher's exact test. Next, potential factors influencing the times to recovery were examined by multiple regression analyses, including the age, drug resistance, severity score at the initial examination, biofilm formation (average of 9 measurements), invasion rate (average of 3 measurements), and improvement in the severity score at 1 week after the initial examination. A forward-backward procedure was employed to construct a regression model. We confirmed the significance of each factor selected by controlling for the influence of sex and age as covariates. A significance level of 0.05 was used in the regression analyses. R statistical environment software (version 2.14.2) was used for all analyses.

TABLE 1 Characteristics of the patients and bacterial strains

Patient characteristic or bacterial strain	Result for patients[a] with:		
	Relapse	Treatment failure	No relapse or treatment failure
No. of patients	19	6	52
Male:female ratio	10:9	3:3	27:25
Age [median (IQR)] (yrs)	1 (0.5–2)	1 (1–1)	1 (0–2)
No. of patients attending day care centers	9	3	24
Haemophilus influenzae strain			
BLNAR	13	4	25
Low BLNAR	1	0	7
BLNAS	4	1	17
BLPAR	0	0	2
BLPACR	1	1	1

[a] There were three patients with both relapse and treatment failure.

RESULTS

Profiles of the patients and bacterial strains. A total of 74 children were enrolled in this study (Table 1). They included 39 boys and 35 girls with a median age of 1 year (interquartile range [IQR], 0.25 to 2 years). Sixty of the children (81.1%) were ≤2 years old. Seventy-four *H. influenzae* strains were isolated from these patients, including 37 (50.0%) BLNAR strains, 8 (10.8%) low-BLNAR strains, 2 (2.7%) BLPAR strains, and 2 (2.7%) BLPACR strains. The results of antimicrobial susceptibility testing are shown in Table 2. The serotypes of 74 *H. influenzae* isolates were all nontypeable.

Biofilm formation and invasion of BEAS-2B cells. The mean OD_{600} at 24 h in the microtiter biofilm assay for the 74 *H. influenzae* isolates was 0.81 ± 0.77 (range, 0 to 3.5). Biofilm formation by the *H. influenzae* isolates was variable, but 70

TABLE 2 Susceptibility profile of *Haemophilus influenzae* isolates

Antimicrobial agent	Dose range (μg/ml)		MIC_{90} (μg/ml)
	Minimum	Maximum	
Ampicillin	≤0.12	≥16	8
Amoxicillin-clavulanic acid	≤0.12	16	8
Piperacillin	≤0.06	≥16	0.25
Cefaclor	≤0.5	≥128	64
Ceftriaxone	≤0.03	0.25	0.25
Cefditoren	≤0.03	0.5	0.25
Meropenem	≤0.03	0.5	0.25
Levofloxacin	≤0.03	1	0.06

(94.6%) out of 74 isolates showed the ability to form biofilms even after 24 h. The mean invasion rate of *H. influenzae* isolates for BEAS-2B cells was 0.29 ± 0.82. Forty-six (62.2%) of the 74 isolates showed the ability to invade BEAS-2B cells, and the highest invasion rate exceeded 5%.

Relationship between time to recovery and initial score or biofilm formation or invasion. The median time to recovery (when the severity score reached 0) was 9.5 days (IQR, 7.3 to 19.0 days). Differences in the recovery times among patients with different initial severity scores are shown in Fig. 1. There was no significant association between the severity score and the recovery time ($P = 0.11$). In addition, there was no relationship between the time to recovery among patients and the ability of the isolates to form biofilms or invade cells (correlation coefficients, −0.06 and 0.01, respectively).

Relationship between clinical outcome and biofilm formation or invasion of BEAS-2B cells. Relapses and treatment failures were observed in 19 (25.7%) and 6 (8.1%) children, respectively, with both relapse and treatment failure occurring in 3 children (4.1%). The invasion rate of *H. influenzae* isolates for BEAS-2B cells was higher among the patients with relapses (0.57 ± 1.41%) than among those without relapses (0.19 ± 0.46%), but there was no significant difference in the invasion rates between relapse and nonrelapse cases. There were also no significant differences in the invasion rates of isolates between patients with or those without treatment failure. Moreover, there was no significant association between biofilm formation by *H. influenzae* and relapse or treatment failure (Fig. 2).

Factors influencing recovery. The relationship between the relapse rates among patients and different severity scores at the initial examinations was not significant ($P = 0.37$, Fisher's exact test). Meanwhile, our regression analysis showed that the improvement in the severity score at 1 week after the initial examination was significantly associated with the time to recovery ($P < 0.0001$). Only the improvement rate of the severity score was selected by the forward-backward procedure, and the association remained significant after adjustment for sex and age. The regression coefficient was −0.26 (95% confidence interval [CI], −0.38 to −0.15), which indicates that a 10% improvement in the severity score at 1 week after the initial examination shortens the time to recovery of a patient by 2.6 days.

DISCUSSION

AOM is a frequent complication of respiratory tract infections in children, and one of the main bacterial pathogens is *H. influenzae*. It was recently reported that AOM has become more difficult to treat with oral antibiotics when BLNAR *H. influenzae* is the causative pathogen (9). The global prevalence of BLNAR isolates of *H. influenzae* remains low, but these isolates have been emerging in some countries, particularly Japan (10). In the present study, 37 (50%) out of 74 *H. influenzae* strains showed intermediate resistance or resistance to AMP (MICs, ≥2 μg/ml). However, there was no significant association between AMP resistance and the time to recovery or the relapse rate. We performed myringotomy and drainage of MEF in all of the patients on day 1, and these procedures might have heavily influenced the improvement in the AOM by antibiotic therapy.

A biofilm is a structured community of bacteria enveloped in a self-produced extrapolymeric matrix that adheres to a surface, and biofilm production is a common cause of persistent and chronic bacterial infections (4). Recently, Torretta et al. investigated nasopharyngeal biofilm-producing pathogens in children with a history of recurrent mild/moderate AOM. They found that biofilm-producing pathogens were more frequently isolated from the nasopharynx in the recurrent AOM group than in the control group, and *H. influenzae* was confirmed to be the main pathogen in the recurrent group (11). In addition, Bakaletz reported that biofilms contribute to both chronic otitis media and recurrent AOM (12). In the current study, nearly all of the *H. influenzae* isolates (94.6%) had the ability to form biofilms. However, there was no significant association between biofilm formation and the clinical findings or the outcome. AOM has a multifactorial etiology, and this may explain why we did not find a significant association between the clinical findings or the outcome and the ability of causative bacteria to form biofilms.

Some reports have suggested that the internalization of *H. influenzae* by epithelial cells has an important role in persistent and chronic infections by this microorganism (13, 14, 15). In the present study, 46 (62.2%) out of 74 isolates demonstrated the ability to invade BEAS-2B cells, indicating that many *H. influenzae* strains isolated from MEF samples of children with AOM can invade the airway epithelial cells *in vivo*. Although there was no significant difference in the invasion rates between patients with or without relapse, the invasion rates of *H. influenzae* isolates for BEAS-2B cells were higher among the patients with relapse than among those without relapse. Presumably, when AOM is due to invasive *H. influenzae*, the epithelial cells of the middle ear mucosa will be

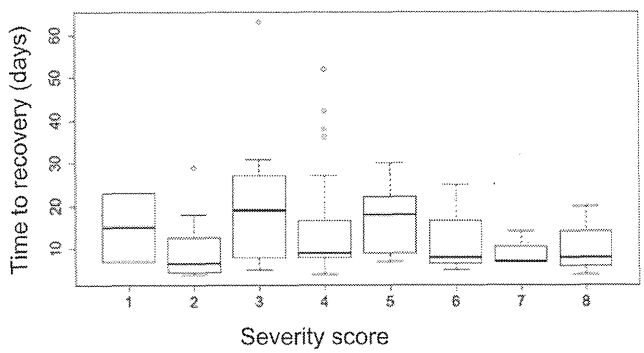

FIG 1 Box-and-whisker plot of times to recovery. The bold lines indicate medians, and the bottom and top of each box indicate the 25th and 75th percentiles, respectively. There was no significant difference in the times to recovery (when the severity scores decreased to 0) between patients with different scores at the initial examination ($P = 0.46$).

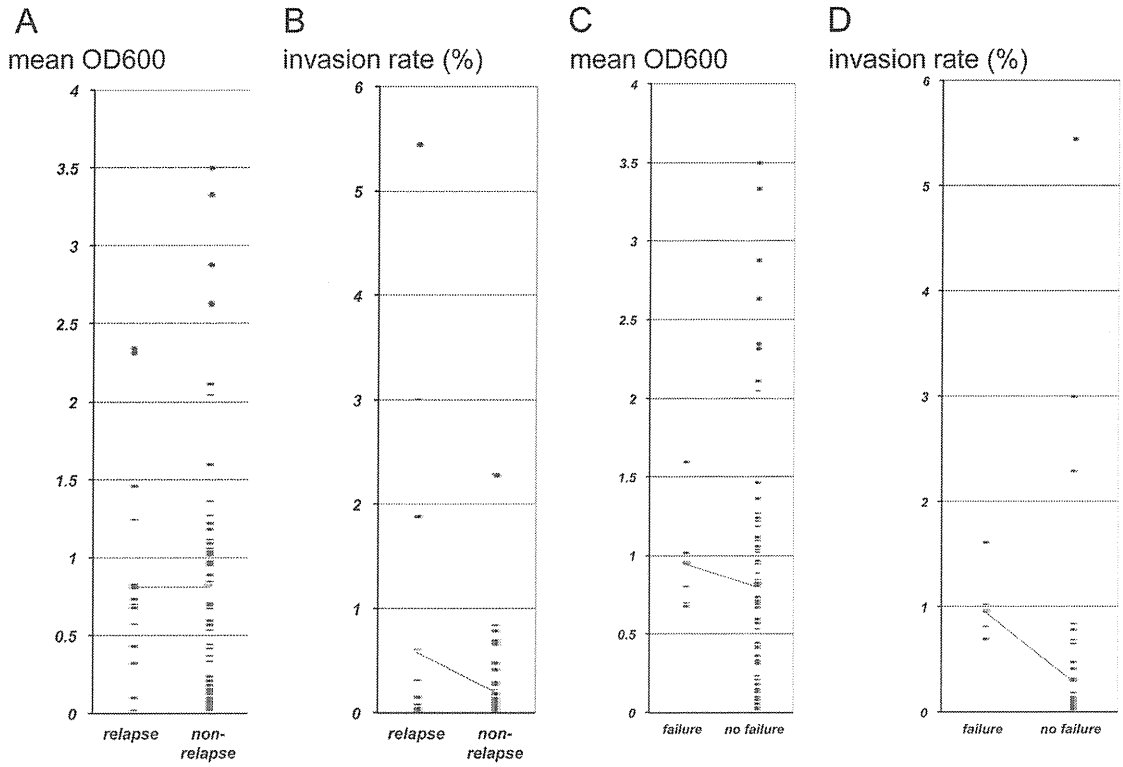

FIG 2 Dot plots of the mean OD_{600} (A) and the invasion rate (%) (B) in patients with or without relapse, and dot plots of the mean OD_{600} (C) and the invasion rate (%) (D) in patients with or without treatment failure. The OD results are measures of the ability of the organisms to form biofilms. Although the invasion rates of *H. influenzae* isolates for BEAS-2B cells were higher among the patients with relapses (0.57% ± 1.41%) than among those without relapses (0.19% ± 0.46%), there was no significant difference. There was no significant association between biofilm formation and relapse or treatment failure.

destroyed and damaged, so AOM tends to relapse if the patient acquires another respiratory tract infection.

This study showed that the improvement rate in the severity score at 1 week after the initial examination was significantly associated with the time to recovery. It is important for physicians and the parents of children with AOM to be able to predict the outcome of AOM at 1 week after the start of treatment. When there is little improvement in the score at 1 week, physicians should consider switching to another antibiotic.

In this study, we did not identify any significant associations between the clinical findings or the outcomes and the bacterial factors. Treatment failures and relapses of AOM in children are influenced not only by microbiological factors, such as antibiotic resistance (10), viral coinfection (16, 17), biofilm formation (11, 12), and invasion of epithelial cells (18), but also by host factors, including immaturity of the immune system (17), lack of breast-feeding (19), tubal dysfunction (20), recent antibiotic usage (21), and multiple episodes of AOM. In addition, environmental factors such as attending a day care center (2) and the presence of siblings (22) influence treatment failure and relapse of AOM. This complexity may explain why we could not identify a significant association with any of the bacterial factors. Further studies in a larger number of AOM patients are needed to more accurately assess the risk factors for this disease.

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The authors declare no conflicts of interest.

REFERENCES

1. Rovers MM, Schilder AG, Zielhuis GA, Rosenfeld RM. 2004. Otitis media. Lancet 363:465–473.
2. Yano H, Suetake M, Kuga A, Irinoda K, Okamoto R, Kobayashi T, Inoue M. 2000. Pulsed-field gel electrophoresis analysis of nasopharyngeal flora in children attending a day care center. J. Clin. Microbiol. 38: 625–629.
3. Jurcisek J, Greiner L, Watanabe H, Zaleski A, Apicella MA, Bakaletz LO. 2005. Role of sialic acid and complex carbohydrate biosynthesis in biofilm formation by nontypeable *Haemophilus influenzae* in the chinchilla middle ear. Infect. Immun. 73:3210–3218.
4. Kaji C, Watanabe K, Apicella MA, Watanabe H. 2008. Antimicrobial effect of fluoroquinolones for the eradication of nontypeable *Haemophilus influenzae* isolates within biofilms. Tohoku J. Exp. Med. 214:121–128.
5. Forsgren J, Samuelson A, Ahlin A, Jonasson J, Rynnel-Dagöö B, Lindberg A. 1994. *Haemophilus influenzae* resides and multiplies intracellularly in human adenoid tissue as demonstrated by in situ hybridization and bacterial viability assay. Infect. Immun. 62:673–679.
6. Okabe T, Yamazaki Y, Shiotani M, Suzuki T, Shiohara M, Kasuga E, Notake S, Yanagisawa H. 2010. An amino acid substitution in PBP-3 in *Haemophilus influenzae* associate with the invasion to bronchial epithelial cells. Microbiol. Res. 165:11–20.
7. Dagan R, Leibovitz E, Fliss DM, Leiberman A, Jacobs MR, Craig W, Yagupsky P. 2000. Bacteriologic efficacies of oral azithromycin and oral cefaclor in treatment of acute otitis media in infants and young children. Antimicrob. Agents Chemother. 44:43–50.

8. **Clinical and Laboratory Standards Institute.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard M7-A7, 7th ed, vol 23. Clinical and Laboratory Standards Institute, Wayne, PA.
9. **Kaczmarek FS, Gootz TD, Dib-Hajj F, Shang W, Hallowell S, Cronan M.** 2004. Genetic and molecular characterization of beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae* with unusually high resistance to ampicillin. Antimicrob. Agents Chemother. **48:**1630–1639.
10. **Hirakata Y, Ohmori K, Mikuriya M, Saika T, Matsuzaki K, Hasegawa M, Hatta M, Yamamoto N, Kunishima H, Yano H, Kitagawa M, Arai K, Kawakami K, Kobayashi I, Jones RN, Kohno S, Yamaguchi K, Kaku M.** 2009. Antimicrobial activities of piperacillin-tazobactam against *Haemophilus influenzae* isolates, including β-lactamase-negative ampicillin-resistant and β-lactamase-positive amoxicillin-clavulanate-resistant isolates, and mutations in their quinolone resistance-determining regions. Antimicrob. Agents Chemother. **53:**4225–4230.
11. **Torretta S, Marchisio P, Drago L, Baggi E, De Vecchi E, Garavello W, Nazzari E, Pignataro L, Esposito S.** 2012. Nasopharyngeal biofilm-producing otopathogens in children with nonsevere recurrent acute otitis media. Otolaryngol. Head Neck Surg. **146:**991–996.
12. **Bakaletz LO.** 2012. Bacterial biofilms in the upper airway—evidence for role in pathology and implications for treatment of otitis media. Paediatr. Respir. Rev. **13:**154–159.
13. **van Schilfgaarde M, Eijk P, Regelink A, van Ulsen P, Everts V, Dankert J, van Alphen L.** 1999. *Haemophilus influenzae* localized in epithelial cell layers is shielded from antibiotics and antibody-mediated bactericidal activity. Microb. Pathog. **26:**249–262.
14. **Ahrén IL, Williams DL, Rice PJ, Forsgren A, Riesbeck K.** 2001. The importance of a β-glucan receptor in the nonopsonic entry of nontypeable *Haemophilus influenzae* into human monocytic and epithelial cells. J. Infect. Dis. **184:**150–158.
15. **Ahrén IL, Karlsson E, Forsgren A, Riesbeck K.** 2002. Comparison of the antibacterial activities of ampicillin, ciprofloxacin, clarithromycin, telithromycin and quinupristin/dalfopristin against intracellular nontypeable *Haemophilus influenzae*. J. Antimicrob. Chemother. **50:**903–906.
16. **Ruohola A, Meurman O, Nikkari S, Skottman T, Salmi A, Waris M, Osterback R, Eerola E, Allander T, Niesters H, Heikkinen T, Ruuskanen O.** 2006. Microbiology of acute otitis media in children with tympanostomy tubes: prevalences of bacteria and viruses. Clin. Infect. Dis. **43:**1417–1422.
17. **Yano H, Okitsu N, Hori T, Watanabe O, Kisu T, Hatagishi E, Suzuki A, Okamoto M, Ohmi A, Suetake M, Sagai S, Kobayashi T, Nishimura H.** 2009. Detection of respiratory viruses in nasopharyngeal secretions and middle ear fluid from children with acute otitis media. Acta Otolaryngol. **129:**19–24.
18. **Hotomi M, Arai J, Billal DS, Takei S, Ikeda Y, Ogami M, Kono M, Beder LB, Toya K, Kimura M, Yamanaka N.** 2010. Nontypeable *Haemophilus influenzae* isolated from intractable acute otitis media internalized into cultured human epithelial cells. Auris Nasus Larynx **37:**137–144.
19. **Abrahams SW, Labbok MH.** 2011. Breastfeeding and otitis media: a review of recent evidence. Curr. Allergy Asthma Rep. **11:**508–512.
20. **Bylander A.** 1986. Pathophysiological aspects on eustachian tube function and SOM. Scand. Audiol. Suppl. **26:**59–63.
21. **Pettigrew MM, Laufer AS, Gent JF, Kong Y, Fennie KP, Metlay JP.** 2012. Upper respiratory tract microbial communities, acute otitis media pathogens, and antibiotic use in healthy and sick children. Appl. Environ. Microbiol. **78:**6262–6270.
22. **Ladomenou F, Kafatos A, Tselentis Y, Galanakis E.** 2010. Predisposing factors for acute otitis media in infancy. J. Infect. **61:**49–53.

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Removal of waterborne pathogens from liver transplant unit water taps in prevention of healthcare-associated infections: a proposal for a cost-effective, proactive infection control strategy

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Abstract

Hospital water supplies often contain waterborne pathogens, which can become a reservoir for healthcare-associated infections (HAIs). We surveyed the extent of waterborne pathogen contamination in the water supply of a Liver Transplant Unit. The efficacy of point-of-use (POU) water filters was evaluated by comparative analysis in routine clinical use. Our baseline environmental surveillance showed that *Legionella* spp. (28%, 38/136), *Pseudomonas aeruginosa* (8%, 11/136), *Mycobacterium* spp. (87%, 118/136) and filamentous fungi (50%, 68/136) were isolated from the tap water of the Liver Transplant Unit. 28.9% of *Legionella* spp.-positive water samples (n = 38) showed high-level *Legionella* contamination ($\geq 10^3$ CFU/L). After installation of the POU water filter, none of these pathogens were found in the POU filtered water samples. Furthermore, colonizations/infections with Gram-negative bacteria determined from patient specimens were reduced by 47% during this period, even if only 27% (3/11) of the distal sites were installed with POU water filters. In conclusion, the presence of waterborne pathogens was common in the water supply of our Liver Transplant Unit. POU water filters effectively eradicated these pathogens from the water supply. Concomitantly, healthcare-associated colonization/infections declined after the POU filters were installed, indicating their potential benefit in reducing waterborne HAIs.

Keywords: Healthcare-associated infection, hospital water supply, infection control, POU water filter, waterborne pathogens
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Introduction

Hospital water supplies have served as reservoirs for waterborne pathogens such as *Legionella* spp., *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Acinetobacter* spp., *Mycobacterium* spp. and fungi [1–5]. The degree of the colonization in water supplies has been correlated with the incidence of healthcare-associated infections (HAIs) [6,7]. Forty-two per cent of ICU patients with *Pseudomonas aeruginosa* harbored isolates with identical genotypes to those found in the taps [8]. Water supplies were recognized as one of the most important and controllable, and yet the most overlooked, sources of HAIs [1,2].

Despite water treatment with chlorination, domestic water supplies may still be contaminated by low concentrations of various microorganisms [9]. Although most of the microorganisms are not harmful to the general public, some opportunistic pathogens pose threats to hospitalized patients. In China, the waterborne pathogen contaminations of water supplies have often been overlooked. In fact, the European Working Group for Legionella Infections (EWGLI) reported in 2009 that China was one of the top 15 countries implicated in cases of travel-associated Legion-

naires' disease [10]. In a study of eight hospital water supplies in Shanghai [11], 43.0% (83/193) of water samples were positive for *Legionella* spp., and 63 water samples exceeded the concentration of 10^3 CFU/L. So, we sought to determine if waterborne pathogens were present in the water supply of our hospital, especially in the Liver Transplantation Unit (LTU), where the patients are most susceptible to opportunistic infections. Furthermore, could removal of these waterborne pathogens reduce the incidence rate of hospital-acquired infections in the LTU? Thus, we performed an infection control intervention by: (i) investigating the baseline frequency of waterborne pathogens in the water supply of the LTU, and (ii) evaluating the efficacy of point-of-use (POU) water filters in removing waterborne pathogens. To our knowledge, this is the first environmental surveillance of waterborne pathogens in a hospital water supply in China.

Materials and Methods

Study site

This study was performed in an 18-bed LTU of a university-affiliated general hospital with 1600 beds in Shanghai, China. The Unit consists of nine patient rooms (two patient beds and one sink/tap in each room), one nurses' station and one doctor's office. The hospital receives its water from a municipal water treatment plant without additional on-site disinfection.

Study design

Cold tap water samples were collected between 2009 and 2011 (June, September and October in 2009, January, July, August, September, October and November in 2010, and March in 2011) from each tap outlet in sterile containers with 0.01% w/v sodium thiosulphate.

Three taps located in one patient room, the nurses' station and the doctor's office were installed with 0.2 μm POU filters (AQ14F1S, Pall Corp., Port Washington, NY, USA) for removal of the waterborne pathogens (Fig. 1). A pre-filtration fixture (pore size, 1.2 μm) was also installed for capturing particulate debris to extend the life of the POU filter. Filters were changed every 2 weeks according to the manufacturer's instructions from July to November 2010 (18 weeks), and water samples were collected and cultured every 3–4 days. The unfiltered tap water sample served as the control, while the water filtered through the pre-filter alone served as the pre-filtered water control. We picked the doctor's office and nurses' station for installation so that all medical staff had access to filtered (pathogen-free) water before and between patients' care.

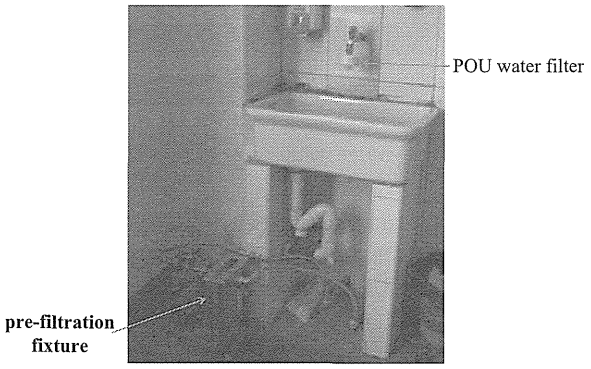

FIG. 1. Tap installed with POU water filter and pre-filtration fixture.

The incidence of Gram-negative bacteria colonization/infection in the LTU was also monitored. We analyzed patient-related data for the same 4-month period before the installation of the water filters (from July to November 2009) and a corresponding 4-month period after outlets had been equipped with filters (from July to November 2010). Patient data were retrieved from the hospital surveillance system. Microbiological cultures from patients were performed only when clinically indicated. No additional control measures were carried out during this period.

Microbiological analysis

Total heterotrophic plate count (HPC) bacteria, cultured on R_2A agar (Oxoid, Basingstoke, UK) at 25°C for 14 days, were enumerated by the standard pour plate method [12]. *Legionella* spp. was monitored using GVPC selected agar (Oxoid) according to ISO 11 731 [13]. Colonies morphologically consistent with *Legionella* spp. were identified by the latex agglutination test (Oxoid). For *Pseudomonas aeruginosa*, filamentous fungi and *Mycobacterium* spp. detection, water samples were filtered (pore size of 0.45 μm, Millipore, USA) and the filter membrane was placed on Cetrimide agar plates, Sabouraud dextrose agar plates containing 25 mg/L penicillin and 400 mg/L chloramphenicol (Oxoid) and Middlebrook 7H10 plates (BD, Franklin Lakes, NJ, USA), and incubated at 35°C for 48 h, 30°C for 28 days and 35°C for up to 8 weeks, respectively.

Statistical analyses

An ANOVA (SPSS ver. 15.0) was used to analyze the bacterial counts in POU-filtered, pre-filtered and unfiltered control samples. Comparison of the incidence of Gram-negative bacterium infection/colonization in the post-filtration period with that in the pre-filtration period was carried out by use of the chi-squared test (SPSS ver. 15.0). The correlation coefficient of temperature and the number of positive water samples were calculated by use of two-tailed Spearman's analysis.

Results

Baseline of waterborne pathogens in the LTU

A total of 136 cold water samples were enumerated for the targeted pathogens. *Legionella* spp, *Pseudomonas aeruginosa*, *Mycobacterium* spp. and filamentous fungi were detected in 38 (27.9%), 11 (8.1%), 118 (86.8%) and 68 (50.0%) water samples, respectively. HPC bacteria were detected in all the water samples, with a mean concentration of 1.1×10^7 CFU/L. Among the isolated *Legionella*, 29 of these (76.3% of positive samples and 21.3% of the total) were identified as *L. pneumophila*. Furthermore, 28.9% of *Legionella* spp.-positive samples were detected with high-level contamination ($\geq 10^3$ CFU/L). More than 18% (7/38) of the samples were positive for both *L. pneumophila* and *Legionella* of other species (Table 1).

Mycobacterium spp. was isolated from almost all water samples throughout the study. However, if we examine the data without considering *Mycobacterium* spp., sampling sites positive for target pathogens were higher in the hot season (from June to October), averaging 5.7 sites positive/month (40/7), compared with the cold season (from November to March), averaging three sites positive/month (9/3), which is almost a two-fold increase. Some pathogens seemed to persist in some outlets for a long time; for example, filamentous fungi were isolated from tap water of room one during the entire study period. We also found that the positive rate of *Legionella* spp. correlated with temperature fluctuations of tap water (correlation coefficient = 0.907; p 0.000), which suggested that cold water temperature below 20°C might be considered protective against *Legionella* contamination (Fig. 2).

Control modality using POU filter

As the water samples were found to be highly contaminated by *Legionella* spp. (10^3–10^4 CFU/L), three POU water filters were installed. From July to November 2010, a total of 190 water samples were collected from these three tap outlets, of which 57 were unfiltered water, 43 were pre-filtered water and 90 were POU-filtered water. No significant difference was observed in *Legionella* isolation between pre-filtered and unfiltered water (Table 2). In contrast, all samples filtered by the POU water filter were culture-negative for any of these pathogens. The difference in isolation between POU-filtered and control water was significant at $p < 0.05$. It is noteworthy that one of 34 (2.9%) water samples tested positive for HPC bacteria after 3 days use of filters, four of eight (42.1%) water samples tested positive after 7 days use, and the positive rate increased to 69.2% after 14 days use. Retrograde contamination may occur during use over time.

The number of Gram-negative bacterium infection/colonization patients per 1000 patient-days of hospitalization in the post-filtration period (1.70 ± 0.95) was significantly lower than that in the pre-filtration period (3.20 ± 1.25; $\chi^2 = 2.119$, p 0.067). Gram-negative bacterium colonizations/infections were reduced by 46.9%.

Discussion

Opportunistic waterborne pathogens can be introduced into a healthcare facility water distribution system. Despite water treatment and a chlorine disinfection process, treated water may still contain low concentrations of various microorganisms, such as *Legionella*, *P. aeruginosa*, non-tuberculous mycobacteria and fungi (e.g. *Aspergillus*). Pathogens can enter the water system of healthcare facilities and can colonize the water supply piping, hot water tanks, sinks, faucet aerators and shower heads. Hospital water distribution systems might be one of the most important sources of HAIs [1]. Thus, the World Health Organization (WHO) published its fourth edition of 'Guidelines for Drinking-Water Quality' [9], which specifically stated the importance of disinfection of the water supply as a control measure to prevent healthcare-associated infections. However, as in healthcare facilities throughout the world, no mandate exists for Chinese healthcare facilities to survey for waterborne pathogens in the water supply of healthcare facilities.

We conducted this prospective surveillance in the absence of any recognized outbreak attributable to waterborne pathogens of *Legionella* spp., *Pseudomonas aeruginosa*, non-tuberculous mycobacteria and filamentous fungi. A high prevalence rate of waterborne pathogens was found in the water supply of the

TABLE 1. Characteristics of pathogen contamination in the cold water samples without filter installation (n = 136)

Parameters	*Legionella* spp.	*Legionella pneumophila*	*Pseudomonas aeruginosa*	*Mycobacterium* spp.	*Filamentous fungi*	HPC bacteria[b]
Positive samples, No. (%)	38 (27.9)	29 (21.3)	11 (8.1)	118 (86.8)	68 (50.0)	136 (100.0)
Samples with $>10^3$ CFU/L, No. (%)	11 (8.1)	8 (5.9)	0	19 (13.9)	0	136 (100.0)
Geometric mean count (CFU/L[a], Mean (Range))	2.9×10^3 (50–5.8×10^4)	3.4×10^3 (100–2.0×10^4)	70.0 (5–3.6×10^2)	5.9×10^2 (2–5.0×10^3)	41.5 (10–62)	1.1×10^7 (1.0×10^4–3.4×10^8)

[a]Only positive samples were included.
[b]HPC, heterotrophic plate count.