showed that PSSP, PISP and PRSP accounted for 49.6, 28.5 and 21.9%, respectively, according to the criteria of the NCCLS [22]. Nasopharyngeal pathogens are well recognized as the causative agents of middle ear infections [23–25]. In most pediatric AOM cases, identical pathogens were isolated from both middle ear and the nasopharynx [26]. Nasopharyngeal pathogens were also closely related with the clinical outcome of AOM [8, 9]. Thus, attempts were made in this study to obtain more clear-cut differences among *S. pneumoniae* isolated from the nasopharynx in pediatric AOM patients by PCR-based genotyping depending on the mechanism of resistances [19]. Briefly, PCR primers to amplify the locus of *pbp* genes highly conserved among the PSSP were designed in this study. The primers failed to amplify the target *pbp* genes when the genes included mutations. PBP1A displays both transglycosylase and transpeptidase activities and synthesizes a peptidoglycan for the long axis of the cell walls. Alterations in PBP1A are most important for the development of resistances to penicillins and increased resistances to both penicillins and cephalosporins with additional mutations in *pbp2b* and *pbp2x* [11, 15, 27, 28]. PBP2B is a transpeptidase for developing the lancet form. Alterations in PBP2B are related with developing low levels of resistance to penicillin [29, 30]. The PBP2X is necessary for septum formations. Alterations in PBP2X mediated the resistance to cephalosporins [31–34].

Three distinct genotypes were identified in the current study. They were the strains with mutations in all *pbp1a*, *pbp2x*, and *pbp2b* genes, the strains with mutations in *pbp2x* gene, and the strains without mutation in the three *pbp* genes. The strains with mutations in all three *pbp* genes were identified in 38.2% of all isolates and 96.5% of PRSP. The strains with mutations in the three *pbp* genes were also highly resistant to cephalosporins [35]. Other reports suggested that the decreased affinity of PBP1A, 2B, and 2X for β-lactams played an important role in the development of resistances [11–15]. The 13.5% of PISP isolates also had mutations in the three *pbp* genes. On the other hand, 55.6% of PSSP strains were without mutations in *pbp* genes. The strains with mutations in *pbp2x* gene were identified in 26.0% of all of the isolates. The strains were also identified in 35.9% of PSSP. Ito et al. [36] reported the high prevalence of PRSP in the nasopharynx of healthy children attending day care centers or a public health examination. About 75% of children attending day care centers had PRSP while only 37% of children who were not attending day care centers had *S. pneumoniae*. The PRSP are highly prevalent in children regardless of acute otitis media. In contrast to this surveillance in Japan, the strains with mutations in *pbp2x* gene were identified less often in the United States and European countries [19, 37]. These discrepancies in the prevalence of the strains with mutations in *pbp2x* gene may depend on the prescription pattern of oral cephalosporins. The strains with mutations in *pbp2x* were usually susceptible to penicillin, but showed decreased susceptibilities to oral cephalosporins. In Japan, physicians usually prescribe oral cephalosporins, while in the United States penicillins are frequently prescribed to outpatients. In this surveillance, a total of 75.8% of nasopharyngeal isolates from children had mutations in *pbp* genes. Although the mechanisms of resistance to macrolides are different from those to β-lactams, about 84% strains with mutations in *pbp* genes were also resistant to macrolides (data not shown).

The annual prevalence of mutations in *pbp* genes showed a rapid decrease in strains without mutations in *pbp* genes. The strains with mutations in *pbp2x* increased in 1998–2000, and then gradually decreased in 2001–2002. Alterations in PBP2X are important in so far as they increase resistance to cephalosporins, while PBP1A mediates resistances to penicillins. The rapid increase in PRSP in Japan was reported to parallel the broad use of oral cephalosporins [37]. The different patterns of prescription of antibiotics, especially oral cephalosporins, to pediatric outpatients in Japan compared with other countries are considered as the cause of the high prevalence of pneumococci with mutations in *pbp2x*. Since 1999, we changed the policy of antimicrobial treatment of pediatric outpatients. Amoxicillin is now the drug of first choice instead of the usual prescriptions of oral cephalosporins for pediatric infectious diseases of the upper respiratory tract. This change in antimicrobial treatment policies might influence the annual prevalence of the strains with mutations in only the *pbp2x* gene. On the other hand, strains with mutations in the three *pbp* genes increased from 2001 to 2002. Induction of mutations in the three *pbp* genes might be different from those in only *pbp2x*. Further precise surveillance is necessary to evaluate the evolution of these mutations.

Our data show that, along with MICs, we can use PCR-based genotyping to characterize and improve our understanding of antimicrobial resistance in pneumococci. When treating pediatric infectious diseases, physicians should pay attention to recent increases in antimicrobial-resistant *S. pneumoniae*.

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NOTE *Virology*

Pathogenicity of H5N1 Influenza A Viruses Isolated in Vietnam between Late 2003 and 2005

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ABSTRACT. Since late 2003, highly pathogenic H5N1 influenza A viruses have spread among poultry and wild aquatic birds in Asian countries. Transmission of these viruses to humans can be lethal. Most human cases of infection with H5N1 viruses have occurred in Vietnam. Therefore, to understand the pathogenicity in mammals of these H5N1 viruses, we took viruses isolated from poultry (5 strains) and humans (2 strains) in Vietnam and tested their virulence in mice. The results showed that the H5N1 viruses from humans were pathogenic in mice and that one avian isolate was also pathogenic. These findings suggested that the H5N1 viruses circulating in poultry adapted during replication in humans or that strains pathogenic in mice were transmitted directly to humans.

KEY WORDS: H5N1 avian influenza virus, pathogenicity, Vietnam.

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In 1997, in Hong Kong, 18 people were infected with highly pathogenic avian influenza (HPAI) viruses of the H5N1 subtype, six of whom died [2, 9]. These were the first recognized cases of HPAI viruses infecting humans with lethal outcomes. The H5N1 viruses isolated from humans during this outbreak varied in their virulence in mice; some replicated systemically with lethal outcomes, whereas others did not [3, 6]. Thus, some HPAI viruses can replicate efficiently in mammalian hosts without adaptation.

Since late 2003, HPAI H5N1 viruses have spread among domestic poultry and wild aquatic birds in Vietnam, Thailand, China, Korea, Japan, Laos, Cambodia, and Indonesia (World Organization for Animal Health [OIE] [http://www.oie.int]). These outbreaks caused devastating damage to domestic poultry populations in these countries and have yet to be controlled in some areas. To date, 63 deaths among 124 humans infected with the H5N1 viruses have been recognized in Asian countries (World Health Organization [WHO] [http://www.who.int/en/]). Of these, 91 infected patients and 41 deaths were reported in Vietnam. Because the majority of the human cases of H5N1 virus infection have occurred in Vietnam, it is important to determine the pathogenic nature of the H5N1 viruses circulating there. Accordingly, we assessed the pathogenicity in mammals of H5N1 viruses circulating in Vietnam, by inoculating mice with H5N1 viruses isolated from poultry and from humans.

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Five viruses isolated from chickens or a duck between January 2004 and August 2005 were propagated in the allantoic cavities of 10-day-old embryonated chicken eggs (Table 1). Two viruses isolated from humans in late December 2003 and January 2004 in Vietnam were also used in this study (Table 1). All five avian and both human isolates contained multiple basic amino acids at the hemagglutinin (HA) cleavage site (Muramoto Y. *et al.*, in press), which is indicative of high virulence in chickens [4, 8]. To characterize the virulence of these viruses in mice, we determined the MLD_{50} (i.e., the dose required to kill 50% of the infected mice) for each virus. Groups (n=3 per group) of 6-week-old female BALB/c mice (Japan SLC, Hamamatsu, Japan) were anesthetized with sevoflurane and infected

Table 1. Virulence in mice of H5N1 influenza viruses isolated in Vietnam from 2003 to 2005[a]

Origin	Virus	Date isolated	MLD_{50} (log_{10} PFU)
Avian	A/chicken/Vietnam/G04/04	Jan. 2004	3.3
	A/chicken/Vietnam/G62/05	Jan. 2005	> 4.0[b]
	A/duck/Vietnam/5004/05	Jan. 2005	> 6.0
	A/chicken/Vietnam/TY9/05	Apr. 2005	> 6.0
	A/chicken/Vietnam/TY31/05	Aug. 2005	> 6.0
Human	A/Vietnam/3028II/03	Dec. 2003	2.5
	A/Vietnam/3040/04	Jan. 2004	0.25

a) Six-week-old BALB/c mice were intranasally infected with virus.
b) 10^4 PFU was the maximum titer in 50 μl virus, due to its inability to grow to a high titer in eggs.
MLD_{50}, the dose required to kill 50% of infected mice; PFU, plaque-forming units.

intranasally with serial 10-fold dilutions of viruses, thereby creating doses ranging from 10^0–10^6 plaque-forming units (PFU). The mice were monitored daily for clinical signs of infection for 14 days postinfection (p.i.). MLD_{50} values were calculated using the method of Reed and Muench [7].

Both H5N1 viruses isolated from human patients were pathogenic in mice, although the MLD_{50} of A/Vietnam/3040/04 was more than 100-fold lower than that of A/Vietnam/3028II/03 (Table 1). By contrast, 4 of the 5 viruses isolated from poultry did not kill the mice, with only A/chicken/Vietnam/G04/04 being lethal, despite its MLD_{50} being higher than those of the human isolates. These results indicate that the H5N1 viruses isolated in Vietnam vary in their pathogenicity in mice.

To understand the basis for the difference between lethal and nonlethal avian H5N1 isolates in mice, we examined viral replication in the organs of mice infected with the lethal A/chicken/Vietnam/G04/04 and the nonlethal A/chicken/Vietnam/TY9/05. Groups (n=3/group) of mice were infected with 10^6 PFU of virus, and three and six days later, lungs, spleen, and brain were collected for virus titration. The tissues were homogenized in cold phosphate-buffered saline to make 10% homogenates of lung tissue and 20% homogenates of spleen or brain tissue. The solid debris was removed by brief centrifugation, and undiluted and 10-fold serially diluted supernatants were used to titrate virus infectivity with MDCK cells (Table 2).

Although nonlethal A/chicken/Vietnam/TY9/05 was detected in lungs at both three and six days p.i., the virus titer was lower on day 3 p.i. compared to day 6. In addition, this virus was not detected in the other organs tested. By contrast, substantially higher titers of virus were detected in the lungs of mice infected with the lethal A/chicken/Vietnam/G04/04 at both day 3 and day 6 p.i. Moreover, this virus was also recovered from the brain of all infected animals on day 6, and from the spleen of some of the animals. These results indicate that the severity of disease caused by A/chicken/Vietnam/G04/04 was likely associated with the efficient replication of the virus in this host and due to broad tissue tropism, including the ability to replicate in brain.

Here, we have identified one avian and two human isolates from Vietnam that are highly pathogenic in mice. The avian strain possesses HA and neuraminidase genes that are closely related to those of H5N1 viruses that were isolated from humans in 2004 in Vietnam (Muramoto Y. *et al.*, in press), indicating that an H5N1 virus capable of causing lethal infection in mammals was circulating in avian species in Vietnam. Finding a virus lethal to mice in chickens raises an interesting possibility. A/goose/Guangdong/1/96, the precursor of the H5N1 viruses isolated to date, and those isolated from birds prior to 2001 were nonpathogenic in mice [1, 5]. Therefore, the H5N1 viruses isolated from humans in 1997 in Hong Kong that were pathogenic in mice were likely selected during replication in humans from viruses nonpathogenic in mice. However, with the isolation of a chicken virus that is lethal to mice in Vietnam, the possibility exists that some of the humans infected in the current outbreak were exposed to H5N1 viruses that were already lethal to mice. In fact, H5N1 avian isolates capable of causing lethal infection in mice have been reported previously [1]. The circulation of these avian isolates may explain the higher mortality rate in humans (>50%) in the 2003 2005 outbreak than was seen in the 1997 outbreak (33%).

It is noteworthy that the virus titers in the lungs of mice infected with the nonlethal avian isolate A/chicken/Vietnam/TY9/05 were higher on day 6 p.i. than on day 3 (Table 2). This finding suggests possible adaptation of this virus in mice. Thus, in addition to the scenario described above (i.e., direct transmission to humans of virus with the potential to cause lethal infection in mammals), nonlethal virus could adapt during replication in humans to become more pathogenic, a circumstance that likely occurred in the 1997 outbreak. In either case, the H5N1 viruses continue to pose a serious threat to public health as long as they circulate in poultry, a situation unlikely to change in the near future. It is clearly essential that we continue to monitor the genetic and biologic changes in these viruses.

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Table 2. Replication of avian H5N1 viruses in mice[a)]

Viruses	Days postinfection	Virus titer (mean $\log_{10}$ PFU ± SD/g) in		
		Lungs	Spleen	Brain
A/chicken/Vietnam/TY9/05	3	2.3, 3.9		
	6	4.2 ± 0.6		
A/chicken/Vietnam/G04/04	3	6.4 ± 0.2	3.1, 3.7	
	6	6.1 ± 0.5	2.0	4.2 ± 0.1

a) Six-week-old BALB/c mice, anesthetized with sevoflurane, were infected intranasally with 10^6 PFU of virus. Three mice from each group were euthanized on day three or six postinfection. Virus titers were determined using MDCK cells. The lower limits of virus detection were < 2.0 $\log_{10}$ PFU per gram of lung tissue and < 1.7 $\log_{10}$ PFU per gram of spleen or brain tissue. When virus was not detected from all three mice, individual titers were recorded. –, virus was not detected from any of the infected mice.

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NOTE *Virology*

Molecular Characterization of the Hemagglutinin and Neuraminidase Genes of H5N1 Influenza A Viruses Isolated from Poultry in Vietnam from 2004 to 2005

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ABSTRACT. Highly pathogenic H5N1 avian influenza A viruses have been spreading among domestic poultry, wild aquatic birds, and humans in many Asian countries since 2003. The largest number of patients, to date, infected with the H5N1 viruses are in Vietnam, where these viruses continue to cause outbreaks in domestic poultry. Here, we molecularly characterized the hemagglutinin and neuraminidase genes of nine H5N1 viruses isolated between January 2004 and August 2005 from domestic poultry in Vietnam. We found that several groups of highly pathogenic H5N1 avian influenza viruses are circulating among these birds, which suggests that H5N1 viruses of different lineages have been introduced into Vietnam multiple times.

KEY WORDS: H5N1 avian influenza virus, hemagglutinin, neuraminidase.

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Influenza A viruses are enveloped viruses covered with surface glycoproteins, including two integral membrane proteins—hemagglutinin (HA), which possesses receptor-binding activity, and neuraminidase (NA), which possesses the sialidase activity required for the release of progeny viruses from the cell surface. Influenza A viruses are classified into subtypes based on their antigenic differences. To date 16 HA subtypes and 9 NA subtypes of viruses have been isolated from aquatic birds, the natural reservoir of influenza A viruses [16]. Among these subtypes are viruses belonging to the H5 or H7 subtype, which are highly pathogenic in domestic poultry.

In 1997, in Hong Kong, a pathogenic avian H5N1 influenza virus infected 18 people, 6 of whom died [2, 15]. All of the viruses isolated from patients contained only avian virus genes and were not reassortants with human viruses [2, 15]. These infections were, therefore, recognized as the first cases of direct transmission of influenza viruses from avian species to humans with lethal outcome. Since late 2003, highly pathogenic H5N1 avian influenza viruses have spread among domestic poultry and wild aquatic birds in many Asian countries (World Organization for Animal Health [OIE] [http://www.oie.int]) [1, 7], devastating domestic poultry populations. As of November 29, 2005, more than 133 humans have been infected with these H5N1 viruses. The high mortality associated with these infections, coupled with the difficulty in controlling these outbreaks, has raised concerns of a possible human pandemic. Vietnam has seen the largest number of patients infected with the H5N1 viruses: 93 patients, with 42 deaths (World Health Organization [WHO] [http://www.who.int/en/]). Outbreaks of the H5N1 virus continue to cause problems there. To better understand the H5N1 viruses circulating in domestic poultry populations in Vietnam, we molecularly characterized the HA and NA of viruses isolated between January 2004 and August 2005.

Tracheal swabs were collected from domestic poultry in different provinces in Vietnam from January 2004 to August 2005 (Table 1 and Fig. 1). Samples were inoculated into embryonated hen's eggs, and viral RNAs extracted from the virus-containing allantoic fluid by using a commercial kit (QIAamp Viral RNA mini kit, Qiagen, Hilden, Germany). HA and NA gene segments were amplified by RT-PCR using reverse transcriptase (SuperScript III Reverse Transcriptase, Invitrogen, CA, U.S.A.), DNA polymerase (PfuUltra High-Fidelity DNA Polymerase, Stratagene, CA, U.S.A.), and primers for the HA and NA genes of H5 viruses (available upon request). These PCR-derived dsDNAs were then cloned into a vector (TOPO vector, Invitrogen, CA, U.S.A.) and two or three clones of each isolate were sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, CA, U.S.A.). All sequences were deposited in the Influenza Sequence Database (http://daphne.lanl.gov/) [8]. Phylogenetic analysis (Fig. 2) was performed by using Clustal W program.

Sequence analysis of the HA genes from the nine isolates

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Table 1. H5N1 avian influenza viruses and the accession numbers of the HA and NA sequences analyzed in this study

Virus	Abbreviations	Date of sample collection	Province of sample collection	Accession no. of gene	
				HA	NA
A/chicken/Vietnam/G04/04	CkG04	Jan. 2004	Hatay	ISDN128306	ISDN128352
A/chicken/Vietnam/G62/05	CkG62	Jan. 2005	Thaibinh	ISDN128307	ISDN128353
A/duck/Vietnam/5001/05	Dk5001	Jan. 2005	Hatay	ISDN128302	ISDN128348
A/duck/Vietnam/5003/05	Dk5003	Jan. 2005	Angiang	ISDN128303	ISDN128349
A/duck/Vietnam/5004/05	Dk5004	Jan. 2005	Tiengiang	ISDN128304	ISDN128350
A/chicken/Vietnam/TY9/05	CkTY9	Apr. 2005	Hagiang	ISDN128308	ISDN128354
A/duck/Vietnam/5082/05	Dk5082	Jun. 2005	Thaibinh	ISDN128305	ISDN128351
A/chicken/Vietnam/TY25/05	CkTY25	Jul. 2005	Ninhbinh	ISDN128309	ISDN128355
A/chicken/Vietnam/TY31/05	CkTY31	Aug. 2005	Hatay	ISDN128310	ISDN128356

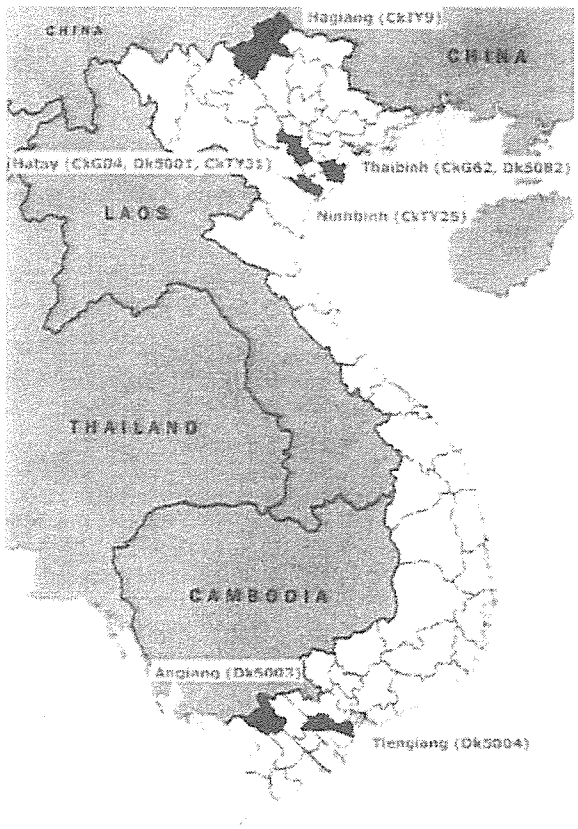


Fig. 1. Location of the Vietnamese provinces where avian H5N1 virus isolate samples were collected.

showed that all the HAs examined contained multiple basic amino acids at the cleavage site, although there were some variations (PQRERRRKKR/G, PQRERRKKR/G, and PQRERRRKR/G). Because HAs with multiple basic amino acids at their cleavage site are readily cleaved by ubiquitous cellular proteases [5, 14], these nine isolates are likely to be highly virulent to chickens.

The phylogenetic analysis of the HA genes showed that H5N1 viruses isolated after 2003, including the nine Vietnamese isolates in this study, can be divided into three clades (Fig. 2A). Six of the Vietnamese isolates sequenced here are closely associated with the H5N1 viruses that have been isolated from poultry and humans in Vietnam since 2004 (clade 3). These viruses may, therefore, be enzootic in Vietnam. A/chicken/Vietnam/G62/05 isolated in early 2005 belongs to the first clade, and A/chicken/Vietnam/TY25/05 and A/chicken/Vietnam/TY31/05 isolated in July and August 2005, respectively, to the second clade. The viruses in clade 2 were isolated in several countries, including Japan, Korea, China, and Indonesia. While the NA genes of H5N1 viruses isolated after 2003 are divided into two clades, the phylogenetic relationship of the NAs of the nine Vietnamese isolates was essentially the same as that of their HA genes except A/chicken/Vietnam/G62/05 belongs to clade 2 (Fig. 2B).

The HAs of avian influenza viruses preferentially bind to receptors that terminate in an α(2,3)-linked sialic acid, whereas those of human viruses preferentially bind to receptors terminating in an α(2,6)-linked sialic acid [11, 13]. In the past three pandemics, the receptor-binding specificity of the pandemic viruses converted from the avian virus-preferred α(2,3)-linked sialic acid receptor to the human virus-preferred α(2,6)-linkage [3, 9]. Thus, for avian viruses to cause a pandemic among humans, the receptor-binding specificity of their HAs may have to change to α(2,6)-linked sialic acid specificity. Here, alignment of the HA amino acid sequences revealed that all of the poultry isolates examined in this study possessed Gln at position 226 and Gly at position 228 (in H3 numbering), which are known to be associated with binding to α(2,3)-linked sialic acids [12]. In addition, the other amino acids of the receptor-binding site (residues Tyr98, Ser136, Trp153, Ile155, His183, Asn186, Glu190, and Leu194) were identical among all nine isolates and to those of the HA of A/Hong Kong/156/97, an H5N1 virus isolated from a patient in Hong Kong in 1997 and known to preferentially bind to α(2,3)-linked but not α(2,6)-linked sialic acids [4, 10]. These results suggest that the HAs of these nine isolates would preferentially recognize α(2,3)-linked sialic acids. Thus, it is unlikely for these isolates to be efficiently transmitted among humans without

A

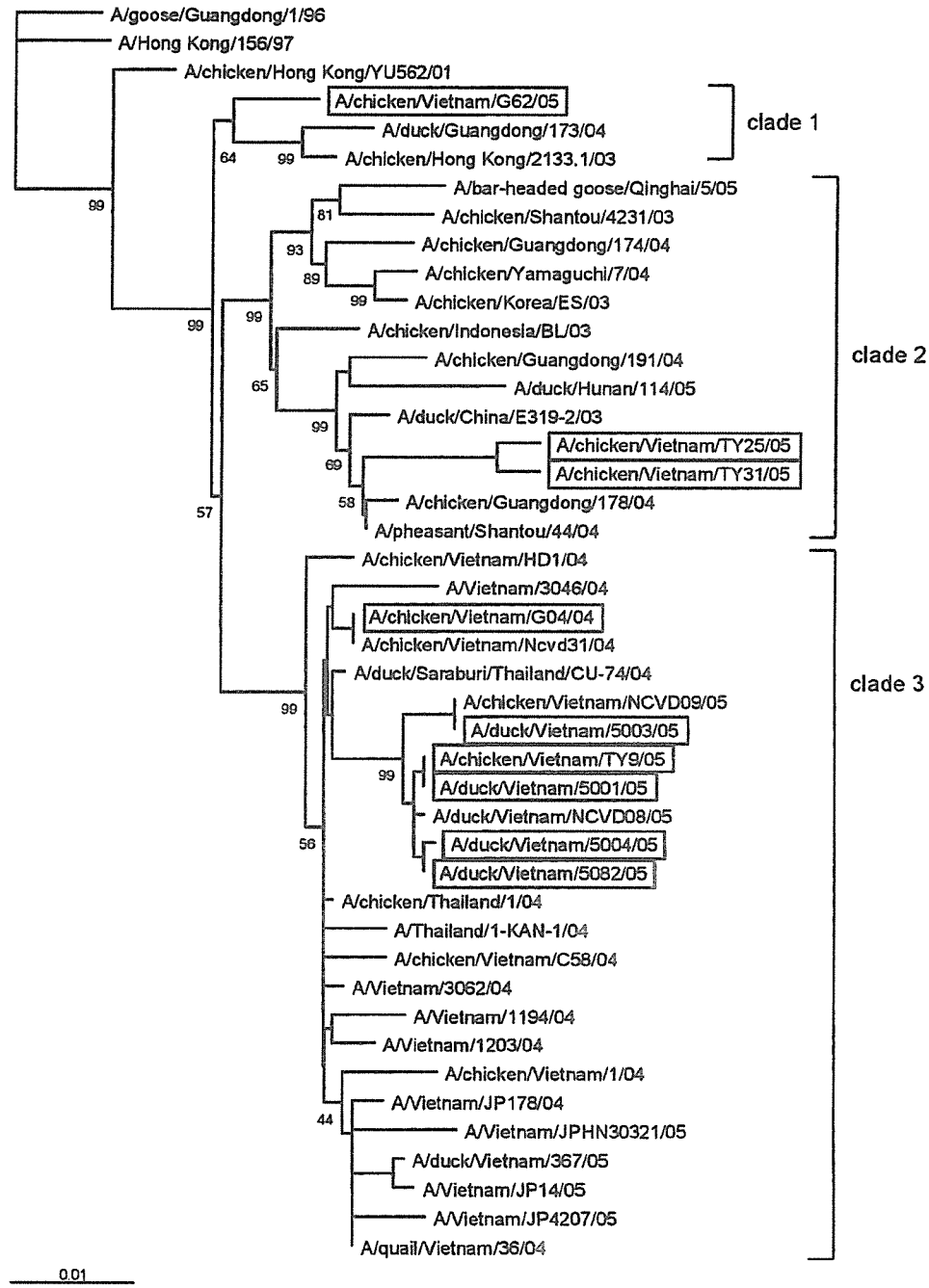
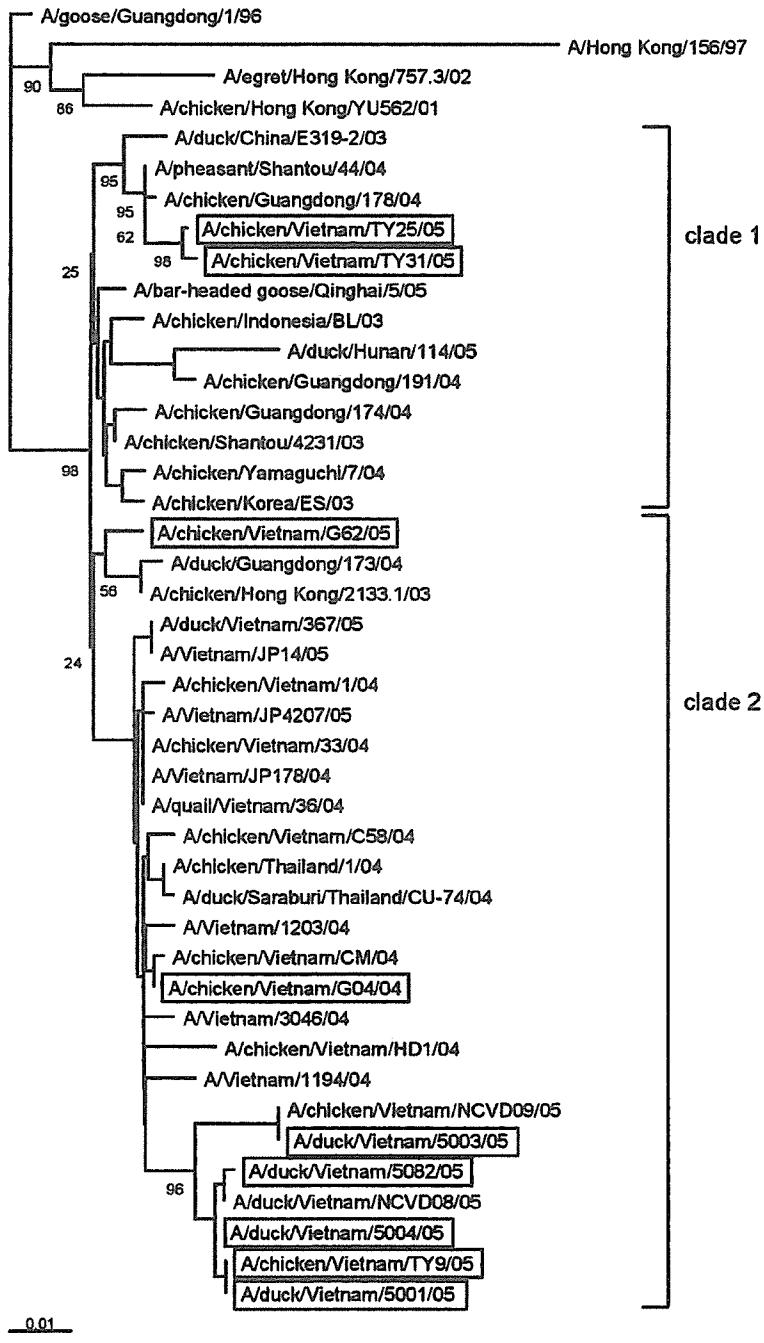


Fig. 2. The phylogenetic trees of the H5 HA (A) and N1 NA (B) genes of influenza A viruses. Viruses analyzed in this study are depicted in boxes. Phylogenetic trees were inferred from nucleotide sequences by the neighbor-joining method. Nucleotides 501 1735 (1235 bases) of the H5 HA genes and 441 1370 (930 bases) of the N1 NA genes were used for the phylogenetic analyses. The scale bar indicates 0.01 nucleotide changes per site. Numbers at the nodes indicate confidence levels of a bootstrap analysis with 1,000 replications as a percentage value. Only the bootstrap values that are critical for defining important groups are shown.

B



additional mutations that would make them recognize α(2,6)-linked sialic acids.

Alignment of the NA amino acid sequences revealed that all nine isolates contain a 20-amino acid deletion in the stalk region. Such a deletion is associated with adaptation of influenza viruses from aquatic birds to chickens [10]. In fact, a similar deletion has been detected in other H5N1 viruses isolated since 2002 from land-based poultry in Viet-

nam and other Asian countries [6] as well as in H5N1 viruses isolated in the 1997 outbreak in Hong Kong (e.g., A/Hong Kong/156/97) [2]. Our findings, therefore, indicate that all of the isolates in our study have been adapted to land-based poultry such as chickens and quail.

Here, we have demonstrated that several groups of highly pathogenic H5N1 avian influenza viruses have been circulating among poultry in Vietnam, suggesting that H5N1 viruses of different lineages have been introduced into Vietnam multiple times. How these viruses were introduced into Vietnamese poultry remains unknown. Several possible routes exist, including introduction by wild birds, virus-contaminated materials, or imported poultry and ducks. Strict control of the introduction of potentially virus-contaminated materials into countries would help limit or prevent the establishment of these viruses in domestic birds; however, since the H5N1 virus is now part of the wild bird population [1, 7], the virus can also be introduced into domestic poultry by wild birds. Continued surveillance of poultry for influenza infection is critical to minimize the magnitude of an outbreak and thus limit the risk of human infection.

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ORIGINAL PAPER

Rapid detection of avian influenza virus A and subtype H5N1 by single step multiplex reverse transcription-polymerase chain reaction

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Abstract Outbreaks of H5N1 highly pathogenic avian influenza (HPAI) virus caused great economic losses to the poultry industry and resulted in human deaths in Thailand and Viet Nam in 2004. Rapid typing and subtyping of H5N1 viruses, especially from clinical specimens, are desirable for taking prompt control measures to prevent the spread of the disease. Here, we developed a set of oligonucleotide primers able to detect, type and subtype H5 and N1 influenza viruses in a single step multiplex reverse transcription-polymerase chain reaction (RT-PCR). RNA was extracted from allantoic fluid or from specimens with guanidinium isothiocyanate reagent. Reverse transcription and PCR were carried out with a mixture of primers specific for influenza viruses of type A, subtype H5 and N1 in a single reaction system under identical conditions. The amplified DNA fragments were analyzed by agarose gel electrophoresis. All the H5N1 viruses tested in the study and the experimental specimens presented three specific bands by the method established here. The results presented here suggest that the method described below is rapid and specific and, therefore, could be valuable in the rapid detection of H5N1 influenza viruses in clinics.

Keywords Avain influenza virus · H5N1 · Typing · Subtyping · Multiplex RT-PCR

Introduction

Influenza viruses are divided into type A, B and C on the basis of antigenic differences in the nuclear and matrix proteins of the virus and type A viruses are further subtyped on the basis of antigenic differences of the surface glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA) proteins [1]. So far, fifteen HA (H1 through H15) and nine NA (N1 through N9) subtypes have been identified [2, 3]. Among the 15 HA subtypes, only H5 and H7 are highly virulent in poultry although not all cause highly pathogenic avian influenza (HPAI). The rest of the viruses cause a much milder, primarily respiratory disease known as low pathogenic avian influenza (LPAI) [4]. Since the outbreaks of the H5N1 HPAI in Hong Kong in 1997, growing evidence has been showing that H5N1 viruses might directly cross the species barrier to infect humans and cause high mortality in both species [5–7]. In Asia, at the beginning of 2004, the outbreaks of the H5N1 HPAI caused not only tremendous economic losses to the poultry industry but also human infections and high mortality in Viet Nam and Thailand [8, 9].

Conventional laboratory diagnosis of avian influenza (AI) is based on virus isolation in tissue culture or embryonated chicken eggs. However, it is time consuming and labor intensive in spite of its high sensitivity and specificity. The enzyme-linked immunosorbant assay (ELISA) has been applied for the rapid detection of influenza virus [10], although it has comparatively poor sensitivity. There is a requirement for the development of rapid and sensitive diagnostic techniques for the verification of

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clinical diagnosis of influenza and improvement of the quality of surveillance systems.

Molecular techniques have enabled major advances in the speed and sensitivity of the laboratory diagnosis of viral infections [11]. The RT-PCR has a higher sensitivity (93%) for influenza A virus than cell culture (80%) and ELISA (62%) [12]. PCR-based methods for identification of subtypes of influenza A viruses [13], distinction between influenza type A, B and C viruses or differentiation of N1 from N2 viruses [14] have been developed. By the inclusion of multiple sets of primers, the multiplex RT-PCR assay offers the possibility for the molecular detection of the presence of more than one gene or genome segment in a single pathogen or in more than one pathogen in a single reaction system [14–16].

As earlier mentioned, although all the HPAI outbreaks have been caused by the H5 and H7 subtype viruses, not all H5 subtype viruses cause natural infection of HPAI in poultry. To date, almost all the H5N1 subtype viruses isolated in nature have been found to be HPAI viruses, except one case in which a low pathogenic H5N1 was reported [17]. Consequently, almost every isolate of H5N1 subtype viruses should be considered as of HPAI. In the present study, a multiplex RT-PCR method was established not only for typing of A, but also for subtyping of H5 and N1 AI viruses directly from the allantoic fluid, organ homogenates or cloacal and pharyngeal swabs. The results showed that the multiplex RT-PCR was a rapid, sensitive and convenient method for the diagnosis and investigation of infections caused by the H5N1 influenza viruses.

Materials and methods

Viruses

Reference viruses of H1 through H15 and N1 through N9 subtypes used in this study are described as before [18]. Ten H5N1 avian influenza viruses were stored in the laboratory. These viruses were cultured in embryonated chicken eggs and characterized by hemagglutinin inhibition (HI) and neuraminidase inhibition (NI) assays. Virus propagation and the extraction of RNA from the viruses were performed in the biosafety level 3 (BSL-3) laboratory.

Other avian viruses used for the specificity tests of the RT-PCR, in this study, included Newcastle disease virus, infectious bronchitis virus, infectious laryngotracheitis virus, infectious bursal disease virus, avian paramyxovirus-2 and EDS-76 virus, which are all available in the repository of viruses in the laboratory.

Type and subtype specific primers

The primers were selected from the conserved regions of target genes specific for NP, H5HA and N1NA, respectively, by the sequence data available in the GenBank. The sequence data was generated using the sequence analysis of the influenza database at http://www.flu.lanl.gov. The three primer sets were analyzed with OLIGO 6.0 primer design software to ensure that they would be used together in a multiplex format. The sequence information of the primers used to amplify each target is shown in Table 1.

Extraction of virus RNA

RNA was extracted from infectious allantoic fluid, lixivium of cloacal and pharyngeal swabs or organ homogenates using the guanidinium isothiocyanate extraction reagent (0.8 M guanidinium isothiocyanate, 0.4 M ammonium thiocyanate, 0.1 M potassium acetate, 5% glycerol, 38% phenol water) by ameliorating the process as described previously [19]. Briefly, 250 μl of the allantoic fluid, lixivium or organ homogenates (containing 50 mg of tissue) was mixed with 750 μl of guanidinium isothiocyanate reagent. After mixing thoroughly and keeping at room temperature for 5 min, the mixture was extracted with 200 μl chloroform. After 5 min at room temperature and centrifugation at 15,000×g for 15 min at 4°C, the RNA in the supernatant was precipitated by adding an equal volume of cold isopropanol. The mixture was then kept in liquid nitrogen for 3 min, after which the RNA precipitate was collected by centrifugation at 15,000×g for 10 min at 4°C. The pellet was then washed by 75% cold ethanol and

Table 1 Properties of the primers for Type A, Subtype H5 and N1

Specific primer		Primer sequences[a]	PCR products (bp)
Type A	NP-1150f	5′-AGRTACTGGGCHATAAGRAC-3′	327
	NP-1457r	5′-ATTGTCTCCGAAGAAATAAG-3′	
Subtype H5	H5-20f	5′-TTACACATGCYCARGACATACT-3′	549
	H5-547r	5′-ATCTYTGRTTYAGTGTTGATGT-3′	
Subtype N1	N1-568f	5′-TGGYTRACAATYGGAATTTC-3′	405
	N1-952r	5′-GTCWCCGAAAACYCCACTGCA-3′	

[a]Codes for mixed bases position: Y=C/T, R=A/G, W=A/T, H=A/C/T

dissolved in 50 μl of RNase-free water containing 1 μl of RNase inhibitor (30 U/μl, Genview).

One step RT-PCR for typing or subtyping only

For the detection of type A influenza viruses, or the H5 or N1 subtype influenza viruses, the amplification reaction contained primers only for the type A, H5 or N1 subtype specific targets. Since the nucleoprotein (NP) gene is relatively highly conserved in all type A influenza viruses [20], the NP primers were used to identify type A influenza viruses. The expected sizes of the RT-PCR products are listed in Table 1. These tests were designed to ascertain the availability of the primers in the one step RT-PCR assay.

RT-PCR was carried out in a reaction mixture (25 μl volume) containing 1× PCR reaction buffer (Promega), 0.25 mM each of four dNTPs (Promega), 2 mM $MgCl_2$ (Promega), 2 U AMV reverse transcriptase (Promega), 12 U RNase inhibitor (Genview), 1 U Taq polymerase (Promega), 0.4 μM of each primer, 1 μl of RNA template and 15.2 μl of RNase-free water. The RT-PCR conditions for the amplification of all three fragments were 42°C for 45 min, 95°C for 3 min, 35 cycles of 95°C for 30 s, 55°C for 40 s and 72°C for 40 s, followed by 72°C for 10 min.

Multiplex RT-PCR for typing and subtyping simultaneously

Multiplex RT-PCR was carried out in a reaction mixture (50 μl volume) containing 1.4× PCR reaction buffer (Promega), 0.325 mM each of four dNTPs (Promega), 2.5 mM $MgCl_2$ (Promega), 6 U AMV reverse transcriptase (Promega), 24 U RNase inhibitor (Genview), 4 U Taq polymerase (Promega), 0.4 μM of each primer for NP-1150f, NP-1457r, H5-20f and H5-547r, 0.6 μM of each primer for N1-568f and N1-952r, 3 μl of RNA template and 18.3 μl of RNase-free water. The multiplex RT-PCR conditions were 42°C for 45 min, 95°C for 3 min, 35 cycles of 95°C for 30 s, 55°C for 40 s and 65°C for 2 min, followed by a final 65°C for 10 min.

Agarose gel electrophoresis

Five microlitres of the amplified products were loaded in an agarose gel (1.0%) containing 0.5 μg/ml ethidium bromide and the electrophoresis was conducted in 1×TAE buffer. After the electrophoresis, the DNA bands were visualized by UV trans-illumination.

Results

Specificity test for the NP, H5 and N1 primers

One step RT-PCR was optimized and evaluated by the amplification with one primer pair of NP, H5 and N1 primers, respectively. Several different primers for the three primer sets were designed, but here, we only report the best primers that presented the ideal amplification results and could be used in any other experiments. Since the nucleotide sequences of the NP gene are relatively highly conserved in all subtypes of AI viruses [20], we designed the NP primers to amplify a 327 bp fragment of the NP genes. To check the specificity, the NP primers were used to amplify the H1 through H15 subtype reference viruses. The results of amplification with the NP primers are shown in Fig. 1 where only one band of 327 bp was visible.

H5 gene primers were designed by alignment of the sequences available in the GenBank and the sequences data in our laboratory. The H5 primers, as was the case with the NP primers, were acquired following thorough testing. All the H5 viruses used in the study yielded a single band (549 bp) after the one step RT-PCR (Fig. 2).

The specificity tests for the H5 primers were done to amplify the reference viruses from H1 to H15 subtypes. As shown in Fig. 3, only the H5 subtype virus gave a specific band, no expected bands were observed in viruses of the other subtypes.

Using the methods employed for the designing and testing of the NP and H5 primers, the N1 primers were developed and used to amplify the NA gene of the ten viruses. As shown in Fig. 4, all the ten viruses produced the

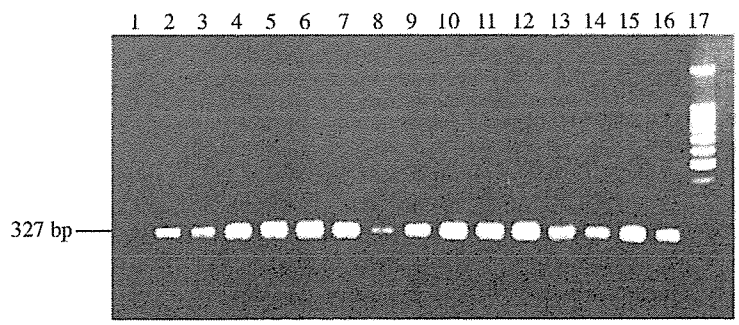

Fig. 1 RT-PCR results with NP primers. Lane 1, negative control; Lanes 2–16, reference influenza A virus strains from H1 through H15; Lane 17, markers (200 bp DNA ladder)

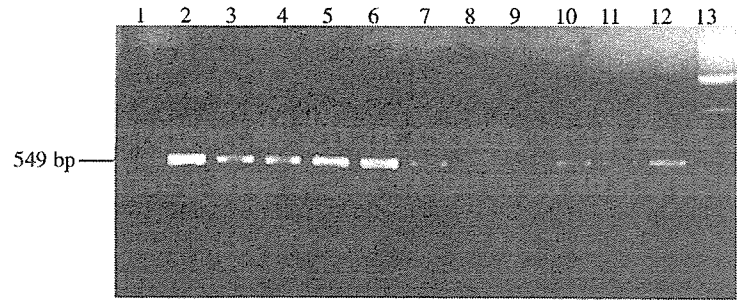

Fig. 2 RT-PCR results of H5N1 viruses with H5 primers. Lane 1, negative control; Lanes 2–11, Ten H5N1 field isolates; lane 12, reference strain A/Duck/HK/820/80(H5N3); Lane 13, size markers (100 bp DNA ladder)

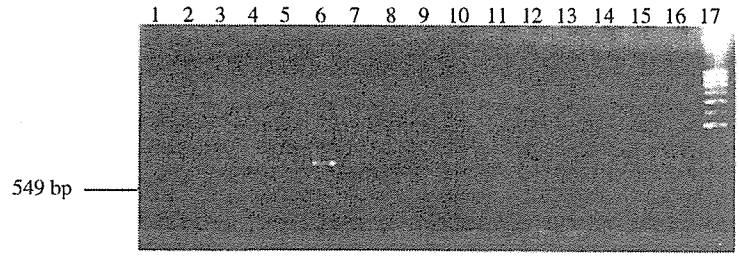

Fig. 3 RT-PCR results of H1 through H15 subtype viruses with H5 primers. Lane 1, negative control; Lanes 2–16, reference strains H1 through H15; Lane 7, size markers (200 bp DNA ladder)

expected single clear bands (405 bp). Subsequently, the specificity of the N1 primers was tested by the amplification of all the subtypes of the N1 to N9 of the reference strains with the one step RT-PCR. Only the strain of the N1 subtype gave the predicted DNA band as shown in Fig. 5.

The identities of these RT-PCR products were then confirmed by sequence analysis with Sanger dideoxy sequencing method and comparison using the BLAST search, which gave a maximum sequence homology of 98–100% when the nucleotide sequence of a RT-PCR product was compared to the sequences published in the GenBank (data not shown).

Multiplex RT-PCR for typing and subtyping simultaneously

The conditions of the multiplex RT-PCR for typing and subtyping, simultaneously, were optimized by modulating the concentration of the reaction buffer and the extension temperature. The results showed that a 1.4 fold concentration of PCR reaction buffer and 65°C of extension temperature were optimum for the reaction. The results of the DNA fragments for the influenza type A, subtype H5 and N1 specific gene fragments amplified simultaneously in a single step multiplex format are shown in Fig. 6. The RNA from all the viruses identified as H5N1 viruses by HI and NI tests in the laboratory was amplified and three specific bands, 549 bp, 405 bp and 327 bp for H5HA, N1NA, and NP, respectively, were produced.

The sensitivity of the multiplex RT-PCR using the three sets of primers was determined by testing a serial dilution of RNA (1 ng to 1 fg) of the H5N1 virus, and the sensitivity was found to be between 10 pg and 1 pg (data not shown). The specificity of the three primer sets was examined by RT-PCR using templates extracted from other avian viruses, including Newcastle disease virus, infectious bronchitis virus, infectious laryngotracheitis virus, infectious bursal disease virus, avian paramyxovirus-2 and EDS-76 virus. None of the above viruses gave the expected RT-PCR products after amplification (data not shown).

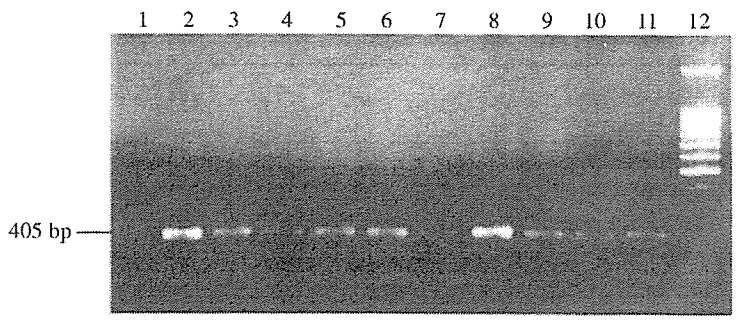

Fig. 4 RT-PCR results of H5N1 viruses with N1 primers. Lane 1, negative control; Lanes 2–11, ten strains of H5N1 viruses; Lane 12, size markers (200 bp DNA ladder)

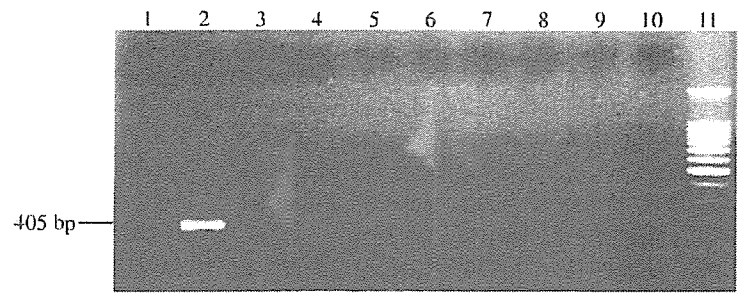

Fig. 5 RT-PCR results of reference viruses with N1 primers. Lane 1, negative control; Lanes 2–10, N1 through N9 reference strains; Lane 11, size markers (200 bp DNA ladder)

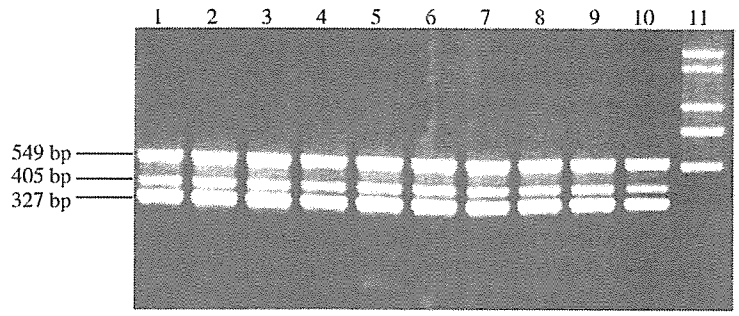

Fig. 6 RT-PCR results of ten H5N1 virus strains with three sets of primers. Lane 1–10, multiplex RT-PCR products of ten H5N1 viruses. Lane 11, size markers (250 bp DNA ladder)

Assay of experimental specimens by multiplex RT-PCR

One H5N1 virus strain was inoculated into 8-week-old SPF chickens by intravenous injection in the BSL-3 laboratory. Cloacal and pharyngeal swabs and the organs including trachea, lung, liver, kidney and spleen were collected and kept in the laboratory (−80°C) until use. The RNA was extracted from 250 µl of the supernatants prepared from the organ homogenates or the cloacal and pharyngeal swabs. The RT-PCR was carried out as outlined above and the results are presented in Fig. 7. All the samples produced the three expected specific bands. The identity of the three fragments was confirmed by sequence analysis with Sanger dideoxy sequencing method and comparison using the BLAST search and the results showed that they were all identical to those of the challenge virus strain.

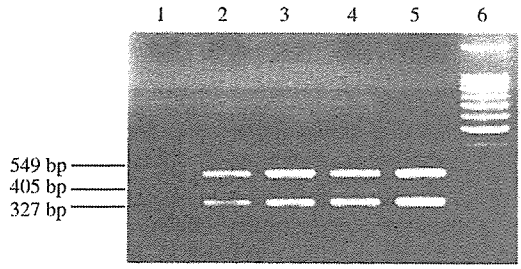

Fig. 7 RT-PCR results of experimental specimens. Lane 1, negative control; Lane 2–5, PCR products from cloacal and pharyngeal swabs, lung, liver and kidney, respectively. Lane 6, size markers (200 bp DNA ladder)

Discussion

Several laboratory methods, including the immunofluorescence, enzyme immunoassay, RT-PCR and TaqMan-PCR, for the rapid detection of influenza viruses have been reported during the past years. PCR assays have been designed for either type A only [21, 22], or for distinguishing between type A, B or C viruses [23, 24] or indeed for the diagnosis of human influenza. Subtype specific primers have been designed to detect single subtypes of type A influenza viruses [25] or to differentiate between subtypes of HAs [18, 26, 27] and NAs [27]. However, few reports show that the H5N1 subtype viruses could be identified by RT-PCR methods, simultaneously. The threat of imminent re-occurrence of outbreaks has existed since the outbreak incidents of the H5N1 subtype influenza in many countries. It is prudent to diagnose the disease with a rapid method for prompt stamping-out to abate any epidemic situation. The study aimed at the establishment of a rapid method for the screening or detection of not only the NP genes, the relatively highly conserved gene in all type A viruses [20], but also the H5HA and N1NA in a single reaction.

In the present study, we designed a multiplex RT-PCR to simultaneously amplify three genome segments in a single step to detect type A and subtype H5 and N1 influenza viruses using the NP, H5 and N1 specific primers. Conventional methods for the subtyping of influenza viruses require the expansion of the viruses in tissue culture or embryonated eggs, followed by subtyping with serological methods (HI and NI test). This demands

considerable efforts and might take a week or more to obtain the results. Here, we designed a one step RT-PCR assay that requires only a 5-h turn-around time making it appropriate for the rapid testing of clinical specimens. If required, sequence analysis of RT-PCR products, followed by sequence comparison and phylogenetic analysis, could be conducted so as to provide more valuable information such as the origin of the AI viruses identified.

The primers of the NP, H5 and N1, designed for the multiplex RT-PCR assay, were first examined separately to ascertain their usage for amplification together in a multiplex reaction under similar conditions. All primers were designed to ensure that the final reaction products could easily be differentiated on the basis of their size in a 1.0% agarose gel. To amplify the desired products, the multiplex RT-PCR conditions required optimization. Henegariu et al. [28] suggested that increasing the amount of primers for the weak loci and decreasing the amount for the strong loci should overcome the phenomenon of uneven amplification with some barely visible products. Here, the concentration of 0.6 mM N1 primers each and 0.4 mM of the other two sets of primers gave the three expected clear bands. Furthermore, the tests also showed that the yield of the RT-PCR products was increased by decreasing the extension temperature from the usual 72°C to 65°C. In addition, primers with short amplification products generally worked better at higher salt concentrations, whereas longer products became harder to denature [28]. Thus, in this study, the reaction buffer concentration was increased to 1.4 fold higher than that of the conventional one fold, thereby, leading to better amplification results. Three bands could be clearly obtained by using allantoic fluid, while, the intensity of the N1 band was lower than that of the other two bands when clinical samples were used in the study as shown in Fig. 7. We speculated that the virus titers in the samples from the challenged chickens could have been lower than those in the allantoic fluid. In addition, some animal organs like the pancreas and liver contain abundant amounts of RNase that consequently could degrade the extracted vRNA to some degree. Thus, the decreased amount of the RNA template would affect the yield of expected gene segments. Bej et al. [29] reported that one amplified gene fragment would create an inhibition effect on another expected gene fragment during the multiplex PCR. Thus, competition and interference among the amplified gene fragments could easily occur in multiplex PCR and the phenomenon may be more remarkable at lower template concentrations. Moreover, there may be less NA RNA than the other two genes, so NA may be produced in lower quantities. Further experiments would probably help to determine this.

The H5 and N1 primers are highly specific when detecting the H5N1 viruses and the adding of NP primers may decrease the sensitivity of the whole reaction to some extent, however, it is necessary to apply the NP primers that are aimed at determining viruses whose subtypes are neither H5 nor N1. It came out as shown in this article that when this method was applied to the detection of clinical specimens as described, it could still detect the viruses from different specimens, including cloacal and pharyngeal swabs and the organs such as trachea, lung, liver, kidney and spleen. Therefore, adding the NP primers to this multiplex RT-PCR reaction is necessary and it will not affect much the usage of multiplex method to detect clinical specimens.

This method is purposed to detect H5N1 influenza viruses of all birds and humans from clinical specimens. The amplification outcome with this multiplex RT-PCR method could be explained only when the negative control is reasonable. The results may be interpreted as demonstrated in Table 2 according to the bands shown by agarose gel electrophoresis. If there is no band of 327 bp corresponding to the NP gene, the results would be of none sense when either of the other two bands or both of them appear.

In order to establish a reliable detecting method with high specificity, we sequenced the amplification products each time, because only the result that the size of the bands is similar to our expected gene segments could not guarantee that the obtained bands are just our expected gene segments. Therefore, it is essential to sequence the amplification products so as to further confirm the specificity of the method.

The high sensitivity and specificity observed with the use of the single step multiplex reverse transcription-polymerase chain reaction described here suggest that the method could be of potential value in the rapid detection of H5N1 influenza viruses in clinics. The specificity and sensitivity of the multiplex RT-PCR established here should be further validated and evaluated on a larger quantity of clinical specimens and compared with that of the conventional virus isolation method using embryonated chicken eggs. This work is in progress and the preliminary results show the potential application of this system for the rapid detection of the H5N1 influenza viruses.

Table 2 Interpretation of the amplification results when detecting clinical specimens with the multiplex RT-PCT method

Bands shown on agarose gel	Determination of virus in specimen
No	Not influenza A virus
327 bp	Influenza A virus
327 bp and 549 bp	Subtype H5 influenza virus, not N1
327 bp and 405 bp	Subtype N1 influenza virus, not H5
327 bp, 405 bp and 549 bp	Subtype H5N1 influenza virus

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Pathogenicity of a highly pathogenic avian influenza virus, A/chicken/Yamaguchi/7/04 (H5N1) in different species of birds and mammals

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Summary. Outbreaks of highly pathogenic avian influenza (HPAI) have been occurring in domestic poultry in Asia since 1996. In the beginning of 2004, HPAI outbreaks were caused by H5N1 virus in two farms and a group of pet chickens in different areas of Japan. In the present study, the pathogenicity of A/chicken/Yamaguchi/7/04 (H5N1), which had been isolated from a dead chicken during the first outbreak in Japan, was assessed in chickens, quails, budgerigars, ducklings, mice, and miniature pigs by experimental infection. The virus was highly pathogenic to all the birds tested. Mice were susceptible to infection with a low mortality rate and miniature pigs were resistant to infection with the virus.

Introduction

A wide variety of species of birds and mammals are susceptible to influenza A virus infection. Viruses of all 16 hemagglutinin (HA) (H1–H16) and 9 neuraminidase (N1–N9) subtypes have been isolated from avian species [1, 6]. Aquatic birds are the natural reservoirs of influenza A viruses [12]. Influenza viruses are perpetuated in nature by continuing to circulate in migratory ducks and frozen lake water [9]. Based on the severity of the disease they cause in chickens, avian influenza viruses are divided into two groups, highly pathogenic and low pathogenic [1]. Low pathogenic avian influenza (LPAI) viruses replicate in limited tissues where host proteases such as trypsin-like enzymes are found. Highly pathogenic avian influenza (HPAI) viruses possess inserted multiple basic amino acid residues at the site of cleavage of their HAs into HA1 and HA2 by ubiquitous proteases such

as furin and PC6 [8, 25]. This cleavage confers infectivity to a greater number of tissues, leading to a severe systemic disease, characterized by high mortality [14]. The HPAI viruses are restricted to subtypes H5 and H7, and viruses of these two subtypes had been believed to be low pathogenic in the reservoir host, ducks, until HPAI H5N1 viruses were isolated from bar-headed geese, brown-headed gulls, and black-headed gulls, 2005, in China [4, 16].

Outbreaks of HPAI in poultry such as chickens and quails around the world have caused high mortality and substantial economic losses, thereby impacting negatively on the poultry industry [1, 27]. Outbreaks have occurred often in the last decade in North America, Europe, and Asia. In Asia, highly pathogenic H5N1 influenza viruses have been recognized since 1996 [28]. In 1997, HPAI viruses were directly transmitted from birds to humans in Hong Kong, signaling the necessity to clarify the ecology of avian influenza virus [26]. HPAI outbreaks again occurred during 2001–2002 in Hong Kong [24]. In 2004, HPAI outbreaks also occurred in Cambodia, China, Indonesia, Malaysia, Japan, Laos, South Korea, Thailand, and Vietnam [15]. The HPAI virus, A/chicken/Yamaguchi/7/04 (H5N1), isolated in Japan, 2004, was lethal to chickens [18]. The pathogenicity of this HPAI virus in birds other than chickens and in mammals is not known. In order to determine the pathogenicity of the virus in chickens, quails, budgerigars, ducklings, mice, and miniature pigs, and to compare the pathogenicity of this HPAI virus in those animals in parallel with that of other H5N1 influenza viruses, experimental infection was carried out in the present study.

Materials and methods

Viruses

Influenza virus strain A/chicken/Yamaguchi/7/04 (H5N1) (Ck/Yamaguchi/04) was isolated from a dead chicken during the first outbreak of HPAI in Japan and was provided by the National Institute of Animal Health (Ibaraki, Japan) [18]. A/duck/Yokohama/aq-10/03 (H5N1) (Dk/Yokohama/03), isolated from duck meat imported from China, was provided by the Animal Quarantine service (Kanagawa, Japan) [13, 19]. R(A/duck/Mongolia/54/01-A/duck/Mongolia/47/01) (H5N1) (R(Dk/Mong-Dk/Mong)) was a reassortant virus generated from A/duck/Mongolia/54/01 (H5N2) and A/duck/Mongolia/47/01 (H7N1) which were isolated in our laboratory from fecal samples of wild ducks in Mongolia [13]. These three viruses were propagated in 10-day-old embryonated chicken eggs for 48 h at 35 °C. The infectious allantoic fluid was used as inoculum for experimental infections of animals and for the preparation of purified virus.

Animals

Chickens (*Gallus gallus*), quails (*Coturnix japonica*), budgerigars (*Melopsittacus undulatus*), ducklings (*Ahas platyhnchos*), mice (*Mus musculus*), and miniature pigs (*Sus scrofa domestic*) were used for the experimental infection study. Specific pathogen-free white leghorn chickens were hatched and raised for four weeks in our laboratory. One-month-old quails and three-month-old budgerigars were purchased from pet shops. Three-day-old ducklings were purchased from a duck farm in Hokkaido, Japan. Six-week-old female BALB/c mice and two-month-old specific pathogen-free male miniature pigs were purchased from Japan SLC, Inc. (Shizuoka, Japan) and Nippon Institute for Biological Science (Yamanashi, Japan).

Animal experiments

Viruses were inoculated intranasally, at a 50% egg infectious dose (EID_{50}) of $10^{8.0}$, into birds and mammals. For the birds and miniature pigs, 0.1 ml of each H5N1 virus containing $10^{8.0}EID_{50}$ was inoculated intranasally. For the mice, 0.03 ml of each H5N1 virus containing $10^{8.0}EID_{50}$ was inoculated intranasally. As a negative control, phosphate buffered saline (PBS) was given to the birds and mammals as much volume as the virus suspension. Birds and mice were sacrificed at 3 and 14 days post-infection (p.i.). When animals were dead or sacrificed, trachea and lung (respiratory organs), liver, spleen, kidneys, colon, brain, heart, pancreas, and blood of each animal were collected aseptically and were used for the titration of virus and histopathological examination. For miniature pigs, nasal swabs were collected in minimal essential medium daily from day 1 p.i. to day 7 p.i., and were used for the titration of virus. Animals were housed in self-contained isolator units (Tokiwa Kagaku, Tokyo, Japan) at a BSL 3 biosafety facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan.

Virus titration

The tissue homogenates from birds and mice were inoculated into 10-day-old embryonated chicken eggs and incubated for 48 h at 35 °C. The titers of virus were calculated by the method of Reed and Muench [22] and expressed as the EID_{50} per gram of tissue. Viral titers of the nasal swab samples of the miniature pigs were calculated as the 50% tissue culture infectious dose ($TCID_{50}$) per ml for swab in MDCK cells.

Antibody detection

Serum samples treated with beta-propiolactone (Wako Pure Chemicals Industries, Ltd., Japan) at 37 °C for 3 h were examined for the presence of antibodies against H5 influenza virus by ELISA. The purified R(Dk/Mong-Dk/Mong) (H5N1) virus was used as antigen for ELISA according to Kida et al. [10]. ELISA titers were expressed as reciprocals of serum dilutions.

Histopathological examination

The tissues of birds and mammals were fixed in 20% formalin in PBS (pH 7.2), sectioned, and stained with hematoxylin and eosin for microscopic examination. For the detection of influenza virus antigens in the tissues, all the sections were stained using the streptavidin-biotin immunoperoxidase complex method (Histofine SAB-PO ®kit, Nichirei Corp., Tokyo) with rabbit anti-A/duck/Pennsylvania/10218/84 (H5N2) hyperimmune serum at a 1:1,000 dilution as the primary antibody.

Results

Chickens

All of the chickens inoculated with Ck/Yamaguchi/04 and Dk/Yokohama/03 died on day 2 and between day 2 p.i. and day 4 p.i. (2–4d), respectively, and virus was recovered from each of the tissues tested (respiratory organs, liver, kidneys, colon, and brain) (Table 1). Higher titers of viruses were detected in four of the five tissues of chickens inoculated with Ck/Yamaguchi/04 than in those with Dk/Yokohama/03. None of the chickens inoculated with R(Dk/Mong-Dk/Mong) had died by day 14 p.i., and virus was not recovered from any of the tissues at