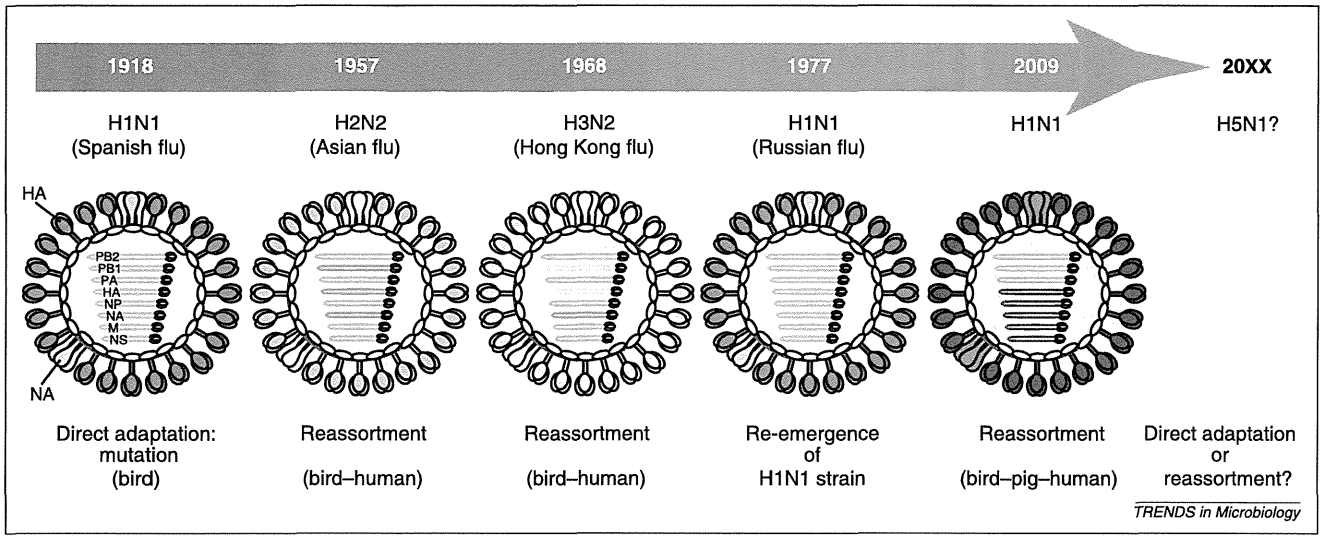


Figure 1. Timeline and evolution of pandemic influenza viruses. Pandemics in the 20th century were caused by infection by an avian influenza virus (1918), an avian–human reassortment virus containing avian HA, NA and PB1 (1957), and an avian–human reassortment virus containing avian HA and PB1 (1968), with the other gene segments from the circulating human virus. Re-emergence of the H1N1 strain that circulated in the 1950s led to a limited pandemic in 1977. The latest pandemic influenza (2009) was caused by an avian–swine–human reassortment producing a virus containing PB2 and PA from an avian virus, PB1 from a human virus, and the other gene segments from two distinct lineages of swine viruses. Future pandemic strains could arise through any of the mechanisms: adaptation by accumulation of mutation(s), and genetic reassortment and re-emergence of a virus that has not infected humans for a generation. Common names of the past pandemics and the virus origins are shown in parentheses at the top and bottom, respectively.

and adapted to replication in humans, the 1957 and 1968 pandemics by reassortments producing human influenza viruses containing HA and PB1 gene (and/or NA gene) segments from an avian virus, and the 1977 pandemic by re-emergence of the H1N1 virus [2,18]. However, the most recent influenza pandemic emerged in 2009 by an avian–swine–human reassortment producing a virus containing PB2 and PA genes from an avian virus, PB1 from a human virus, and the other gene segments from two distinct lineages of swine viruses [7,19].

Aside from the past human pandemics, five AI subtypes are known to have produced sporadic human disease after direct transmission from an avian host: HPAI viruses H5N1, H7N3 and H7N7, and LPAI viruses H7N2, H7N3, H7N7, H9N2 and H10N7 [3]. The mechanism(s) that determine the transmissibility to humans and pandemic potential of these AI viruses have still not been fully elucidated [7,18]. Three criteria for a new influenza virus strain to have the potential to produce a pandemic are: (i) emergence (or re-emergence) of an influenza virus HA subtype that has not infected humans for at least one generation, (ii) high infectivity in humans, and (iii) sustainable transmission among humans (Figure 2a) [3,9]. However, because viral adaptation in different hosts is multifactorial [9], no clear genetic pattern has emerged to define the changes necessary for the evolution of a pandemic human virus [7]. It is uncertain which route the next pandemic influenza virus will take (Figure 1).

The HPAI H5N1 virus is not currently a pandemic virus and remains an avian virus [20,21], but it is undergoing continual evolution [22]. Outbreaks of HPAI H5N1 viruses were first recorded in poultry in Guangdong, China in 1996 [23]. Since its emergence the A/goose/Guangdong/1/96 (Gs/GD) virus lineage has probably become the best-studied HPAI virus (Figure 3, upper panel). This virus has gradually become endemic in poultry in different regions of China, developing into genetically and antigenically distinct sublineages.

Influenza virus surveillance in China has revealed that since 1997, the Gs/GD virus lineage has undergone frequent reassortment with different AI viruses that were circulating in the region and has generated many different viruses with a genetic shift (or genotype) [2,24]. These reassortment viruses had considerable variability in their combination of internal genes, although each had a Gs/GD virus-derived HA. Based on their internal genes, the genotypes were designated A, B, C, D, E, V, W, X0-3, Y, Z and Z+ [2,24]. Furthermore, a novel genotype, designated P, emerged in the Lao People's Democratic Republic (PDR) in 2007, marking the first case of reassortment between the Gs/GD virus lineage and another AI virus outside China [25]. Continuing outbreaks of the Gs/GD virus lineage have also led to the accumulation of point mutations in the viral HAs, generating genetic and antigenic changes. According to the current classification, HA genes are grouped phylogenetically into clades 0–10 [4]. These HA clades correlate well with antigenic differences [4]. Thus, reassortment of Gs/GD internal genes and antigenic evolution of its HA by point mutations have been interactively involved in the emergence of dominant variants of the Gs/GD virus lineage [26].

The ecology of the Gs/GD virus lineage has varied over time [4,27]. Historically, HPAI has emerged transiently, mainly in localized areas in populations of gallinaceous species, owing to mass die-offs due to the high mortality of HPAI infections (Figure 4a) [28]. HPAI viruses were also thought not to persist in wild bird populations [28]. In fact, there had been little evidence of Gs/GD virus lineage infections in wild birds, even during the early endemic infections in land poultry in 1996–2001 (Figure 4b) [4]. However, this situation has changed dramatically since 2002 because aquatic and terrestrial wild birds died following infection

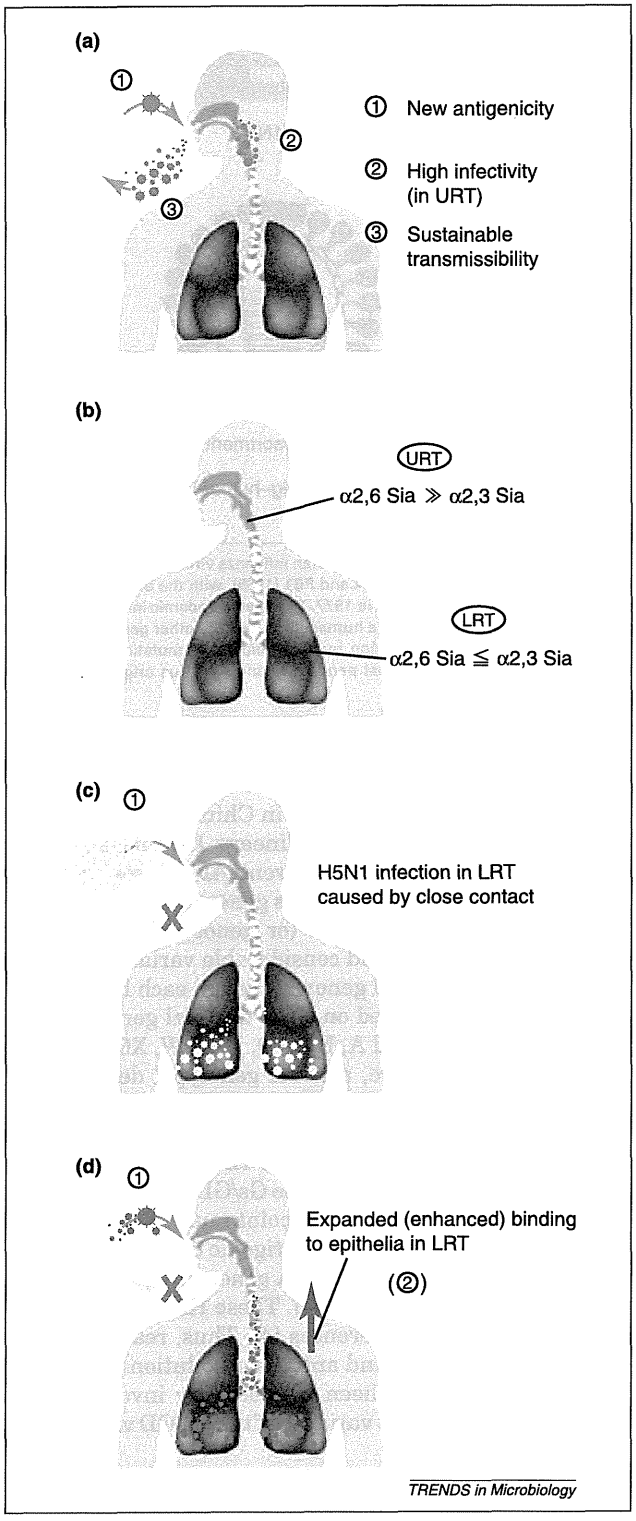


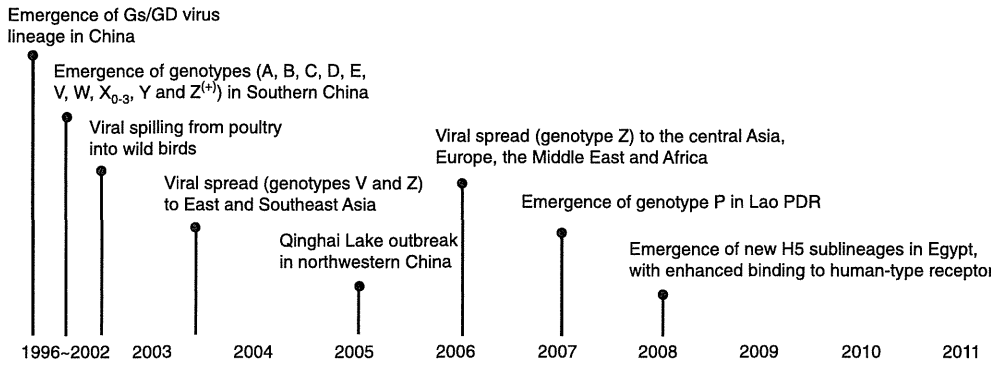
Figure 2. Influenza virus adaptation in humans for a pandemic. **(a)** Criteria for influenza virus to be a pandemic strain. The three criteria shown here must be met for a new pandemic to arise. URT, upper respiratory tract. **(b)** Distribution of α2,3 and α2,6 sialylglycans in the human respiratory tract. The URT contains abundant α2,6 sialylglycan (α2,6 Sia; human-type receptor) and less α2,3 sialylglycan (α2,3 Sia; avian-type receptor). By contrast, the lower respiratory tract (LRT) contains substantial α2,3 Sia. This distribution pattern imposes a primary species barrier for AI viruses. **(c)** Pathology of the Gs/GD virus lineage (H5N1) in humans. The virus lineage only has binding affinity for α2,3 sialylglycan and needs to reach the LRT [52,53]. The pandemic criterion met is marked as a circled number. **(d)** Pathology of the H5 sublineage of the Gs/GD virus lineage that has emerged in Egypt. Expansion of receptor usage (increased α2,6 sialylglycan binding) enables the new sublineage to bind more efficiently to epithelia in the LRT than the parental virus lineage, but this new sublineage still lacks binding affinity to the URT and sustainable transmissibility in humans.

with Gs/GD lineage viruses (Figure 4c) [1,4]. Large outbreaks of genotype Z viruses at Qinghai Lake in northwestern China in 2005 characterized the strikingly varied phenotypes of this virus lineage, with high mortality in a variety of wild aquatic birds [24,29]. The viruses then spread unexpectedly over large geographic areas, apparently via wild bird migration in winter 2006, spreading westward to Central Asia, Europe, the Middle East and Africa [30]. Several experimental infection studies also suggested a role of wild birds in the spread and maintenance of recent Gs/GD lineage viruses [6,31]. As of August 2011, 7030 poultry and/or wild-bird outbreaks have been reported from over 50 countries and HPAI has become endemic in birds in at least four countries (China, Indonesia, Vietnam and Egypt) (World Organization for Animal Health; http://www.oie.int/animal-health-in-the-world/update-on-avian-influenza/). Although large outbreaks in wild birds have rarely been reported since 2006, the virus appears to have been maintained in wild bird populations, providing opportunities for spread back to domestic poultry that make it difficult to control. Such intertwined ecology of the Gs/GD virus lineage has recently generated multiple H5 sublineages in endemic areas. Among them, a number of phylogenetically and antigenically distinct H5 sublineages have been isolated in Egypt, but none has yet become dominant, and these viruses are currently co-circulating in the local bird population [32]. Co-circulation of different H5 sublineages has also allowed reassortment among the sublineages in Vietnam [33] and Nigeria [34]. Therefore, the Gs/GD virus lineage has reached epizootic levels in both domestic and wild bird populations across Eurasia and Africa.

Compared to earlier H5N1 viruses, the recent Gs/GD virus lineage has shown the ability to cross the species barrier and infect a broad range of mammalian species, including humans [32,35–37]. The cumulative number of confirmed human cases of H5N1 infection reported to the World Health Organization (WHO; http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/) to date is 566 with 60% mortality (Figure 3, lower panel). More than 80% of the total human H5N1 influenza cases have been reported in AI endemic areas, indicating hotspots for bird-to-human transmission. In addition to infections in humans, other mammals such as tigers, leopards, dogs, cats and a stone marten have recently been symptomatically infected with an HPAI H5N1 virus by feeding on bird carcasses [27]. Palm civets [35], donkeys [36] and pigs [38] were also shown to be naturally infected with this virus lineage. Furthermore, experimental infections have been established in mice, ferrets, monkeys, cattle and foxes [27]. Thus, the current Gs/GD virus lineage has exceptionally strong zoonotic properties.

Receptor binding

Influenza virus infection requires binding of viral HA to host glycans or gangliosides that terminate in sialic acids (Sias), but there are distinct differences in the forms of



Country	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths
China	1	1	0	0	8	5	13	8	5	3	4	4	7	4	2	1	0	0	40	26
Vietnam	3	3	29	20	61	19	0	0	8	5	6	5	5	5	7	2	0	0	119	59
Thailand	0	0	17	12	5	2	3	3	0	0	0	0	0	0	0	0	0	0	25	17
Cambodia	0	0	0	0	4	4	2	2	1	1	1	0	1	0	1	1	8	8	18	16
Indonesia	0	0	0	0	20	13	55	45	42	37	24	20	21	19	9	7	8	6	179	147
Azerbaijan	0	0	0	0	0	0	8	5	0	0	0	0	0	0	0	0	0	0	8	5
Turkey	0	0	0	0	0	0	12	4	0	0	0	0	0	0	0	0	0	0	12	4
Egypt	0	0	0	0	0	0	18	10	25	9	8	4	39	4	29	13	32	12	151	52
Nigeria	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1
Other	0	0	0	0	0	0	4	2	6	3	1	0	0	0	0	0	2	0	13	5
Total	4	4	46	32	98	43	115	79	88	59	45	33	73	32	48	24	50	26	566	332

From WHO, updated on 10th October 2011.

TRENDS in Microbiology

Figure 3. Timeline of Gs/GD virus lineage epidemiology. (Upper panel) Major events since 1996 in the Gs/GD virus lineage (H5N1), which is epizootic in bird species. (Lower panel) Confirmed human H5N1 infection cases reported to the World Health Organization (WHO; http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/). It is noteworthy that emergence of a new H5 sublineage in Egypt, with enhanced binding to human-type receptors, caused that country to have the highest number of human infections worldwide after 2009.

these molecules that are recognized (Figure 5a) [10,39]. Virus affinity for different sialyl sugar structures is an important determinant of virus host range and pathogenicity [2,10].

Sias are a family of sugars with a nine-carbon backbone that are typically found attached to the end of glycoconjugate chains [Figure 5a(i)] [10,39]. There are over 50 natural modifications of the Sia core structure, including esterification with acetyl, glycolyl, lactyl, sulfate and phosphate groups [39]. Sias bind to cell-membrane sugars through an α2,3, α2,6, α2,8 or α2,9 linkage catalyzed by sialyltransferases that are expressed in a tissue- and species-specific manner [Figure 5a(ii)] [40]. The most common sialyl terminal substituents are *N*-acetylneuraminic acid-α2,3-galactose (NeuAcα2,3 Gal) and *N*-acetylneuraminic acid-α2,6-galactose (NeuAcα2,6 Gal) [10]. Human influenza viruses preferentially bind to sugar chains ending in NeuAcα2,6 Gal, whereas most avian viruses preferentially bind to sugar chains ending in NeuAcα2,3 Gal [41,42]. As expected from this specificity, human upper-airway epithelia express mostly NeuAcα2,6 Gal [43], whereas duck intestinal epithelia express mostly NeuAcα2,3 Gal [41,44]. This provides an interspecies barrier preventing avian viruses from easily infecting humans. However, swine tracheal epithelial cells contain both NeuAcα2,3 Gal and NeuAcα2,6 Gal [45,46], which explains why pigs are highly susceptible to both human and avian viruses and serve as an intermediate host acting as a 'mixing vessel' [47].

Surprisingly, it was recently found that this restriction mechanism(s) is not a complete barrier to interspecies transmission [48]. An HPAI H5N1 virus that only binds to an α2,3 Sia linkage emerged in China in 1997 and has been occasionally been transmitted directly from birds to humans [20,49]. Recent lectin histochemistry analyses demonstrated that the NeuAcα2,3 Gal receptor for AI viruses is more widely expressed in the human respiratory tract than was previously thought [43]. Although NeuAcα2,6 Gal oligosaccharides are dominant on ciliated and non-ciliated cells in human upper-airway epithelia, NeuAcα2,3 Gal oligosaccharides are also located on ciliated cells [50,51] and alveolar epithelia (Figure 2b) [52,53]. The expression profile of NeuAcα2,6 Gal and NeuAcα2,3 Gal on cell surfaces also changes depending on the disease and host age [11,54,55]. These findings suggest that the potential of direct bird-to-human transmission of avian viruses with a NeuAcα2,3 Gal preference may be greater than previously supposed [56,57].

Chandrasekarn *et al.* recently revised this linkage paradigm by showing that the topology of sialylated pentasaccharides, widely present in the human upper airway, can modulate the receptor-binding properties of influenza A viruses beyond specific α2,3 and α2,6 Sia linkages [Figure 5a(iii)] [51]. Mass spectrometry analyses have shown a remarkable diversity of sialylated *N*-glycans in human upper respiratory epithelia, with long α2,6 oligosaccharide branches (i.e. pentasaccharide or longer) predominating. Although this observation suggests a high

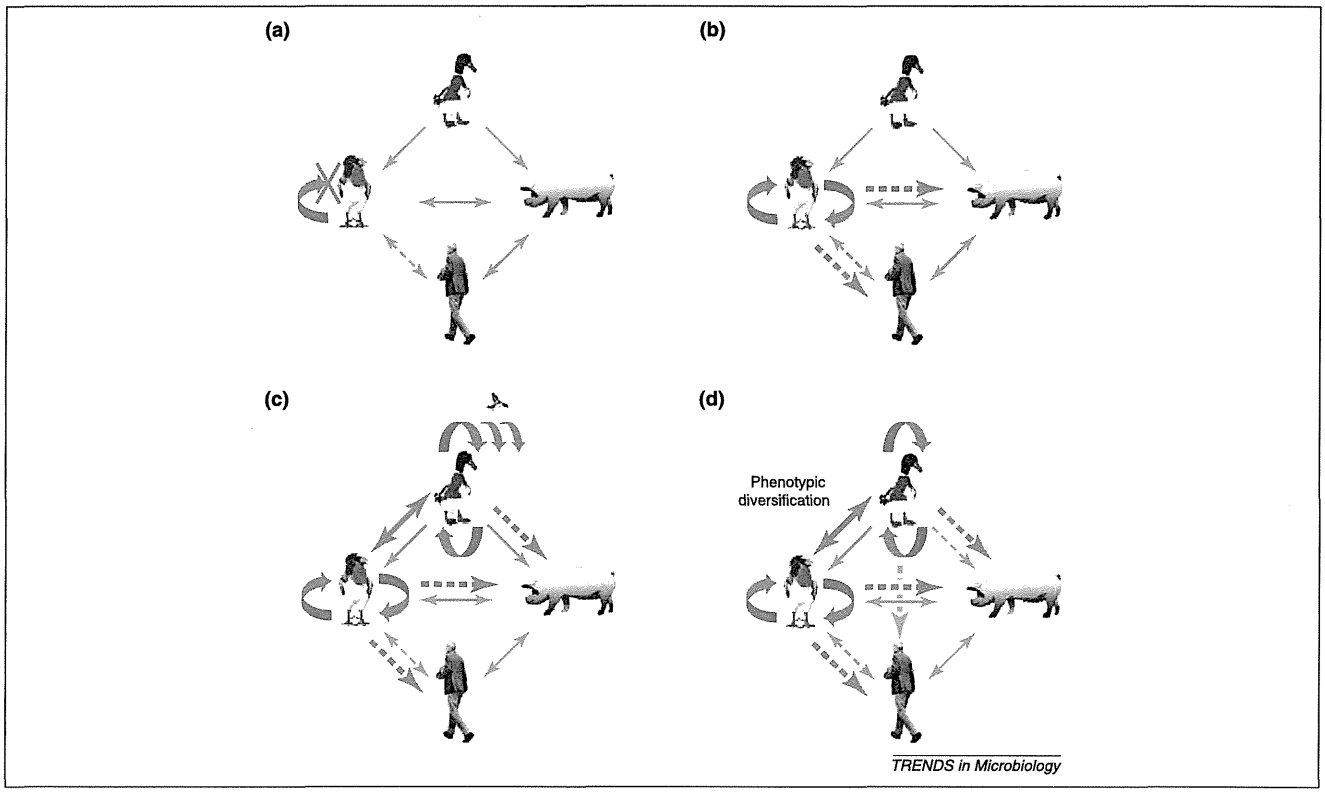


Figure 4. Changing ecology and interspecies transmission of the Gs/GD virus lineage. Schematic of interspecies transmission between wild waterfowl, land-based poultry, pigs and humans. Grey arrows represent the infectious routes of influenza A virus. Red arrows show dissemination of the Gs/GD virus lineage (H5N1). Frequent viral cross-species transmission is depicted as a broken arrow or a dotted arrow. **(a)** Before the emergence of Gs/GD virus in 1996, highly pathogenic avian influenza had emerged transiently in land-based poultry in localized areas (traditional outbreak dynamics). **(b)** In 1997–2001, the Gs/GD virus lineage became endemic in land-based poultry in China and caused sporadic infections in humans and pigs. **(c)** In 2002–2006, several genotypes of the Gs/GD virus lineage infected a variety of wild birds and caused severe outbreaks. The Gs/GD virus lineage unexpectedly spread outside China in 2006 and thereafter spread over large distances through bird migration. **(d)** Since 2007, virus persistence in a wide range of birds has allowed phenotypic diversification in endemic areas and has led to the emergence of a new H5 sublineage in Egypt with enhanced binding affinity to the human lower respiratory tract. Also, a higher risk of human H5N1 infection due to contact with infected waterfowl has been reported recently in Egypt [32]. Thus, these results suggest expanding possibilities for viral transmission from birds to humans.

complexity of HA ligands in the respiratory tract, glycan–HA co-crystal structures showed that long human α2,6 sialylated glycans have a bent topology, whereby even distant carbohydrate groups contribute to HA binding via numerous contacts between carbohydrate residues 1–5 and the HA molecules (Figure 5b, lower panel) [51,58]. However, α2,3 and short α2,6 *N*-glycans invariably form a narrow topology for HA binding, and their interaction with the HA primarily involves contact between amino acids and only the first and second sugars (Figure 5b, upper panel). Indeed, HAs from human-adapted H1N1 and H3N2 viruses, but not from AI viruses, specifically bind to long α2,6 sialylated glycans, whereas only short *N*-glycans are receptors for avian strains regardless of the Sia linkage type [51,59]. These results suggest that recognition of the characteristic topology of long human α2,6 glycans, but not the α2,6 linkage itself, might be crucial for adaptation and pandemic spread of AI viruses in humans.

Influenza viruses recognize the further complexity generated by the sugar composition of glycan internal carbohydrate units [Figure 5a(iii)] [60]. Screening of HAs on a glycan microarray showed that the receptor specificity of different HAs differs markedly not only for Sia linkages but also for other internal glycan modifications, such as sulfation [61]. Furthermore, a study of H5N1 virus infection in *ex vivo* cultures of human nasopharyngeal tissues, on which Siaα2,3 Gal was not detected by a linkage-specific lectin, indicated that current HPAI H5N1 viruses could use other receptors via currently undetermined glycan topology to infect human upper airway epithelia [62]. In summary, it appears that influenza viruses recognize more complex glycan topologies on their target cells, which have not been fully elucidated, and different viruses use a different range of glycan receptors for viral entry [Figure 5a(i)–(v)], although human and avian virus HAs have primary specificity for α2,6- and α2,3-linked sialosides, respectively.

Spread of strains with increased affinity for α2,6 Sia

Almost all H5N1 HPAI viruses isolated from humans thus far display preferential binding for α2,3 Sia (Figure 2b,c) [44]. Several cases have been reported in Asia in which changes in viral amino acid residues surrounding the HA receptor-binding domain appear to have been positively selected in patients infected with HPAI H5N1 viruses [63,64]. These findings indicated that adaptation of this virus lineage to humans can take place by modification of receptor specificity. Indeed, several amino acid substitutions in H5 HAs conferring enhanced binding to α2,6 Sia have been described in virus isolates from patients or have

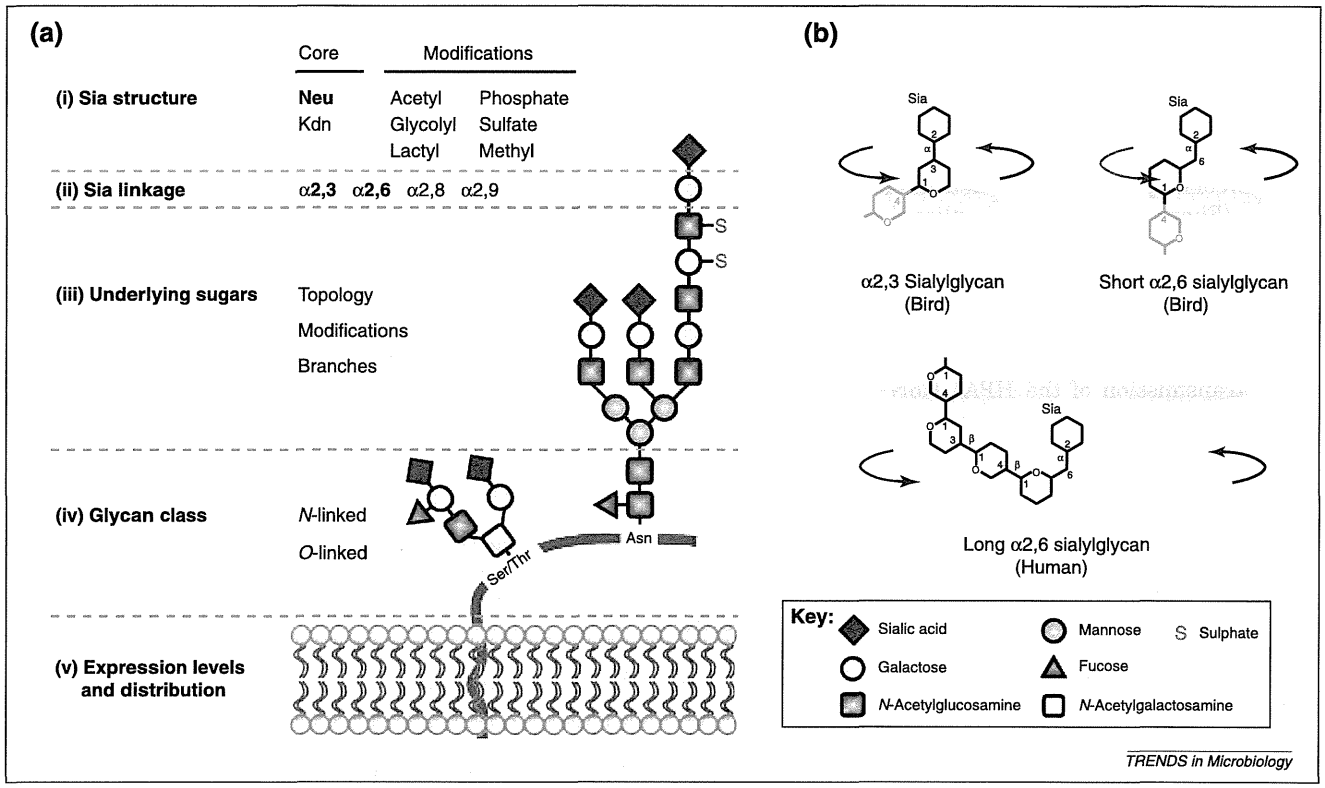


Figure 5. Complexity of sialylglycans recognized by influenza A virus. **(a)** Model of sialylglycan for analysis of hemagglutinin (HA) binding to receptors. **(i)–(v)** Representative structures and modifications of the glycans. The structural information in the entire glycan chain influences viral binding specificity, although human and avian virus HAs have a primary specificity for either terminal NeuAcα2,6 Gal or NeuAcα2,3 Gal, respectively (bold). The figure was adapted, with permission, from [39]. **(b)** Sialylglycan structure required for human adaptation of influenza A virus. Distinctly different topologies are adopted by α2,3 and short α2,6 sialylglycans (characteristic in birds) and long α2,6 sialylglycans (characteristic in humans) for binding HA. α2,3 and short α2,6 sialylglycans interact with HA with the first and second sugars in a narrow 3D topology. By contrast, long α2,6 sialylglycans, which are widely present in human upper airway epithelia [e.g. glycans containing the lactosamine repeat $(Gal\beta1,4GlcNAc\beta1,3)_n$] contact HA via their first to fifth carbohydrate residues in a wide 3D topology. AI viruses need to adapt to the characteristic human long α2,6 sialylglycan topology to infect humans efficiently. The figure was adapted, with permission, from [58].

been introduced experimentally [32,63,65,66]. However, the impact of such modifications on virus transmissibility to humans has not been fully elucidated, and there have been only limited reports of these mutations in H5N1 in human influenza cases [63,64].

By contrast, recent epidemiological studies of H5N1 found diverse populations of H5N1 viruses with altered receptor affinity in birds. In the Lao PDR, the novel genotype P virus emerged in 2007, with reduced affinity for α2,3 Sia [25]. More recently, some new sublineages of genotype Z have emerged in local bird populations in Egypt with enhanced α2,6 Sia receptor affinity and binding affinity for α2,3 Sia [32]. It was noted that these sublineages had increased attachment to and infectivity in the human lower respiratory tract. The emergence of this sublineage in 2008 coincided with an increase in human H5N1 virus infection cases in Egypt, causing this country to have the highest number of human H5N1 cases worldwide since 2009 (Figure 3). These findings suggest that viral acquisition of α2,6 Sia binding during viral diversification in birds can contribute to an increase in bird-to-human transmission efficiency (Figure 4d) [32]. Maintenance of the Gs/GD virus lineage in birds has clearly driven such phenotypic variation in the field, and this should be considered in all countries where H5N1 is endemic [25]. Because H5 variants have emerged and are circulating in some bird populations [32] that have habitats in human rural and urban areas, the emergence of such viral mutations in bird species could be important risk factors for human infections. The mechanism underlying the emergence of H5N1 viruses in Egypt with both α2,3 Sia and α2,6 Sia binding affinity is unclear. Geographic and cultural factors inherent in this region could provide different evolutionary pressures affecting the epidemiology of H5N1 viruses, leading to complex evolution of H5N1 viruses in nature with differing Sia-binding affinities [4].

As described above, some strains of the Gs/GD virus lineage have acquired binding affinity for the α2,6 linkage [32,66], but still have inefficient transmissibility to humans. One explanation for this restriction might be that a complete change in receptor specificity from α2,3 to α2,6 is necessary for virus replication to adapt to the human upper respiratory tract and for efficient viral transmission among humans [27,52]. In fact, most variant H5N1 strains have retained binding affinity for avian-type receptors [32,63,65,66], in contrast to the earliest isolates from the 1918, 1957 and 1968 pandemics, which had binding affinity for NeuAcα2,6 Gal even though their HAs were derived from an avian virus (Figure 2a,b) [67]. Another explanation could be that acquisition of HA binding to long α2,6 sialyl glycans, characteristic of human upper-airway epithelia, is a necessary condition for virus adaptation for human

infections, but loss of the ability to bind to an α2,3 linkage is not necessary for efficient transmission among humans [51,59], as observed for the human H1N1 A/Texas/36/91 strain [68]. The new H5 sublineages in Egypt, with significantly enhanced binding affinity for α2,6-linked sialylglycopolymers, have increased attachment to human tracheal and alveolar epithelia, but undetectable attachment to the larynx (Figure 2d) [32]. This implies that the Egyptian H5 sublineage recognizes different glycan topologies in human upper- and lower-airway epithelia, which is still unexplained. All H5N1 variants with some α2,6 Sia-binding affinity [32,63,66] probably have not yet acquired receptor binding affinity to long human α2,6 glycans. In fact, efficient transmission of the HPAI H5N1 virus has not occurred in humans or in experimental mammalian models of transmission [69,70].

However, the increasing host range for the Gs/GD virus lineage and repeated infections could drive mutations conferring efficient human-to-human transmission [71]. Indeed, Chutinimitkul *et al.* have recently reported that some HA mutations, introduced experimentally, can cause a complete switching of receptor specificity in an Indonesian AI virus [72]. Enhanced binding to α2,6 Sia is an important initial step for adaptation of avian viruses to infect humans [9,53]. Such a step in the Gs/GD virus lineage has probably occurred, at least partially, in Egypt, where new H5 sublineages have expanded their receptor specificity from alveoli to trachea in the human airway, as described above (Figure 2d and 4d) [32]. It may not be necessary for this virus lineage to use an intermediate (mixing vessel) host to form a pandemic virus strain because its increased attachment to human airway eptithelia would enable reassortment between avian and human influenza viruses or adaptation of avian viruses directly in humans. It is also noteworthy that the new H5 sublineage in Egypt was reported, according to bioinformatic analysis, to have evolved toward a Spanish flu virus-like receptor-specificity for human infection [73]. Furthermore, the currently widespread genotype Z viruses, including the Egyptian H5N1 viruses, have viral polymerase PB2 with mammalian-type PB2-627Lys [29], which enhances the host range and virulence of influenza A viruses [14, 74–76], implying an increased potential for evolution to a pandemic virus. Thus, the current Gs/GD virus lineage is diversifying (Table 1) in a way that might increase its human pandemic potential. However, it is not known how long it would take for a strain with pandemic potential to acquire human transmissibility and whether the Gs/GD virus lineage could overcome the species barrier in the future (Box 1) by further optimizing its replicative machinery for humans, which is probably needed for overall viral fitness [9]. Finally, it should be noted that not only HA but also other viral gene products (e.g. PB2, PB1-F2, NA and NS1) confer viral adaptation to mammals, interacting with host factors during various steps in viral infection, including replication, nuclear export, assembly, budding, and antagonism of the host antiviral response (reviewed in [2,7,12,13]).

Box 1. Outstanding questions

- Could the Gs/GD virus lineage (H5N1) be the next pandemic strain?
- What is the mechanism(s) necessary for the current H5N1 virus to adapt further in humans?
- Could full switching of HA receptor specificity from avian- to human-type receptors or acquisition of binding affinity for long α2,6-sialylsaccharides evolve in bird species?
- How complex and diverse is the glycan topology in the respiratory tract of humans and pigs and in the gastrointestinal tract of birds?
- How long does it take for a potential pandemic influenza virus strain to acquire human transmissibility?
- Would the H5N1 virus retain its high pathogenicity in humans if it fully adapts to infect human upper-airway epithelia and would it produce a pandemic?
- Are there any distinct animal species in nature that play an intermediate role in the emergence of H5N1 variants as a source of new viruses able to produce future human pandemics?
- How widely is the current H5N1 virus distributed in nature, especially in animal species for which epidemiological surveys have not been done previously?
- What would be the most promising strategy for H5N1 control in the current situation where H5N1 is disseminated widely in both poultry and wild waterfowl?

Possible advances in the development of diagnostic testing and vaccines

In countries where the HPAI H5N1 virus is active, rapid diagnostic assays that distinguish influenza virus subtypes are needed to enable rapid initiation of appropriate therapy and infection-control measures, and timely epidemiological investigations [27,77]. Antigen-detection tests of clinical specimens are widely used for rapid diagnosis of seasonal human influenza, but are less useful for H5N1 influenza viruses because of their low sensitivity and the inability of

Table 1. Summary of the changing nature of the Gs/GD virus lineage (H5N1)

Category	Changing nature	Refs.
Gene	Emergence of multiple genotypes (combination of internal genes); A, B, C, D, E, P, V, W, X0-3, Y, Z and Z+[a]	[2,24,25]
Antigenicity	Emergence of multiple clades (HA gene); 0–10[b]	[4]
Ecology	Establishment of endemic infections in land-based poultry	[3,4]
	Persistence in a range of wild water and terrestrial birds	[4,30]
	Geographically large spread apparently by bird migration	[6,24,29]
Phenotype	Strong zoonotic properties	[27]
	Alteration of receptor-binding specificity	[25,32,66]
	Drug resistance (e.g. amantadine and oseltamivir)	[27]
Other	Acquisition of mammalian-type PB2-627Lys (commonly detected in a sublineage of genotype Z)	[29]

[a]Viruses with genotypes A, C, D and E have not been detected since 2002.

[b]Clades are further defined to the third order (e.g. clade 2.2.1).

validated tests to distinguish between influenza subtypes [27,77]. Several rapid diagnostic systems are being developed, including an H5-specific immunochromatography kit and high-speed real-time reverse transcriptase PCR (RT-PCR), with the latter as microfluidic continuous-flow RT-PCR [78]. However, the presence of multiple sublineages of current H5N1 viruses [22] and their changing nature pose challenges because assays need to be targeted at genes and epitopes that are continually evolving.

Furthermore, as the complex dynamics of influenza viruses in birds is constantly challenging species barriers, new technologies are required to determine whether a particular AI virus strain is adapting to human receptors [60]. Several new techniques have recently emerged for studying interactions of influenza viruses with host cell receptors. These include a dose-dependent direct binding assay [32,66] and a glycan microarray [60,61], allowing quantitative and high-throughput analysis, respectively. Although such technological developments still require improvements in simplicity and convenience for detection, future portable kits and instruments should allow field testing, adding a new dimension for characterizing and assessing AI outbreaks [60].

Mass vaccination of human populations with an appropriate vaccine is an effective way to reduce the spread, morbidity and mortality of pandemic viruses [2,27]. The new H5 sublineage in Egypt could be considered an important seed-virus candidate for such a vaccine [32]. However, the most important strategy is to minimize the potential for emergence of a pandemic strain and to limit the opportunity for humans, poultry and pigs to be infected by AI viruses. The main control for HPAI outbreaks is identification and depopulation of infected and exposed flocks. This 'stamping-out' approach is very effective in settings where the virus is still not widespread or is maintained in only confined poultry populations [4]. However, once a virus becomes endemic in wild bird populations, control and eradication become greatly complicated, especially in rural areas where complete confinement cannot be controlled and random rearing of backyard flocks is common [4]. Current genetic diversification of the Gs/GD sublineage has caused antigenic variation among different clades and even within the same clade. Thus, antigenically distinct viruses are now co-circulating in an endemic area, as observed in Egypt, where the crossreactivity of different sublineage viruses was too low for one of the viruses to be considered as a seed virus for vaccine antigen, even though the viruses were all isolated in the same geographic area [79].

Vaccination of poultry is now considered to be a preventive or adjunct control measure in several countries [27,80]. Proper vaccination can substantially help to control AI outbreaks only when administrated as part of an integrated control program that includes intensive surveillance, stamping out, quarantine, animal movement control and improved biosecurity [27,80]. New vaccines are being developed, including a reverse genetics vaccine, viral vectored vaccines, a virus-like particle vaccine, and a plasmid DNA vaccine [77,81]. The most promising method for responding rapidly to a possible outbreak and pandemic is the use of a plasmid-based reverse genetics system to construct a vaccine seed virus [81]. However, seed-virus selection must be revised periodically to produce well-matched and efficacious vaccines.

Concluding remarks

Although the current H1N1 pandemic [7,19] may have diverted attention from the continuing worldwide circulation of the H5N1 virus, the pandemic threat of H5N1 is still alarming. With the important role of wild birds in the epidemiology of current HPAI H5N1 viruses [3,4], continuing large-scale surveillance of AI viruses in wild birds, as well as in humans, poultry, and pigs, is crucial to an understanding of the evolution and global spread of these viruses [3,4,25,32]. Geographically widespread and complex dynamics of current H5N1 viruses also make control with a unified regime more difficult. Public health guidelines for control need to be tailored to meet local agricultural practices and people's awareness in the region.

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Letter to the Editor

Misinterpretation in virus clearance studies of biological products due to an uncommon discrepancy between cytopathic effects and infectivity of human immunodeficiency virus (HIV)

We report a case of false-positive HIV infectivity detected by the cytopathic effect (CPE) assay, and we present data showing how to confirm such false-positive results. CPE are apparent cellular responses against viral infections, such as morphological changes in cells and apoptosis. The CPE assay is a useful standard method to determine the presence of infectious viruses during process evaluation for biological products as recommended in guidelines for viral clearance studies [1,2]. Characteristic syncytium formation by infection of specific cells, such as C8166 or MT2 cells, with human immunodeficiency virus (HIV), for example LAI, RF, or HTLV-IIIB strains of HIV-1, has also been established as a sensitive and conventional CPE assay [3–5].

Surprisingly, atypical syncytium-like CPE of infected cells, induced by the filtrate of HIV-spiked samples even after use of a virus removal filter with a pore size of 15 nm (15-nm filter), were observed in our virus clearance study, despite it having been reported that a 15-nm or 35-nm filter can effectively capture intact HIV particles of 80–100 nm in diameter [3,4]. However, two different researchers have also reported similar phenomena (R. Cameron, personal communication). In the following study, we confirmed that these rare syncytium-like CPE can be reproduced experimentally and that non-infectious HIV components of less than 15 nm could penetrate through a 15-nm filter and induce CPE.

The LAI strain of HIV was sonicated twice using a Bioruptor UCD-200TM (CosmoBio, Tokyo, Japan) at 200 W for 10 min (1-min interval) and pre-filtered with PLANOVA™75N (mean pore size, 72 ± 4 nm; Asahi-Kasei Medical, Tokyo, Japan). The resulting disrupted HIV solution, which included both whole HIV particles and their disrupted components, was filtered with PLANOVA™15N (mean pore size, 15 ± 2 nm; Asahi-Kasei Medical) at a constant pressure of 20 kPa at RT, and all of the filtrate was collected as substances that had passed through a 15-nm filter. The disrupted HIV solution contained 5.05 Log_{10} copies/mL of HIV RNA and the concentration of the viral components group-specific antigen p24 of the capsid protein (p24) and envelope glycoprotein (gp120) was 1056.1 ng/mL and 63.5 ng/mL, respectively. Substantial levels of HIV RNA, p24, and gp120 were also detected in the filtrate from a 15-nm filter, with values of 4.19 Log_{10} copies/mL, 1015.1 ng/mL (yield: 96.1%), and 54.1 ng/mL (yield: 85.2%), respectively.

Next, cultured C8166 cells at a concentration of 1.0×10^5 cells/mL were placed in 96-well plates (0.1 mL/well) and 0.1 mL/well of assay sample was added. During the incubation period of 14 days at 37 °C, 50 μL of fresh complete medium was added every 3–5 days. Positive CPE appearance was defined by reference to a report by Ongradi et al. [5]. For the immunofluorescence (IF) assay, cultured C8166 cells 14 days post-infection (dpi) fixed in cold acetone were incubated successively with the primary antibody consisting of anti-HIV polyclonal antibody wt0062 derived from HIV patient sera (1:300), and the secondary antibody consisting of anti-human IgG polyclonal antibody labeled with FITC (1:50, Dako, Glostrup, Denmark). After a 14-day incubation with C8166 cells, the disrupted HIV solution induced typical HIV-specific CPE and virus-specific IF signals, correlating with increases in viral gene expression up to 8.27 Log_{10} copies/mL. CPE were also found in the filtrate of a 15-nm filter at low frequencies: three syncytia or multinucleated giant cells were observed in 1.8×10^5 cells/well. However, IF signals and viral RNA were not detected in the culture fluid (Fig. 1).

Therefore, to confirm the presence or absence of infectious viruses, we carried out three subsequent blind passages after the first cultivation of samples with C8166 cells. For the blind passage, 1.0×10^5 cells of cultured C8166 14 dpi and an equal number of naive C8166 cells were seeded in 24-well plates and cultured in 2 mL/well of complete medium at 37 °C. Three blind passages were performed at 3- to 5-day intervals. Treatment with the disrupted HIV solution led to maintenance of or small increases in substantial amounts of HIV RNA and HIV antigens, and typical syncytia and IF signals were observed in every blind passage. C8166 cells treated with the filtrate from a 15-nm filter had no signs of these viral parameters as a negative control (Fig. 1B). These observations indicated that the presence of infectious HIV in the filtrate of a 15-nm filter could be clearly ruled out. This is the first finding that non-infectious HIV components penetrated through a 15-nm filter to induce syncytium-like CPE. The action of the HIV components may involve indirect and complex processes or a mechanism by agents other than a well-known factor, such as gp120, because a long incubation period of 14 days was needed for syncytial formation. While activation of cellular signal transduction and induction of syncytia by HIV envelope protein-expressing cells have been reported [6], further detailed investigation will be needed to clarify this unexplained result.

Notably, syncytium-like CPE induced by non-infectious HIV components could lead to misinterpretation of inactivation or removal abilities in manufacturing processes. If an atypical and/or unreasonable morphological change such as CPE is observed,

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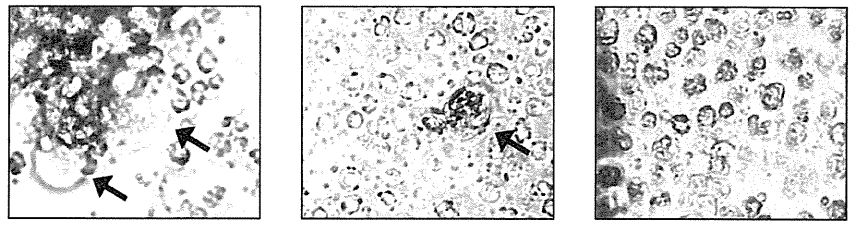
A



		Disrupted HIV solution	The filtrate of 15-nm	Medium (Negative control)
CPE		++	+	-
IF assay		++	-	-
HIV RNA	(Log_{10} copies/mL)	8.27	n.d.	n.d.
p24 /gp120 antigen	(ng/mL)	337.2 / 5.4	138.9 / 1.1	n.d. / n.d.

B

CPE		++	-	-
IF assay		++	-	-
HIV RNA	(Log_{10} copies/mL)	8.46	n.d.	n.d.
p24 /gp120 antigen	(ng/mL)	647.1 / 19.0	n.d. /n.d.	n.d. /n.d.

Fig. 1. Images of morphological changes (phase contrast) and results of IF signals, as well as amounts of HIV RNA, p24 antigen, and gp120 after incubation with disrupted HIV solution, the filtrate of a 15-nm filter, and medium as assay samples, respectively. The arrows represent a syncytium-like typical morphological change or atypical morphological change. (A) The results of CPE, IF signals in cultured cells, and amounts of HIV RNA, p24, and gp120 antigen in culture fluid (at 14 dpi). (B) The results after three serial blind passages. The cultured cells (at 14 dpi) described above were subsequently used for blind passage. ++ = strongly positive, + = positive/weakly positive, − = negative, n.d. = not detected.

careful interpretation of the phenomenon is needed. Consequently, CPE may be induced both by intact HIV and by noninfectious components, and IF assay and blind passages are useful tools for specific identification of infectious HIV, even if samples coexist with such non-infectious components that cause morphological changes in cells.

Conflict of interest

We declare that we have no conflicts of interest. This work was conducted on the basis of collaborative research projects between Osaka University, BioReliance Ltd., Asahi Kasei Medical Co., Ltd., and Benesis Corporation, and the research fund used in this study was provided by Benesis Corporation. Takeru Urayama and Mikihiro Yunoki are employees of Benesis Corporation (Japan Blood Products Organization).

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Reliability of a Newly-Developed Immunochromatography Diagnostic Kit for Pandemic Influenza A/H1N1pdm Virus: Implications for Drug Administration

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Abstract

Background: For the diagnosis of seasonal influenza, clinicians rely on point-of-care testing (POCT) using commercially available kits developed against seasonal influenza viruses. However, POCT has not yet been established for the diagnosis of pandemic influenza A virus (H1N1pdm) infection due to the low sensitivity of the existing kits for H1N1pdm.

Methodology/Principal Findings: An immunochromatography (IC) test kit was developed based on a monoclonal antibody against H1N1pdm, which does not cross-react with seasonal influenza A or B viruses. The efficacy of this kit (PDM-IC kit) for the diagnosis of H1N1pdm infection was compared with that of an existing kit for the detection of seasonal influenza viruses (SEA-IC kit). Nasal swabs (n = 542) were obtained from patients with flu-like syndrome at 13 clinics in Osaka, Japan during the winter of 2010/2011. Among the 542 samples, randomly selected 332 were further evaluated for viral presence by reverse transcriptase polymerase chain reaction (RT-PCR). The PDM-IC kit versus the SEA-IC kit showed higher sensitivity to and specificity for H1N1pdm, despite several inconsistencies between the two kits or between the kits and RT-PCR. Consequently, greater numbers of false-negative and false-positive cases were documented when the SEA-IC kit was employed. Significant correlation coefficients for sensitivity, specificity, and negative prediction values between the two kits were observed at individual clinics, indicating that the results could be affected by clinic-related techniques for sampling and kit handling. Importantly, many patients (especially influenza-negative cases) were prescribed anti-influenza drugs that were incongruous with their condition, largely due to physician preference for patient responses to questionnaires and patient symptomology, as opposed to actual viral presence.

Conclusions/Significance: Concomitant use of SEA-IC and PDM-IC kits increased the likelihood of correct influenza diagnosis. Increasing the credibility of POCT is anticipated to decrease the inappropriate dispensing of anti-influenza drugs, thereby minimizing the emergence of drug-resistant H1N1pdm strains.

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Introduction

Swine-origin pandemic influenza A virus (subtype H1N1pdm) emerged in April, 2009 and rapidly spread across the globe, becoming one of the most common human influenza A viruses in the world [1]. Seasonal influenza virus A subtype H1N1, by contrast, had all but disappeared from most countries by the 2009/2010 winter influenza season. However, H1N1pdm was replaced by a mixed population of H1N1pdm and seasonal

influenza A subtype H3N2 during the 2010/2011 winter influenza season [2].

The H1N1pdm virus contains a triple-reassortant genome that includes a combination of avian, human, and swine influenza virus gene segments. The H1N1pdm genome encodes polymerase basic protein 2 (PB2) and polymerase subunit A (PA), both derived from the North American avian lineage; polymerase subunit B1 (PB1), derived from human seasonal influenza A H3N2; neuraminidase protein (NA) and matrix (M) proteins, derived from the Eurasian swine lineage; and hemagglutinin (HA), nucleoprotein (NP), and nonstructural (NS) proteins, derived from the North American classical swine lineage [3].

Based on epidemiological data from Mexico, where the estimated case fatality ratio in 2009 was 1.2% overall and 5.5% among individuals over 60 years of age [10,11,12], as well as on animal studies of influenza virus infection [13], the pathogenicity of H1N1pdm was initially thought to be relatively high. Subsequent estimates of case fatality ratios were, however, significantly lower than the initial estimates [14,15]. In particular, the case fatality ratio in Japan was only 0.1% [16,17]. It is likely that one of the reasons for the low case fatality ratio is an established system in Japan for the rapid diagnosis of influenza virus and the subsequent administration of anti-influenza drugs.

Several groups are at elevated risk for H1N1pdm infection, including pregnant women, individuals with diabetes, and the obese, elderly, and very young [4]. However, rapid diagnosis of the infection followed by administration of appropriately prescribed anti-viral drugs considerably attenuates disease severity and duration, even in patients belonging to these high-risk groups. The majority of H1N1pdm strains are susceptible to oseltamivir (Tamiflu), although H1N1pdm oseltamivir-resistant strains are on the rise and account for 0.5–1.0% of all cases in most countries [5–8]. On the other hand, the H1N1pdm virus carries an S31N mutation in the M2 gene and is therefore resistant to treatment with adamantanes [9].

Rapid immunologic diagnosis of H1N1pdm was attempted soon after its emergence in 2009 by using immunochromatography (IC) test kits previously developed for the detection of seasonal influenza viruses (SEA-IC kits). Nonetheless, these SEA-IC kits could not reliably differentiate H1N1pdm from seasonal influenza A viruses H1N1 and H3N2. Indeed, subsequent analysis via reverse transcriptase polymerase chain reaction (RT-PCR) indicated that existing SEA-IC test kits showed significantly low sensitivity for H1N1pdm [18,19,20,21,22,23,24,25,26]. Consequently, we developed murine monoclonal antibodies that were specific for H1N1pdm, which exhibited no cross-reactivity with seasonal influenza A (H1N1 or H3N2) or seasonal influenza B viruses. These monoclonal antibodies were then employed to develop a new IC test kit for the high-sensitivity detection of H1N1pdm [27].

Our previous research with clinical samples confirmed the efficacy of the newly-developed H1N1pdm IC test kit (PDM-IC kit) [27]. This work included H1N1pdm-confirmed clinical samples that were obtained during the 2009/2010 winter influenza season from patients at a single clinic in Osaka, Japan. Clinical samples containing other influenza subtypes obtained before 2009 were used as controls to evaluate the specificity of the PDM-IC test kit.

In the current study, the PDM-IC test kit was evaluated for the rapid diagnosis of H1N1pdm infection using clinical samples obtained during the 2010/2011 winter influenza season in Osaka, Japan. A total of 542 clinical samples collected from patients at 13 clinics were involved in the evaluation. The PDM-IC kit was assessed relative to a previously-developed SEA-IC kit for its accuracy and limitations of sensitivity, specificity, positive predictive values (PPVs), and negative predictive values (NPVs). Subsets of the samples were also subjected to RT-PCR and virus isolation (VI) analyses. The data were then compared with clinical data regarding symptoms and drug prescriptions so as to determine the general applicability and reliability of rapid diagnosis kits for point-of-care testing (POCT) at clinics.

Materials and Methods

IC Rapid Test Kits

The Prime Check Flu (H1N1) 2009 (Alfresa Pharma Corporation, Osaka, Japan) for the rapid detection of the NP protein derived from H1N1pdm [27] was employed in this study and was referred to as the "PDM-IC kit". An IC kit currently in use for the rapid detection of NP proteins derived from seasonal influenza A and B viruses (Check Flu A+B kit; Alfresa Pharma Corporation, Osaka, Japan) was employed as the control. This kit was referred to as the "SEA-IC kit". Importantly, the same solution for the suspension of swab samples could be used for tests performed with both kits.

Sample Collection

Samples of nasal or nasopharyngeal fluid were collected from patients with influenza-like symptoms (n = 542) at 13 clinics (termed "A" through "M" clinics) under the guidance of the Higashinari-ku Medical Association in Osaka, Japan during the winter of 2010–2011 (November, 2010 through April, 2011). Two swabs were taken from each patient; one sample was tested using the PDM-IC and SEA-IC kits, and the other was tested using RT-PCR and/or VI.

Viruses and Cells

The following virus strains were used: A/Suita/01/09 (H1N1pdm virus); A/New Caledonia/20/99 (seasonal influenza A virus H1N1); A/Wyoming/2/03 (seasonal influenza A virus H3N2); and B/Malaysia/2506/04 (seasonal influenza B virus). Madin-Darby canine kidney (MDCK) cells were used for propagation of the viruses. The MDCK cells were maintained in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) in a humidified incubator (5% CO_2/95% air) at 37°C.

RT-PCR Analysis

RT-PCR-based analysis for the highly sensitive detection of viral RNA and genotyping of influenza viruses in the clinical specimens was conducted as described previously [27]. For RT-PCR analysis, 332 samples were randomly selected from the initial 542 swab samples. Briefly, viral RNA was extracted from the clinical samples using a QIAamp® Viral RNA Mini kit (QIAGEN, Tokyo, Japan). RT-PCR was then performed with a QIAGEN OneStep RT-PCR kit for H1N1pdm and a SuperScriptTM III One-Step RT-PCR System with Platinum® Taq High Fidelity (Invitrogen, Carlsbad, CA) for the seasonal influenza viruses. The RT-PCR conditions were as follows: 50°C for 30 min and 94°C for 3 min, followed by 40 cycles at 94°C for 30 sec, 54°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 7 min.

VI Analysis

For VI analysis, 263 samples were further selected from the 332 swab samples that were subjected to RT-PCR. The 263 samples included all inconsistent cases (n = 79) between the two kits or between the kits and RT-PCR. The additional 184/263 samples were randomly selected from the original 332 RT-PCR

samples. To isolate the influenza viruses from the clinical samples, MDCK cells were inoculated with clinical specimens and incubated at 37°C in Dulbecco's Modified Eagle Medium (DMEM) containing Nutrient Mixture F-12 (DMEM/F12, Invitrogen) and 1% v/v Antibiotic-Antimycotic liquid (Invitrogen), 0.4% w/v bovine serum albumin, and 2 μg/mL Trypsin Acetylated from Bovine Pancreas, Type V-S (Sigma-Aldrich, St. Louis, MO). Cell were incubated for 3 weeks, or until cytopathic effects were observed.

Virus Identification by Peroxidase-anti-peroxidase (PAP) Staining

Isolated viruses were identified by PAP staining using two influenza virus A subtype-specific murine monoclonal antibodies, C179 (H1-specific) and F49 (H3-specific), and the influenza virus B HA-specific murine monoclonal antibody, 7B11 (unpublished, Yoshinobu Okuno, Osaka Prefectural Institute of Public Health, Osaka, Japan), as described previously [27]. Briefly, MDCK cells in MEM were inoculated with the isolated viruses and incubated at 37°C for 16 h. After fixation with absolute ethanol, the cells were incubated with the monoclonal antibodies described above followed by rabbit anti-mouse immunoglobulin (1:1,000; Organon Teknika, Malvern, PA). The cells were treated successively with goat anti-rabbit immunoglobulin G antibody (1:500; Organon Teknika) and PAP (rabbit anti-peroxidase) complex (1:5,000; Organon Teknika). Finally, a peroxidase reaction was conducted by incubating the cells with 3,3′-diaminobenzidine tetrahydrochloride substrate for 5 min at room temperature. The stained cells were observed under an inverted optical microscope.

Statistical Analysis

This study used the results of RT-PCR as the "gold standard". Based on RT-PCR, the results of the test kits were divided into four groups, i.e., "true-positive (TP)", "true-negative (TN)", "false-positive (FP)", and "false-negative (FN)" cases. Results of the SEA-IC kit were divided into TP, TN, FP, and FN cases, as follows: TP ("A+B−" with seasonal H1N1, H1N1pdm, or H3N2 by RT-PCR; or "A−B+" with influenza B virus by RT-PCR); TN ("A−B−" with "−" by RT-PCR); FP ("A+B−" with "−" by RT-PCR); and FN ("A−B−" with seasonal H1N1, H1N1pdm, H3N2, or influenza B virus by RT-PCR). Results of the PDM-IC kit were divided into TP, TN, FP, and FN cases, as follow: TP ("pdm+" with H1N1pdm by RT-PCR); TN ("pdm− " with "−", seasonal H1N1, H3N2, or influenza B virus by RT-PCR); FP ("pdm+" with "−", seasonal H1N1, H3N2, or influenza B virus by RT-PCR); and FN ("pdm−" with H1N1pdm by RT-PCR). Statistical analysis was performed for sensitivity values, specificity values, positive predictive values (PPV), and negative predictive values (NPV) which were calculated according to the following formulas: sensitivity = TP/(TP+FN); specificity = TN/(FP+TN); PPV = TP/(TP+FP); and NPV = TN/(FN+TN). All results were assessed by correlation analysis, Student's t-test, or the chi-square test, performed using SPSS version 18 (SPSS, Chicago, IL). The level of statistical significance was set at $P<0.05$.

Ethics

The research protocol for the collection of human samples was approved by the Ethics Committee of the Research Institute for Microbial Diseases, Osaka University, Japan. Informed consent was obtained from all patients in writing before enrollment in the study.

Results

Patient Background

Swab samples (n = 542 duplicate samples) were simultaneously collected from 542 patients with influenza-like symptoms presenting at 13 clinics (clinics "A" through "M") within Higashinari-ku, Osaka, Japan from November, 2010 through April, 2011 (Table 1). The study included 271 males and 266 females (gender unknown in five cases), with a mean age of 30.5 years (range, 0–88 years; age unknown in 14 cases) and an average of 1.2±1.2 days between the onset of clinical signs and sampling (onset time unknown in 18 cases) (Table 1).

Reliability of Data Yielded by Rapid Test Kits, RT-PCR, and VI Studies

Of the two swab samples collected from each patient, one sample was immediately tested using the PDM-IC kit and the SEA-IC kit, and the other was kept at −80°C and subsequently used for RT-PCR and VI analyses. The PDM-IC and SEA-IC kits were designed for the rapid detection of NP proteins derived from the H1N1pdm virus and the seasonal influenza A and B viruses, respectively.

The results from the SEA-IC kit were as follows: 44.7% (242/542) of the cases tested positive for influenza A virus; 3.5% (19/542) of the cases tested positive for influenza B virus; and the remaining 51.8% (281/542) of the cases tested negative for either influenza A or B virus (Figure 1A). The PDM-IC kit identified 38.4% (208/542) of the cases as positive for the H1N1pdm virus (Figure 1A). To evaluate the sensitivity and specificity of the two IC test kits for H1N1pdm, 332 randomly selected swab samples out of the 542 original swab samples were analyzed by RT-PCR as a "gold standard". The results for the influenza virus-positive cases are summarized in Figure 1B according to the data obtained from each type of IC kit (PDM-IC versus SEA-IC), and RT-PCR.

The results of the RT-PCR analysis were as follows: 48.5% (161/332) of the cases tested positive for H1N1pdm; 8.1% (27/332) of the cases tested positive for seasonal influenza A virus H3N2; 4.8% (16/332) of the cases tested positive for influenza B virus; and 38.6% (128/332) of the cases tested negative for any type of influenza virus (Figure 1). No seasonal H1N1-positive cases were detected.

The SEA-IC kit showed 89.2% sensitivity, 80.5% specificity, 87.9% PPV, and 82.4% NPV based on the data for influenza virus-positive cases obtained by RT-PCR (332 cases) (Table 2). By contrast, the PDM-IC kit showed 92.5% sensitivity, 95.3% specificity, 94.9% PPV, and 93.1% NPV among 332 cases (Table 2). Interestingly, the SEA-IC kit and PDM-IC kit results from individual clinics showed significant correlations in terms of sensitivity, specificity, and NPV (correlation coefficients 0.706, 0.657, and 0.784; P values 0.007, 0.020, and 0.003, respectively). These results suggest that the rapid IC kit scores could be affected by clinic-related techniques for swab sampling and kit handling. The SEA-IC kit results showed a tendency toward variations in sensitivity, specificity, PPV, and NPV between different age groups (<10, 10–19, 20–29, 30–39, 40–49, 50–59, and ≥60 years) (Figure 2A), while the PDM-IC kit results showed higher overall scores and no clear variations between age groups (Figure 2B). Thus, the PDM-IC kit tended to yield stable results regardless of patient age.

Further analysis regarding sampling time after the onset of clinical signs was next performed. The accuracy of the results obtained using the SEA-IC kit significantly decreased for samples collected on the third or more day after onset (4.2±2.2 days) compared with those collected on the previous days (Figure 2C).

Table 1. Background information for patients with influenza-like symptoms from whom clinical specimens were collected.

Clinic	Number of samples	Gender			Age		Specimen collection	
		Male	Female	Unknown[a]	Mean years (Range)	Unknown[a]	Days after onset	Unknown[a]
A	40	18	21	1	26.4 (1–69)	1	0.8±0.6	1
B	10	6	4	0	28.3 (10–51)	0	1.2±0.4	1
C	79	39	40	0	29.7 (9–76)	0	1.0±0.7	0
D	70	28	40	2	24.5 (2–62)	2	1.1±0.9	3
E	17	6	11	0	30.8 (4–88)	2	1.9±2.8	0
F	39	16	23	0	27.4 (3–63)	1	1.1±0.7	1
G	30	18	12	0	31.8 (2–73)	0	1.5±0.7	0
H	48	24	23	1	25.5 (0–86)	0	0.8±0.7	1
I	38	21	16	1	34.1 (12–62)	0	1.3±0.9	2
J	39	20	19	0	33.6 (9–60)	7	1.0±0.6	6
K	22	11	11	0	31.3 (20–45)	0	1.7±0.6	0
L	48	30	18	0	40.7 (21–78)	1	1.7±1.8	0
M	62	34	28	0	34.7 (2–87)	0	1.5±1.5	3
Total	542	271	266	5	30.5 (0–88)	14	1.2±1.2	18

[a]Information could not be obtained from the answers to the questionnaires.

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This was in contrast to the results obtained using the PDM-IC kit, which showed similar scores for all of the samples collected during the study period, even at the third or more days after the onset of clinical symptoms (Figure 2D). Consequently, although the exact reason for the variable results among the individual age groups (Figure 2A) is not clear, it seems to be due, at least in part, to the duration from disease onset to POCT. In this regard, the SEA-IC kit compared with the PDM-IC kit afforded more false-positive cases (as assessed by specificity and PPV) and false-negative cases (as assessed by sensitivity and NPV) among the RT-PCR-positive samples obtained from patients with a longer duration from disease onset to POCT. For example, an increased number of false-positive cases was observed for patients aged 20–29 years and ≥60 years with a duration from disease onset to POCT of 2.0±1.65 and 2.50±0.71 days, respectively. Similarly, an increased number of false-negative cases was observed for patients aged 20–29 years and 40–49 years with a duration from disease onset to POCT of 1.71±0.76 and 1.67±1.15 days, respectively (Table S1).

As summarized in Figure 1, the two IC test kits afforded inconsistent results in several cases (2.2%, 12/542), the two IC test kits afforded inconsistent results: 11 samples tested negative for influenza viruses by SEA-IC, but tested positive for H1N1pdm by PDM-IC and RT-PCR; and one sample tested negative for influenza viruses by SEA-IC and RT-PCR, but tested positive for H1N1pdm by PDM-IC. Furthermore, several inconsistent cases among the 332 samples subjected to RT-PCR were also identified between the SEA-IC kit and RT-PCR (n = 47: 22 false-negative cases consisting of 20 "A−B−"/"H1N1" cases by SEA-IC/RT-PCR and two "A−B−"/"H3N2" by SEA-IC/RT-PCR; and 25 false-positive cases consisting of 22 "A+B−"/"−"by SEA-IC/RT-PCR and three "A−B+"/"−" by SEA-IC/RT-PCR); as well as between the PDM-IC kit and RT-PCR (n = 20: 12 false-negative cases consisting of "pdm−"/"H1N1pdm" by PDM-IC/RT-PCR; and eight false-positive cases consisting of one "pdm+"/"H3N2" by PDM-IC/RT-PCR and seven "pdm+"/"−" by PDM-IC/RT-PCR).

Further, the 263 samples, including the above-described samples showing inconsistent results between two IC test kits (n = 12), between the SEA-IC kit and RT-PCR (n = 47), and between the PDM-IC kit and RT-PCR (n = 20), were analyzed by VI. As summarized in Figure 1, the results of the VI analysis indicated that 41.1% (108/263) of the cases were positive for H1N1pdm; 3.8% (10/263) of the cases were positive for H3N2; 0.8% (2/263) of the cases were positive for both H1N1pdm and H3N2; and 5.3% (14/263) of the cases were positive for influenza B virus. The remaining 49.0% (129/263) of the cases tested negative for influenza virus infection. Thus, there were several cases that tested positive by VI, but negative by RT-PCR (n = 8; seven for H1N1pdm and one for H3N2), and vice versa (n = 29; 12 for H1N1pdm, 16 for H3N2, and one for influenza B virus).

Relationship between Clinical Symptoms and IC Test Kit Results

To understand the relationship between clinical symptoms and IC test kit results, we used the combined results from the two IC test kits. Based on the results of POCT, the samples obtained from the patients were classified into five groups: 1) "A+B−" by SEA-IC and "pdm−" by PDM-IC for seasonal influenza A virus infection; 2) "A−B+" by SEA-IC and "pdm−" by PDM-IC for seasonal influenza B virus infection; 3) "A+B−" by SEA-IC and "pdm+" by PDM-IC for H1N1pdm virus infection; 4) "A−B−" by SEA-IC and "pdm+" by PDM-IC for possible H1N1pdm virus infection; and 5) "A−B−" by SEA-IC and "pdm−" by PDM-IC for the absence of influenza virus infection. Analysis of the relationship between clinical symptoms and POCT results revealed that there was a significant difference between the symptoms in influenza-positive and influenza-negative patients (Table 3). For example, 53.1% of influenza-positive patients versus 44.2% of influenza-negative patients (P = 0.039) presented with an upper respiratory tract inflammation; 27.1% of influenza-positive versus 19.0% of influenza-negative patients (P = 0.024) presented with muscular pain; 3.7% of influenza-positive versus 23.0% of influenza-negative patients presented with pharynx pain

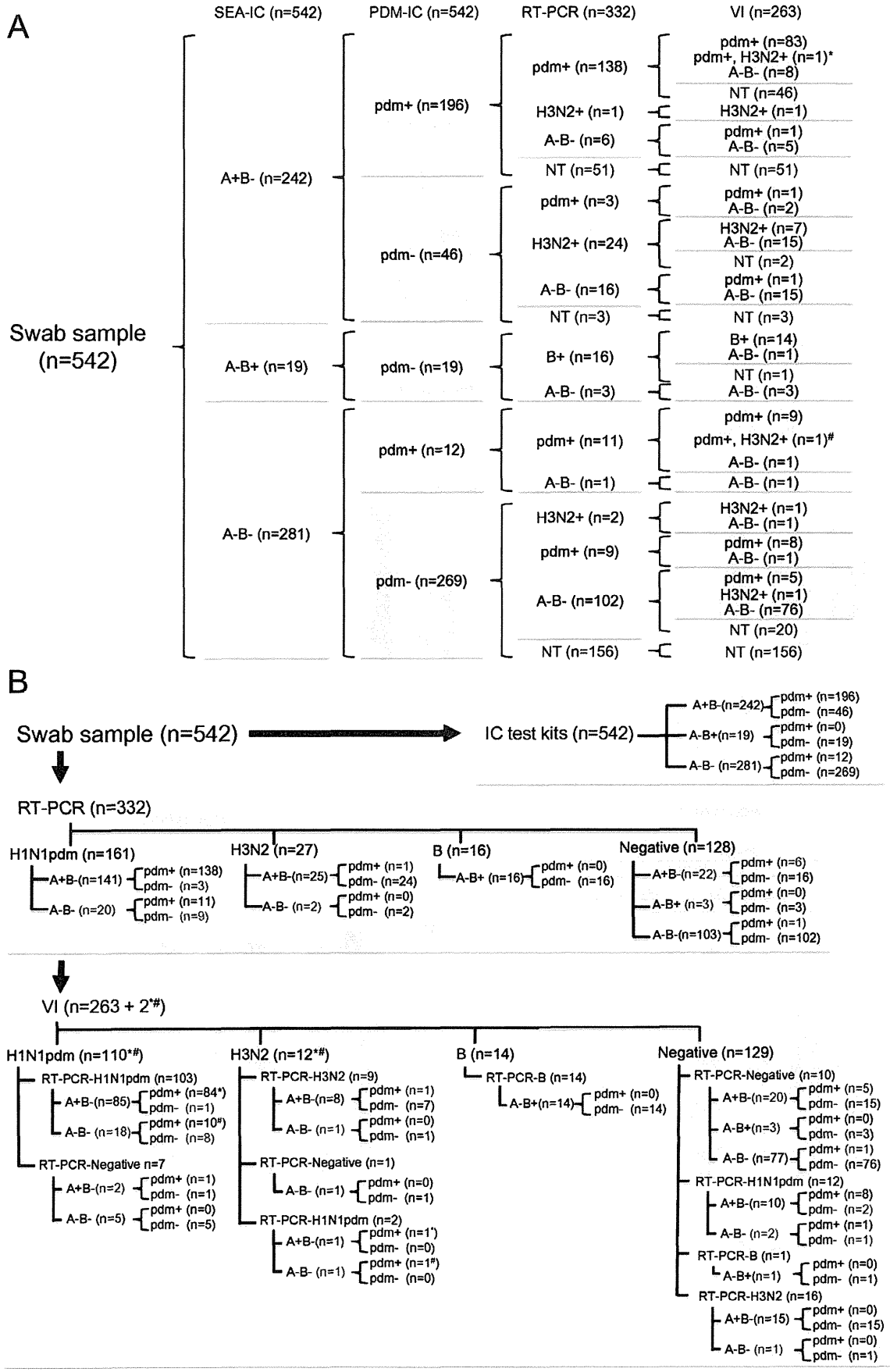
A
SEA-IC (n=542)
PDM-IC (n=542)
RT-PCR (n=332)
VI (n=263)
Swab sample (n=542)
A+B- (n=242)
pdm+ (n=196)
pdm+ (n=138)
pdm+ (n=83)
pdm+, H3N2+ (n=1)*
A-B- (n=8)
NT (n=46)
H3N2+ (n=1)
H3N2+ (n=1)
A-B- (n=6)
pdm+ (n=1)
A-B- (n=5)
NT (n=51)
NT (n=51)
pdm- (n=46)
pdm+ (n=3)
pdm+ (n=1)
A-B- (n=2)
H3N2+ (n=24)
H3N2+ (n=7)
A-B- (n=15)
NT (n=2)
A-B- (n=16)
pdm+ (n=1)
A-B- (n=15)
NT (n=3)
NT (n=3)
A-B+ (n=19)
pdm- (n=19)
B+ (n=16)
B+ (n=14)
A-B- (n=1)
NT (n=1)
A-B- (n=3)
A-B- (n=3)
A-B- (n=281)
pdm+ (n=12)
pdm+ (n=11)
pdm+ (n=9)
pdm+, H3N2+ (n=1)#
A-B- (n=1)
A-B- (n=1)
A-B- (n=1)
pdm- (n=269)
H3N2+ (n=2)
H3N2+ (n=1)
A-B- (n=1)
pdm+ (n=9)
pdm+ (n=8)
A-B- (n=1)
A-B- (n=102)
pdm+ (n=5)
H3N2+ (n=1)
A-B- (n=76)
NT (n=20)
NT (n=156)
NT (n=156)
B
Swab sample (n=542)
IC test kits (n=542)
A+B-(n=242)
pdm+ (n=196)
pdm- (n=46)
A-B+(n=19)
pdm+ (n=0)
pdm- (n=19)
A-B- (n=281)
pdm+ (n=12)
pdm- (n=269)
RT-PCR (n=332)
H1N1pdm (n=161)
A+B-(n=141)
pdm+ (n=138)
pdm- (n=3)
A-B- (n=20)
pdm+ (n=11)
pdm- (n=9)
H3N2 (n=27)
A+B-(n=25)
pdm+ (n=1)
pdm- (n=24)
A-B- (n=2)
pdm+ (n=0)
pdm- (n=2)
B (n=16)
A-B+ (n=16)
pdm+ (n=0)
pdm- (n=16)
Negative (n=128)
A+B-(n=22)
pdm+ (n=6)
pdm- (n=16)
A-B+ (n=3)
pdm+ (n=0)
pdm- (n=3)
A-B-(n=103)
pdm+ (n=1)
pdm- (n=102)
VI (n=263 + 2*#)
H1N1pdm (n=110*#)
RT-PCR-H1N1pdm (n=103)
A+B-(n=85)
pdm+ (n=84*)
pdm- (n=1)
A-B- (n=18)
pdm+ (n=10#)
pdm- (n=8)
RT-PCR-Negative n=7
A+B-(n=2)
pdm+ (n=1)
pdm- (n=1)
A-B- (n=5)
pdm+ (n=0)
pdm- (n=5)
H3N2 (n=12*#)
RT-PCR-H3N2 (n=9)
A+B-(n=8)
pdm+ (n=1)
pdm- (n=7)
A-B- (n=1)
pdm+ (n=0)
pdm- (n=1)
RT-PCR-Negative (n=1)
A-B- (n=1)
pdm+ (n=0)
pdm- (n=1)
RT-PCR-H1N1pdm (n=2)
A+B-(n=1)
pdm+ (n=1*)
pdm- (n=0)
A-B- (n=1)
pdm+ (n=1#)
pdm- (n=0)
B (n=14)
RT-PCR-B (n=14)
A-B+(n=14)
pdm+ (n=0)
pdm- (n=14)
Negative (n=129)
RT-PCR-Negative (n=10)
A+B-(n=20)
pdm+ (n=5)
pdm- (n=15)
A-B+(n=3)
pdm+ (n=0)
pdm- (n=3)
A-B- (n=77)
pdm+ (n=1)
pdm- (n=76)
RT-PCR-H1N1pdm (n=12)
A+B-(n=10)
pdm+ (n=8)
pdm- (n=2)
A-B- (n=2)
pdm+ (n=1)
pdm- (n=1)
RT-PCR-B (n=1)
A-B+(n=1)
pdm+ (n=0)
pdm- (n=1)
RT-PCR-H3N2 (n=16)
A+B-(n=15)
pdm+ (n=0)
pdm- (n=15)
A-B- (n=1)
pdm+ (n=0)
pdm- (n=1)

Figure 1. Summarized results of 524 swab samples analyzed by POCT with two IC test kits (SEA-IC and PDM-IC) kits, RT-PCR, and VI. A, a total of 542 swab samples were subjected to POCT at 13 clinics. Subsequently, 332 of the 542 samples were subjected to RT-PCR, and 263 of the 332 samples analyzed by RT-PCR were subjected to VI. The SEA-IC results for influenza A virus ("A+B−") or influenza B virus ("A−B+"), as well as the PDM-IC results for pandemic influenza A virus H1N1pdm("pdm+" and "pdm−"), are shown. Both H1N1pdm and H3N2 were isolated from two samples, as shown by the (*) and (#). B, The results of (A) are summarized for the individual diagnostics procedures (IC test kits, 542 samples; RT-PCR, 332 samples; and VI, 263 samples). The samples that yielded false-positive and false-negative results following analysis with the SEA-IC and PDM-IC kits (based on the RT-PCR results as the gold standard) are shown in blue and red, respectively.
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(P = 0.000); 29.7% of influenza-positive versus 15.6% of influenza-negative patients presented with a wet cough (P = 0.000); and 35.2% of influenza-positive versus 13.0% of influenza-negative patients presented with a dry cough (P = 0.000).

No significant differences were observed in terms of other symptoms, such as fever, lower respiratory tract inflammation, arthralgia, or diarrhea. In particular, most of the patients with influenza-like syndrome who were enrolled in this study presented with a high fever, and there was no significant difference (P = 0.463) between patients diagnosed as influenza-positive (98.9%) versus influenza-negative (98.1%). Table 3 illustrates that upper respiratory tract inflammation was discerned more frequently in patients infected with seasonal influenza A virus ("A+B−" by SEA-IC and "pdm−" by PDM-IC, 71.7%), compared with other influenza virus-positive groups ("A−B+" by SEA-IC and "pdm−" by PDM-IC, 42.1%; "A+B−" by SEA-IC and "pdm+" by PDM-IC, 50.5%; and "A−B−" by SEA-IC and "pdm+" by PDM-IC, 41.7%) (P = 0.006). Moreover, malaise was documented more frequently in possible H1N1pdm-positive patients ("A−B−" by SEA-IC and "pdm+" by PDM-IC, 41.7%) relative to the other influenza virus-positive groups (8.7–13.0%) or influenza-negative patients (16.4%) (P = 0.034) (Table 3).

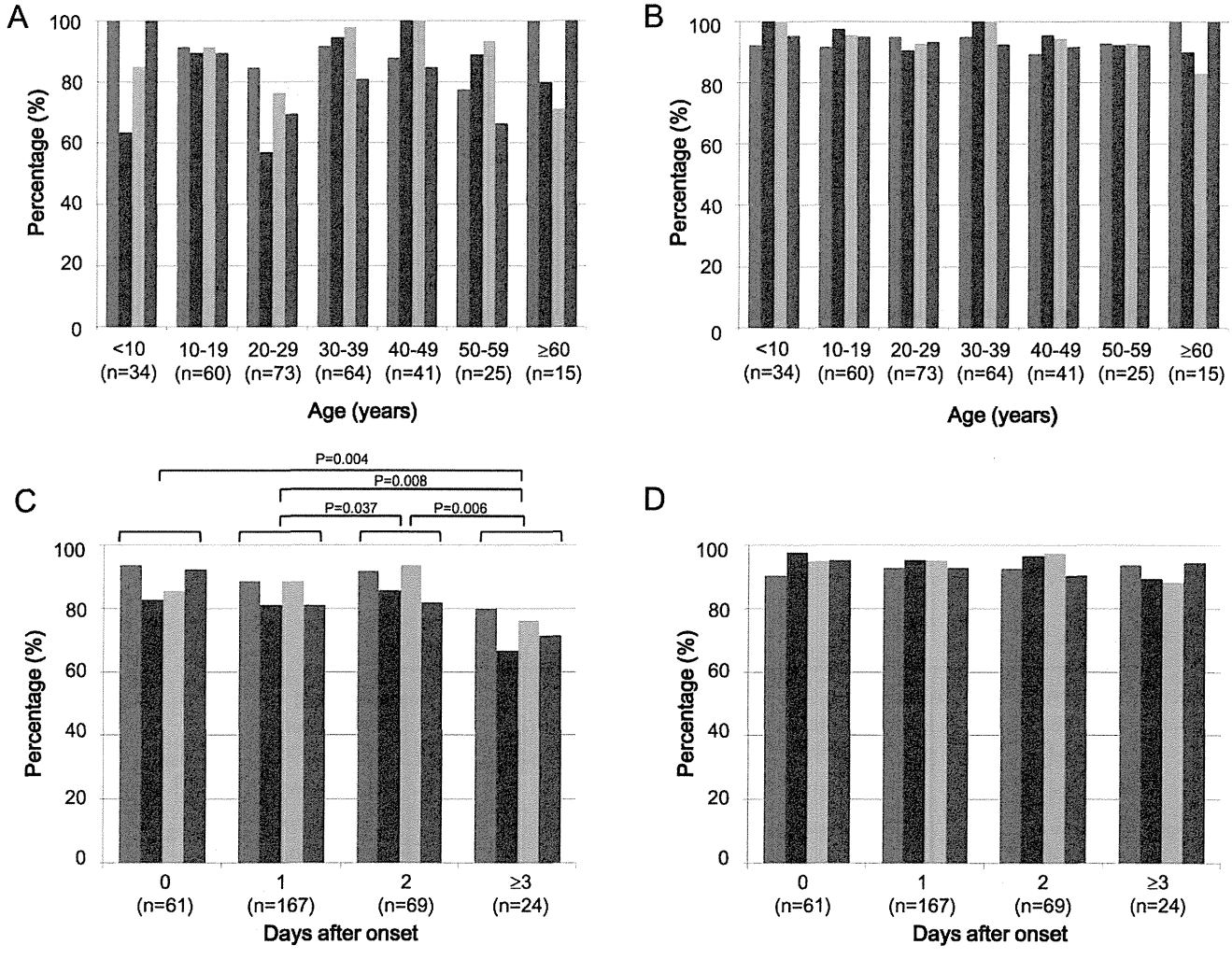

Figure 2. Effect of patient age and duration from disease onset to collection of samples on rapid test kit results. The SEA-IC test kit (A, C) and the PDM-IC test kit (B, D) were independently evaluated for sensitivity (blue), specificity (red), PPV (green), and NPV (purple) according to patient age (from <10 to ≥60 years old) (A, B) and duration from disease onset to sampling (1, 2, and ≥3 days) (C, D). P values in (C) indicate significant differences between groups. The 332 samples with information regarding patient age were used for (A) and (B), and the 321 samples with information regarding the number of days after disease onset were used for (C) and (D).
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Table 2. Evaluation of rapid test kits for POCT at each clinic.

Clinic	Sensitivity		Specificity		PPV[a]		NPV[b]	
	SEA-IC	PDM-IC	SEA-IC	PDM-IC	SEA-IC	PDM-IC	SEA-IC	PDM-IC
A (n = 15)	90.9	83.3	100	100	100	100	80.0	90.0
B (n = 10)	50.0	60.0	100	100	100	100	57.1	71.4
C (n = 56)	100	100	89.3	94.7	90.3	90.0	100	100
D (n = 60)	82.2	90.5	100	94.4	100	97.4	65.2	81.0
E (n = 11)	100	75.0	80.0	100	85.7	100	100	87.5
F (n = 16)	100	100	100	85.7	100	90.0	100	100
G (n = 21)	88.2	93.3	25.0	66.7	83.3	87.5	33.3	80.0
H (n = 28)	90.9	87.5	76.5	100	71.4	100	92.9	95.2
I (n = 16)	100	100	NA[c]	100	75.0	100	NA	100
J (n = 15)	75.0	60.0	85.7	100	85.7	100	75.0	83.3
K (n = 16)	100	100	66.7	100	92.9	100	100	100
L (n = 32)	95.5	100	60.0	92.3	84.0	95.0	85.7	100
M (n = 36)	71.4	91.7	81.8	100	71.4	100	81.8	96.0
Total (n = 332)	89.2	92.5	80.5	95.3	87.9	94.9	82.4	93.1
Correlation coefficient	0.706		0.657		0.180		0.784	
P value	0.007		0.020		0.555		0.003	

[a]Positive predictive value.
[b]Negative predictive value.
[c]Not applicable.
doi:10.1371/journal.pone.0050670.t002

Anti-viral Drug Prescription

As shown in Figure 3 and described above, a total of 542 swab samples were subjected to two IC test kits for POCT and stratified into five groups according to the type of virus infection. To understand the relationship between anti-viral drug prescription and the IC test kit results, we used the combined results of the two IC test kits. No information was available for 18 of these samples regarding drug prescription. Therefore, the remaining 524 samples were further grouped according to the presence or absence of drug prescription. Of these, 317 samples were subjected to RT-PCR analysis, 229 collected from individuals who received anti-influenza drug prescriptions (oseltamivir to 154 patients, zanamivir to 63 patients, laninamivir to 8 patients, and peramivir to 4 patients as shown in Table S2) based on POCT results and patient responses to questionnaires, and 88 from individuals who did not (Figure 3).

Of 179 cases confirmed as influenza virus-positive by RT-PCR except 11 cases of "A−B−, pdm+" by the IC test kit results, 95.5% (171/179) were appropriately prescribed anti-influenza drugs based on positive IC test results. However, 8 influenza virus-positive patients out of 179 did not receive prescriptions due to negative IC kit results. An additional 47 cases were prescribed drugs despite influenza virus-negative RT-PCR results due to 18 false-positive SEA-IC kit results, six false-positive results stemming from both kits, and 23 true negative results stemming from both kits. On the other hand, 27 patients at six clinics were prescribed anti-viral drugs even though they tested negative for influenza virus by POCT. Of these 27 patients, three cases of H1N1pdm infection and one case of H3N2 infection were subsequently detected by RT-PCR. Importantly, seven patients who did not receive drug prescriptions because of negative IC results later tested positive for H1N1pdm (six cases) or H3N2 (one case) by RT-PCR.

Discussion

The present study evaluated a newly-developed PDM-IC kit for its ability to expedite POCT for the detection of H1N1pdm. The study employed 542 duplicate swab samples collected during the 2010/2011 winter influenza season in one ward of Osaka city and two types of IC test kits, the previously-developed SEA-IC kit and the newly-developed PDM-IC kit. Comparison of the data with those obtained by RT-PCR analysis using the same samples revealed that the PDM-IC kit was significantly more sensitive and specific for H1N1pdm than the SEA-IC kit.

The establishment of POCT as a widely accepted method for the diagnosis of influenza depends on physician access to commercially available rapid diagnostic test kits. POCT can easily be performed with such kits within minutes for the detection of seasonal influenza A and B viruses, and the obtained results generally show quite high sensitivity and specificity for these viruses. However, detection of H1N1pdm by test kits that have been developed for seasonal influenza A and B viruses is unreliable, with comparatively low sensitivity and specificity [18,19,20,21,22,23,24,25,26]. Therefore, several IC test kits have recently been developed for the selective diagnosis of H1N1pdm [28,28,30,31].

In this regard, Choi et al. [30] reported 44.0% sensitivity and 99.9% specificity for the SD Bioline Influenza Antigen Test® (The Clinical Usefulness of the SD Bioline Influenza Antigen Test® for Detecting the 2009 Influenza A (H1N1) Virus). Kawachi et al. [29] similarly reported 73.0% sensitivity and 97.9% specificity for their influenza A H1N1 2009 virus test kit. Our previous study revealed 85.5% sensitivity and 100% specificity for a PDM-IC test kit employed using a total of 42 and 126 swab samples collected at one clinic in Osaka before and after the appearance of H1N1pdm, respectively [27]. The current study expanded on our previous

Table 3. Clinical symptoms and pre-existing diseases in study participants.

Symptom/pre-existing disease		Seasonal influenza A virus A+B−, pdm− (n = 46)	Seasonal influenza B virus A−B+, pdm− (n = 19)	H1N1pdm A+B−, pdm+ (n = 196)	Possible H1N1pdm A−B−, pdm+ (n = 12)	Positive seasonal A/B and H1N1pdm (n = 273)	Negative A−B−, pdm− (n = 269)
Fever		44 (95.7%)	18 (94.7%)	196 (100%)	12 (100%)	270 (98.9%)	264 (98.1%)
Upper respiratory inflammation		33 (71.7%)	8 (42.1%)	99 (50.5%)	5 (41.7%)	145 (53.1%)	119 (44.2%)
Lower respiratory	Pneumonia	2 (4.3%)	1 (5.3%)	20 (10.2%)	1 (8.3%)	24 (8.8%)	17 (6.3%)
inflammation	Asthma	0 (0%)	0 (0%)	1 (0.5%)	0 (0%)	1 (0.4%)	0 (0%)
Cough	Wet	15 (32.6%)	6 (31.6%)	57 (29.1%)	3 (25.0%)	81 (29.7%)	42 (15.6%)
	Dry	13 (28.3%)	4 (21.1%)	75 (38.3%)	4 (33.3%)	96 (35.2%)	35 (13.0%)
Arthritis		22 (47.8%)	5 (26.3%)	100 (51.0%)	5 (41.7%)	132 (48.4%)	117 (43.5%)
Muscular pain		11 (23.9%)	3 (15.8%)	58 (29.6%)	2 (16.7%)	74 (27.1%)	51 (19.0%)
Diarrhea		4 (8.7%)	1 (5.3%)	2 (1.0%)	0 (0%)	7 (2.6%)	13 (4.8%)
Vomiting or nausea		2 (4.3%)	1 (5.3%)	9 (4.6%)	1 (8.3%)	13 (4.8%)	16 (5.9%)
Malaise		6 (13.0%)	2 (10.5%)	17 (8.7%)	5 (41.7%)	30 (11.0%)	44 (16.4%)
Pharynx pain		1 (2.2%)	0 (0%)	9 (4.6%)	0 (0%)	10 (3.7%)	62 (23.0%)
Abdominal pain		1 (2.2%)	0 (0%)	2 (1.0%)	0 (0%)	3 (1.1%)	5 (1.9%)
Headache		3 (6.5%)	1 (5.3%)	24 (12.2%)	0 (0%)	28 (10.3%)	21 (7.8%)
Nasal discharge		1 (2.2%)	0 (0%)	11 (5.6%)	0 (0%)	12 (4.4%)	12 (4.5%)
Hypertension		0 (0%)	0 (0%)	2 (1.0%)	1 (8.3%)	3 (1.1%)	7 (2.6%)
Diabetes		0 (0%)	0 (0%)	1 (0.5%)	0 (0%)	1 (0.4%)	1 (0.4%)
Asthma		2 (4.3%)	0 (0%)	5 (2.6%)	0 (0%)	7 (2.6%)	2 (0.7%)
Renal disease		0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (0.4%)
Dialysis		0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Allergy		0 (0%)	1 (5.3%)	3 (1.5%)	0 (0%)	4 (1.5%)	2 (0.7%)
Pregnancy		1 (2.2%)	0 (0%)	0 (0%)	0 (0%)	1 (0.4%)	0 (0%)

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results by comparing the efficacy of the PDM-IC and SEA-IC test kits for the diagnosis of H1N1pdm, and also by using a much larger number of swab samples collected at 13 clinics after the appearance of H1N1pdm. The 542 samples contained both influenza Aand B viruses collected after the appearance of H1N1pdm, whereas the previous study employed only H1N1pdm-confirmed samples collected after the appearance of H1N1pdm.

The results presented herein, using 332 swab samples further analyzed by RT-PCR, showed 92.5% sensitivity and 95.3% specificity for the PDM-IC kit, relative to 89.2% sensitivity and 80.5% specificity for the SEA-IC kit. The high sensitivity and specificity of this and other newly-developed IC test kits for the rapid diagnosis of H1N1pdm can potentially be attributed to the characteristics of the monoclonal antibodies and the cut-off points chosen by the kit development company/personnel. In other words, the company that develops each kit can adjust the sensitivity and specificity of the kit according to its intended use. Users of these test kits should therefore be educated to select the type of kit most appropriate for their needs.

An advantage of the PDM-IC test kit described in this study is that it could be employed with the same swab samples as the SEA-IC kit. Therefore, we were able to obtain results from both test kits using the same samples at the same time. Thus, the PDM-IC kit can be used as a POCT tool to supplement SEA-IC kit data in clinics. Comparison of the data obtained at 13 individual clinics showed significant correlations regarding sensitivity, specificity, and NPV between the SEA-IC kit and the PDM-IC kit. This indicated that the corresponding scores of the IC kits were most likely affected by the skills of the medical and co-medical staff at each clinic. Therefore, increased application of rapid diagnosis test kits for infectious diseases necessitates training in their proper use, and not just selection of the appropriate test kit.

No apparent differences were observed between different age groups in terms of the results yielded by the PDM-IC kit, while some inter-age differences were detected with the SEA-IC kit. Of note, the accuracy of the results of the SEA-IC kit apparently deteriorated at ~3 days after the onset of clinical symptoms. Similarly, a previous report showed that the sensitivity of another IC test kit for H1N1pdm abruptly declined when using the swab samples collected from patients on the third day after the onset of fever [27]. These results are reasonable given that the number of virus particles excreted by a patient generally decreases at 3–4 days after the onset of symptoms [32]. Nevertheless, the current PDM-IC kit demonstrated high sensitivity when used with samples collected on day third or more days versus day 1 and day 2, further demonstrating the reliability of the kit.

Several cases (12/542 = 2.2%) in this study gave inconsistent results when analyzed with the two different IC test kits. In addition, the IC kit results and RT-PCR results were inconsistent for 14.2% (47/332) of the cases. Although both kits yielded several false-negatives and false-positives, overall, the PDM-IC kit demonstrated better specificity for H1N1pdm than the SEA-IC kit. IC kit-derived false-negatives (IC kit-negative, but RT-PCR-