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

Table 1. Virus recovery and antibody response from chickens inoculated with avian influenza virus

Inoculated virus	No. of animals	Days p.i.		Virus recovery[a]						Antibody response[b]
				Respiratory organs	Liver	Kidneys	Colon	Brain	Blood	
Ck/Yamaguchi/04 (H5N1)	6	2d	dead	6 (8.4)	6 (7.4)	6 (7.6)	6 (7.3)	6 (7.1)	ND[c]	ND
Dk/Yokohama/03	5	2–4d	dead	5 (7.1)	5 (5.8)	5 (6.4)	5 (5.8)	5 (7.7)	ND	ND
(H5N1)	1	3d	sacrificed	1 (6.8)	1 (6.5)	1 (7.2)	1 (7.2)	1 (8.0)	1 (7.3)	–
R(Dk/Mong-Dk/Mong)	3	3d	sacrificed	0	0	0	0	0	0	–
(H5N1)	3	14d	sacrificed	0	0	0	0	0	0	–

[a]Digit: number of animals in which each virus was isolated. Parenthesis: average of virus titers ($logEID_{50}/g$). 0 indicates no virus was isolated from animals

[b]Antibody detection was examined by ELISA. –: ELISA titer was below 40

[c]Not determined

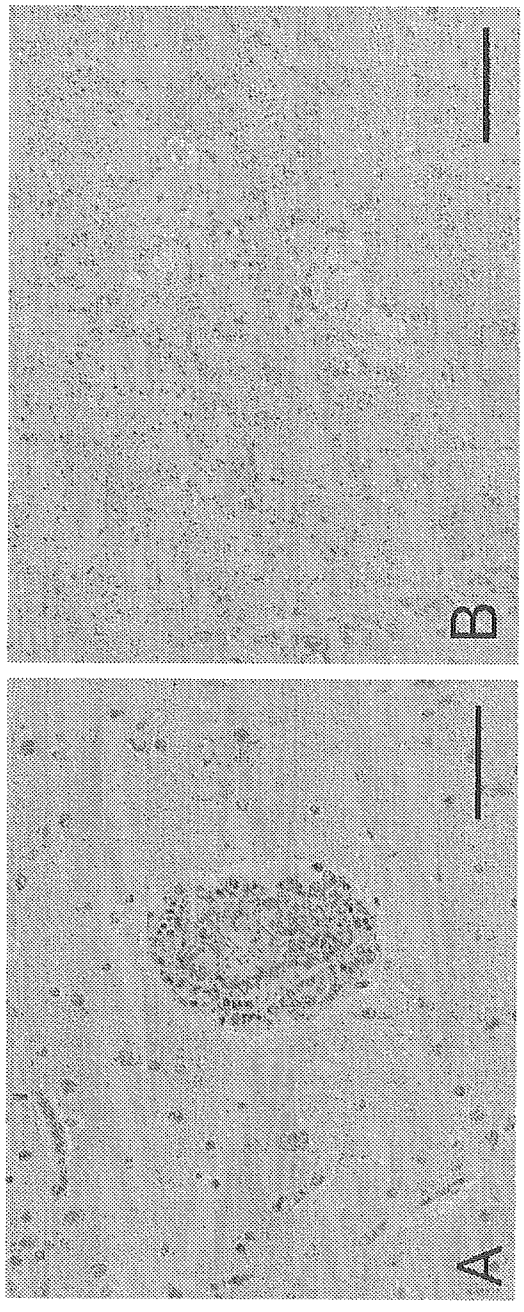

Fig. 1. Histopathological examination in chickens (**A**) and quails (**B**) inoculated with Dk/Yokohama/03. Photomicrographs of hematoxylin and eosin-stained tissue sections. **A**: Perivascular cuffing, swelling of endothelial cells, infiltration and proliferation of microglia in the brain (cerebrum) of the chickens inoculated with Dk/Yokohama/03 on day 4 p.i. **B**: Laminar encephalomalaccia (necrosis) in the brain (cerebellum) of the quails inoculated with Dk/Yokohama/03 on day 4 p.i. Bar, 50 μm

days 3 and 14 p.i. Sero-conversion to H5 influenza virus was not detected in any birds, indicating that the chickens were not infected with R(Dk/Mong-Dk/Mong). In the histopathological examination, influenza virus antigens were detected in the brain, liver, spleen, kidneys, heart, lungs, pancreas, and colon of chickens inoculated either with Ck/Yamaguchi/04 or with Dk/Yokohama/03. Since severe virus encephalitis with perivascular infiltration in the brain affected one chicken inoculated with Dk/Yokohama/03 (Fig. 1A) and higher titers were detected in the brains of chickens inoculated with Dk/Yokohama/03 than with Ck/Yamaguchi/04, it was found that infection with Dk/Yokohama/03 caused severer lesions than infection with Ck/Yamaguchi/04 in the brain.

Quails

All of the quails inoculated with Ck/Yamaguchi/04 and Dk/Yokohama/03 died between day 2 p.i. and day 3 p.i. (2–3d) and between day 3 p.i. and day 4 p.i. (3–4d), respectively, and virus was recovered from each of the tissues tested (Table 2). Disease signs characterized by severe nervous disorders were observed in 2 out of 6 quails infected with Dk/Yokohama/03. Higher titers of viruses were detected in all the tissues of quails inoculated with Ck/Yamaguchi/04 compared to those inoculated with Dk/Yokohama/03. None of the quails 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 day 3 and 14 p.i. Sero-conversion to H5 influenza virus was detected in the quails inoculated with R(Dk/Mong-Dk/Mong) at day 14 p.i., indicating that these quails were infected with R(Dk/Mong-Dk/Mong). In the histopathological examination, in the brain of the quail inoculated with Dk/Yokohama/03, severe virus encephalitis with laminar encephalomalacia (necrosis) was observed (Fig. 1B). Antigens to influenza viruses were detected in the brains and hearts of birds infected either with Ck/Yamaguchi/04 or with Dk/Yokohama/03.

Budgerigars

All of the budgerigars inoculated either with Ck/Yamaguchi/04 or with Dk/Yokohama/03 died by day 5 p.i., and the virus was recovered from each of the tissues tested (Table 3). Disease signs such as severe nervous disorders were observed in 3 out of 7 budgerigars infected with Dk/Yokohama/03. None of the budgerigars 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 days 3 and 14 p.i. Sero-conversion to H5 influenza virus was not detected in any budgerigars inoculated with R(Dk/Mong-Dk/Mong) at day 14 p.i., indicating that the budgerigars were not infected with R(Dk/Mong-Dk/Mong).

Ducklings

Two of the ducklings inoculated with Ck/Yamaguchi/04 died on day 6 p.i. and day 7 p.i. (6–7d), and virus was recovered from each of the tissues including

Table 2. Virus recovery and antibody response from quails inoculated with avian influenza virus

Inoculated virus	No. of animals	Days p.i.		Virus recovery[a]						Antibody response[b]
				Respiratory organs	Liver	Kidneys	Colon	Brain	Blood	
Ck/Yamaguchi/04 (H5N1)	7	2–3d	dead	7 (7.4)	7 (7.1)	7 (8.8)	7 (7.2)	7 (8.4)	ND[c]	ND
Dk/Yokohama/03	4	3–4d	dead	4 (6.8)	4 (4.4)	2 (5.7)	3 (6.4)	4 (8.3)	ND	ND
(H5N1)	2	3d	sacrificed	1 (7.2)	2 (6.0)	2 (8.0)	0	1 (5.8)	1 (3.8)	−
R(Dk/Mong-Dk/Mong)	3	3d	sacrificed	0	0	0	0	0	0	−
(H5N1)	2	14d	sacrificed	0	0	0	0	0	0	+

[a]Digit: number of animals in which each virus was isolated. Parenthesis: average of virus titers ($logEID_{50}/g$). 0 indicates no virus was isolated from animals

[b]Antibody detection was examined by ELISA. −: ELISA titer was below 40. +: ELISA titer was over 40

[c]Not determined

Table 3. Virus recovery and antibody response from budgerigars inoculated with avian influenza virus

Inoculated virus	No. of animals	Days p.i.		Virus recovery[a]					Antibody response[b]
				Respiratory organs	Liver	Kidneys	Colon	Brain	
Ck/Yamaguchi/04 (H5N1)	7	3–4d	dead	7 (6.6)	7 (4.3)	7 (7.1)	3 (3.8)	7 (7.4)	ND[c]
Dk/Yokohama/03 (H5N1)	4	3d	sacrificed	4 (5.0)	3 (3.5)	4 (5.4)	4 (2.9)	4 (6.2)	ND
	3	5d	dead	3 (5.3)	3 (2.6)	3 (4.9)	2 (2.9)	3 (8.0)	ND
R(Dk/Mong-Dk/Mong) (H5N1)	3	3d	sacrificed	0	0	0	0	0	ND
	3	14d	sacrificed	0	0	0	0	0	–

[a]Digit: number of animals in which each virus was isolated. Parenthesis: average of virus titers ($logEID_{50}/g$). 0 indicates no virus was isolated from animals

[b]Antibody detection was examined by ELISA. –: ELISA titer was below 40

[c]Not determined

Table 4. Virus recovery and antibody response from ducklings inoculated with avian influenza virus

Inoculated virus	No. of animals	Days p.i.		Virus recovery[a]					Antibody response[b]
				Respiratory organs	Liver	Kidneys	Colon	Brain	
Ck/Yamaguchi/04 (H5N1)	3	3d	sacrificed	3 (5.8)	3 (5.3)	3 (5.2)	3 (3.0)	0	–
	2	6–7d	dead	2 (3.9)	1 (5.5)	1 (5.7)	1 (2.5)	1 (5.3)	ND[c]
	1	14d	sacrificed	0	0	0	0	0	+
Dk/Yokohama/03 (H5N1)	6	3–4d	dead	6 (7.1)	6 (7.1)	6 (5.7)	6 (4.7)	6 (8.1)	ND
R(Dk/Mong-Dk/Mong) (H5N1)	3	3d	sacrificed	0	0	0	0	0	–
	3	14d	sacrificed	0	0	0	0	0	+

[a]Digit: number of animals in which each virus was isolated. Parenthesis: average of virus titers ($logEID_{50}/g$). 0 indicates no virus was isolated from animals

[b]Antibody detection was examined by ELISA. –: ELISA titer was below 40. +: ELISA titer was over 40

[c]Not determined

the brain (Table 4). One of the ducklings survived for 14 days, and from this duckling, specific serum antibodies against H5 influenza virus were detected. All of the ducklings inoculated with Dk/Yokohama/03 died between day 3 p.i. and day 4 p.i. (3–4d), and the virus was recovered from each tissue. None of the ducklings 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 days 3 and 14 p.i. Seroconversion to H5 influenza virus was detected in the ducklings inoculated with R(Dk/Mong-Dk/Mong) at day 14 p.i.

Table 5. Virus recovery and antibody response from mice inoculated with avian influenza virus

Inoculated virus	No. of animals	Days p.i.		Virus recovery[a]					Antibody response[b]
				Respiratory organs	Liver	Spleen	Kidneys	Brain	
Ck/Yamaguchi/04	2	3–4d	dead	2 (6.3)	0	1 (3.3)	1 (2.3)	0	ND[c]
(H5N1)	2	3d	sacrificed	2 (6.7)	0	0	0	0	–
	4	14d	sacrificed	0	0	0	0	0	+
Dk/Yokohama/03	4	3d	sacrificed	3 (4.5)	0	0	0	0	–
(H5N1)	4	14d	sacrificed	0	0	0	0	0	+
R(Dk/Mong-	3	3d	sacrificed	3 (4.5)	0	0	0	0	ND
Dk/Mong) (H5N1)	3	14d	sacrificed	0	0	0	0	0	ND

[a]Digit: number of animals in which each virus was isolated. Parenthesis: average of virus titers ($logEID_{50}/g$). 0 indicates no virus was isolated from animals

[b]Antibody detection was examined by ELISA. –: ELISA titer was below 40. +: ELISA titer was over 40

[c]Not determined

Mice

Two of the mice inoculated with Ck/Yamaguchi/04 died on day 3 p.i. and day 4 p.i. (3–4d) (Table 5). Virus was recovered only from the respiratory organs in all except one mouse, which was dead at day 4 p.i. In this mouse, the virus was recovered not only from the respiratory organs but also from the spleen and kidneys. The other four mice survived for 14 days, and specific antibodies against H5 influenza virus were detected. All of the mice inoculated with Dk/Yokohama/03 and R(Dk/Mong-Dk/Mong) survived for 14 days. The virus was recovered only from the respiratory organs of the mice at day 3 p.i. It was found that the pathogenicity of these two viruses in mice was relatively low.

Table 6. Virus recovery and antibody response from miniature pigs inoculated with avian influenza virus

Viruses	Virus titers ($logTCID_{50}/ml$)							Antibody response[a] on 14 days p.i.
	1 day	2 day	3 day	4 day	5 day	6 day	7 day	
Ck/Yamaguchi/04 (H5N1)	–[b]	–	–	–	–	–	–	–
Dk/Yokohama/03 (H5N1)	–	–	–	–	–	–	–	–
R(Dk/Mong-Dk/Mong) (H5N1)	–	2.7	2.5	1.7	–	–	–	+

[a]Antibody detection was examined by ELISA. –: ELISA titer was below 40. +: ELISA titer was over 40

[b]–: < 1.5 $logTCID_{50}/ml$

Miniature pigs

All of the miniature pigs inoculated with the three H5N1 viruses survived for 14 days. No virus was detected in the nasal swabs of the miniature pigs inoculated either with Ck/Yamaguchi/04 or Dk/Yokohama/03 from day 1 p.i. to day 7 p.i. (Table 6). In these two miniature pigs, sero-conversion to H5 influenza virus was not detected at day 14 p.i. In another experiment with miniature pigs inoculated with Ck/Yamaguchi/04, the virus was not recovered from any of the tissues at days 3 and 14 p.i. (data not shown). These results indicated that miniature pigs were not infected with Ck/Yamaguchi/04 and Dk/Yokohama/03. Although there were no disease signs in the miniature pig inoculated with R(Dk/Mong-Dk/Mong), viruses were recovered from the nasal swabs between day 2 p.i. and day 4 p.i. (2–4d). Sero-conversion to H5 influenza virus was detected in the miniature pig inoculated with R(Dk/Mong-Dk/Mong).

Discussion

The present study was conducted to determine the pathogenicity of Ck/Yamaguchi/04 in chickens, quails, budgerigars, ducklings, mice, and miniature pigs. Two H5N1 avian influenza viruses, Dk/Yokokama/03 and R(Dk/Mong-Dk/Mong), were compared in terms of pathogenicity with Ck/Yamaguchi/04. The intravenous pathogenicity index (IVPI) in 6-week-old chickens for Ck/Yamaguchi/04, Dk/Yokohama/03, and R(Dk/Mong-Dk/Mong) was 3.0, 2.7, and 0.0, respectively (data not shown). Based on the present results, Ck/Yamaguchi/04 and Dk/Yokohama/03 were classified as HPAI viruses and R(Dk/Mong-Dk/Mong) as a non-pathogenic virus by the OIE criteria [2]. Ck/Yamaguchi/04 and Dk/Yokohama/03 caused systemic infections in birds, but showed little or no pathogenicity in mammals. The slightly longer mean death time in chickens inoculated with Dk/Yokohama/03 allowed for the development of cyanosis of the wattle, typical signs of HPAI. The tendency was shown that virus of higher titer was recovered from chickens inoculated with Ck/Yamaguchi/04 than those inoculated with Dk/Yokohama/03.

The pathogenicity of Ck/Yamaguchi/04 and Dk/Yokohama/03 in quails and budgerigars was as high as that of the HPAI virus, Ck/Hong Kong/220/97 (H5N1), which caused an acute and lethal infection [21]. Notably, the pathogenicity of Ck/Yamaguchi/04 in the quails seemed to be higher than that of Dk/Yokohama/03, as evidenced by the mean death times (Ck/Yamaguchi/04 vs Dk/Yokohama/03, $P = 0.05$) and the tissues from which the viruses were recovered. This difference may be due to the adaptation of isolated HPAI viruses from different hosts (chicken and duck) to quails. The greater susceptibility of quails to the virus originating from duck than from chickens is consistent with previous reports [17]. In our another experiment, Ck/Yamaguchi/04 and Dk/Yokohama/03 caused systemic infections in wild starlings (*Sturnus cineraceus*) (data not shown), indicating that feral birds could play a role as intermediates in virus transmission among poultry flocks, thereby contributing to the spread of avian influenza virus as in the outbreaks in Australia [20]. During the outbreaks of H5N1 HPAI in Japan, 2004, viruses were

isolated not only from chickens but dead crows [18]. The possibility remains that avian influenza virus is spread by the contact of wild birds with chickens.

The pathogenicity of Ck/Yamaguchi/04 and Dk/Yokohama/03 for five-week-old ducks was not high compared to that for chickens and Dk/Yokohama/03 replicated more rapidly and efficiently in the multiple organs than Ck/Yamaguchi/04 in ducks [13]. In the present study, virus was recovered from multiple tissues of three-day-old ducklings inoculated either with Ck/Yamaguchi/04 or with Dk/Yokohama/03, and some of these ducklings were dead, indicating that the pathogenicity of these two viruses in three-day-old ducklings was high.

In the present study, virus was recovered from multiple tissues of only one mouse, which died at day 4 p.i. Two mice died after the inoculation of Ck/Yamaguchi/04 at an EID_{50} of $10^{8.0}$ and the mortality rate of mice was only 33% ($n = 6$). In the latest publication, the 50% lethal dose of the same strain in mice was 5×10^5 EID_{50} under the same conditions (6-week-old female BALB/c mice via the intranasal route), and virus was also recovered from the brain [18]. The difference in pathogenicity may be due to the passage history of Ck/Yamaguchi/04 since the virus obtained from the National Institute of Animal Health (Japan) was propagated twice in embryonated chicken eggs before the present animal experiments. The pathogenicity of the H5N1 viruses isolated from humans in Hong Kong, 1997, was extremely high in mice [5, 7]. In the present study, more than half of the mice inoculated with Ck/Yamaguchi/04 survived the infection, indicating that the 50% mouse lethal dose was over $10^{8.0}$ EID_{50}. In conclusion, the pathogenicity of Ck/Yamaguchi/04 and Dk/Yokohama/03 in mice was much lower than that of the H5N1 viruses isolated from humans in Hong Kong, 1997.

Miniature pigs showed susceptibility to influenza virus, similarly to domestic pigs [3]. Miniature pigs were not susceptible either to Ck/Yamaguchi/04 or to Dk/Yokohama/03, but limited viral replication was observed in upper respiratory tissues in the miniature pigs inoculated with R(Dk/Mong-Dk/Mong). Therefore, the pigs may not play a major role in the maintenance and spread of Ck/Yamaguchi/04 and Dk/Yokohama/03. In contrast, H5N1 viruses isolated in 1997 from a boy (Hong Kong/156/97) and chicken (Ck/Hong Kong/258/97) replicated in pigs, although transmission through contact was not detected [24]. These results suggest that the susceptibility of pigs to avian influenza viruses has no relation to the pathogenicity of the strains in chickens or their subtypes, indicating that possible factors involved in host range restriction may be located in some gene segment(s) other than the HA gene [11, 23].

In conclusion, Ck/Yamaguchi/04 is highly pathogenic to birds and cause systemic infection, including brain. The results indicate that the susceptibility of pigs to this HPAI virus is very low, and that the possibility of genetic reassortments with this HPAI virus in pigs is not a concern.

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(H5N1) and A/duck/Yokohama/aq-10/03 (H5N1) influenza viruses, respectively. We also thank Dr. A. S. Mweene for discussing the contents of and English in this paper.

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Rapid Diagnostic Method for Detection of Mumps Virus Genome by Loop-Mediated Isothermal Amplification

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Most mumps patients are clinically diagnosed without any virological examinations, but some diagnosed cases of mumps may be caused by other pathogens or secondary vaccine failure (SVF). To clarify these issues, a sensitive, specific, and rapid diagnostic method is required. We obtained 60 salivary swabs from 34 patients with natural infection during the course of the illness, 10 samples from patients with vaccine-associated parotitis, and 5 samples from patients with SVF. Total RNA was extracted and subjected to reverse transcription-PCR (RT-PCR) and loop-mediated isothermal amplification (LAMP) for genome amplification. We detected mumps virus RNA corresponding to 0.1 PFU by LAMP within 60 min after RNA extraction, with the same sensitivity as RT-nested PCR. Mumps virus was isolated in 30 of 33 samples within day 2, and mumps virus genome was amplified by LAMP in 32 of them. The quantity of virus titer was calculated by monitoring the time to reach the threshold of turbidity. The viral load decreased after day 3 and was lower in patients serologically diagnosed as having SVF with milder illness. Accuracy of LAMP for the detection of mumps virus genome was confirmed; furthermore, it is of benefit for calculating the viral load, which reflects disease pathogenesis.

Mumps virus is a single-stranded negative-sense RNA virus which belongs to a member of the genus *Rubulavirus* of the family *Paramyxoviridae*. It encodes seven main proteins: the nucleocapsid, phospho, membrane, fusion (F), small hydrophobic (SH), hemagglutinin-neuraminidase (HN), and large proteins (1). The F and HN proteins are envelope glycoproteins and the HN protein plays a role in the initial step of viral attachment to the cellular sialic acid receptor and subsequent membrane fusion, thus allowing viral penetration (26). The SH protein is present in infected cells but its function is not clear, although it is not essential for virus infection, transcription, or replication (25).

Mumps virus is still circulating throughout the world but in the United States nationwide acceptance of the measles, mumps, and rubella (MMR) combined vaccine has reduced the number of mumps patients (2, 18). With high vaccine coverage, several authors reported mumps patients suffering from secondary vaccine failure (SVF) (20). Among mumps virus infections, a few mumps patients are hospitalized because of aseptic meningitis. Encephalitis occurs in 1 of 5,000 to 6,000 cases, and deafness is an irreversible complication (19). In Japan, MMR vaccine was introduced in 1989, but it was discontinued in 1993 because of the unexpectedly high incidence of aseptic meningitis after MMR vaccination containing the Urabe strain (9, 23, 27). Since 1993, a monovalent mumps vaccine has been used, but vaccine coverage is now estimated to be <20%. Annual mumps outbreaks occur with different magnitudes every year (5, 8, 16, 24).

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The diagnosis of virus infection is typically performed by virus isolation and serological examinations, but these methods are time consuming and not appropriate for clinical settings. Most mumps cases are clinically diagnosed, and acute parotitis is also caused by several virus infections other than mumps virus, bacterial infection, and the obstruction of salivary ducts (1, 19). Mumps virus can be isolated from approximately 60% of salivary swabs obtained within 5 days of illness (1), but Reina et al. (21) reported that a more sensitive shell vial method increased the isolation rate from clinical samples. More-precise laboratory-based surveillance would be required for the control of mumps. In some virus laboratories, molecular-based diagnostic methods are employed using reverse transcription-PCR (RT-PCR) and hybridization. RT-PCR is more sensitive than conventional virus isolation and takes several hours to obtain the results, which requires special equipment and skillful experiences (7). Recently, rapid diagnostic kits have been introduced for many kinds of viruses. Most kits are based on an enzyme-linked or photometric immunoassay for the detection of a specific viral protein(s), but no rapid diagnostic kit is available for the diagnosis of mumps infection.

A sensitive and specific method for DNA amplification method was developed by Notomi et al. (15) and termed loop-mediated isothermal amplification (LAMP). This method employs a DNA polymerase with strand displacement activity and a set of four specifically designed primers that recognize six different sequences on the targeted DNA. The key reaction is the formation of 5′ and 3′ end loop dumbbell DNA stem-loop structures. The LAMP reaction is characterized by strand displacement DNA synthesis, yielding the original loop DNA structure and new stem-loop DNA products that are twice as long. Through repetitive reactions, the multibranched stem-loop products are amplified. Distinctive features of LAMP

FIG. 1. Primer design for RT-LAMP for the detection of mumps virus. Sequence data of the partial HN gene from genome position 7931 to 8180. Six primers were synthesized (F3, B3, FIP, BIP, F loop, and B loop). The arrow shows the direction of primer extension. The underlined sequence at genome position 8121 to 8126 is the Alw44I site.

include its rapidity, high sensitivity, high specificity, and simplicity, which are required for rapid diagnosis. We developed a new method for the detection of mumps virus genome RNA by LAMP and compared its sensitivity to that of virus isolation and nested RT-PCR.

MATERIALS AND METHODS

Clinical samples. We obtained 75 salivary swab samples from patients clinically diagnosed with mumps infection from January 2003 to July 2003 to compare the sensitivity of LAMP. To investigate the viral load in different pathophysiological conditions of mumps infection, we used 60 salivary swab samples stocked at −70°C from 34 natural mumps infections, 10 samples from patients with cases of acute parotitis after mumps vaccination identified as vaccine adverse events, and 5 samples from patients with SVF confirmed by an avidity test with an enzyme immunoassay kit (Denka Seiken, Tokyo, Japan). Salivary swabs were soaked in minimum essential medium supplemented with appropriate antibiotics, 5% fetal calf serum, and 1% gelatin. They were then transferred to the Kitasato Institute.

Virus isolation. Salivary swabs were centrifuged at 6,000 rpm for 10 min, filtered, and subjected to virus isolation, RT-PCR, and LAMP. The supernatants were inoculated on a monolayer of Vero cell cultures, and we considered virus isolation negative for the samples which did not show a cytopathic effect after two passages (21).

For the determination of virus titer, virus isolates were serially diluted at the ratio of 1:10, and 100 μl of each dilution was placed on a monolayer of Vero cells in 24-well plates in duplicate. The wells were overlaid with 0.5% agar with minimum essential medium, and the plates were kept at 37°C for 7 days in 5% CO_2. They were then stained, and the number of plaques was counted. Himeji 89/JPN.00, Takamatsu 1/JPN.00, Sapporo K-4/JPN.00, and Tokyo S-III-10/JPN.01 strains were used as representative strains of genotypes B, G, J, and L, respectively (5).

RNA extraction and RT-PCR. Total RNA was extracted from 200 μl of a salivary swab sample by a magnetic bead-RNA purification kit (Toyobo Co., Ltd., Osaka, Japan), and the RNA pellet was suspended in 25 μl of distilled water. It was subjected to RT-PCR and LAMP. Virus genomic RNA material (5 μl) was converted to cDNA by AMV reverse transcriptase (Life Sciences, Inc.) at 50°C for 1 h with MpF921+ (5′-TCTATAATTCAATTGCCAGA-3′). The first PCR was performed with the primers MpF5+ (5′-ATAGCAGGGAGTTATATGAG-3′) and MpL1− (5′-AACCCGTTCTAGACCATCAC-3′). For nested PCR of the HN gene, 548 nucleotides were amplified from genome positions 7,791 to 8,338 with the primers MpHN3+ (5′-GATCCTAGTTACAAATTGCG-3′) and MpHN6− (5′-ACCTGCAGTGATAGTCAATCTGGTTAG-3′) (5, 7, 24). PCR was performed with 1.25 U of *Taq* DNA polymerase (TaKaRa BioMedicals, Tokyo, Japan) with the TaKaRa thermal cycler (TaKaRa BioMedicals) and 30 rounds of thermal cycling conditions: denaturation at 93°C for 1 min, reannealing at 58°C for 1 min, and extension at 72°C for 2.5 min. PCR products were confirmed by electrophoresis through a 1.5% agarose gel stained with ethidium bromide (5, 7, 24).

Mumps virus reverse trascription-LAMP (RT-LAMP). LAMP characterized by autocycling strand displacement DNA synthesis was performed with Bst DNA polymerase (New England Biolabs) with high activity on strand displacement and by a specially designed set of primers, as specified in the software program for LAMP primer design (Eiken Chemical Co. Ltd., Tokyo, Japan). The LAMP primers were designed in the HN region similar to the RT-PCR region; the results are shown in Fig. 1. We synthesized six primers from genome position 7931 to 8180 (Fig. 1); two outer primers (F3 and B3), two inner primers, a forward inner primer (FIP), backward inner primer (BIP), and two loop primers (loops F and B). FIP contains a complementary alignment to F1 linked with the F2 sequence, and BIP contains a B1 sequence linked with a complementary sequence to B2. These four primers amplified the target DNA. We synthesized two additional loop primers: primer F, located between F1 and F2, and loop primer B, located between B1 and B2. The addition of two loop primers enhanced the specificity and reactivity (14). For the LAMP reaction, a LAMP mixture was made in 25 μl of reaction mixture containing 40 pmol (each) of FIP and BIP, 5 pmol (each) of F3 and B3, 20 pmol (each) of loop F and loop B, 1.4 mM each deoxynucleoside triphosphate, 0.8 M betaine, 20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH4)_2SO_4$, 8 mM $MgSO_4$, 0.1% Tween 20, 0.5 U of AMV reverse transcriptase, 8 U of Bst DNA polymerase, and 5 μl of sample RNA. The reaction mixture was subjected to a real-time turbidimeter LA200 (Teramecs, Tokyo, Japan) (12).

A diagram of LAMP is shown Fig. 2. Genome RNA is first converted to cDNA with the FIP primer (Fig. 2, panel 1), and the F3 primer extends the cDNA synthesis with displacement of RNA and the cDNA double strand (panel 2). The RT process produces two kinds of structures, an RNA and cDNA complex from F3 to the B3 portion and single-strand cDNA primed by the FIP primer (panel 3). This cDNA forms a 5′ end loop structure. The BIP primer anneals to the 3′ end of cDNA and extends DNA synthesis (panels 3 and 4). The B3 primer attaches itself to the outer portion of B3 and detaches the double-strand DNA (panel 5). Thereafter, double-stranded DNA and the dumbbell loop structure of single-stranded DNA are produced (panel 6). This dumbbell loop structure is the basic structure for further extension of the LAMP reaction, and the FIP primer binds to the 3′ end of the single-strand loop region (panel 7). Similar DNA synthesis with displacement activity continues with cycling reactions (panels 8 to 12), and multibranched loop structures are synthesized, as reported by Notomi et al. (15).

As the LAMP reaction progresses, the reaction by-products (pyrophosphate ions) bind to magnesium ions and form a white precipitate of magnesium pyrophosphate. Light (650 nm) emitted by light-emitting diodes passes through PCR tubes containing the LAMP solution and illuminates the photodiode on the opposite side. The turbidity is calculated based upon the ratio between the intensity of light received by the photodiode and the emitted light intensity. Thus, measurement of the turbidity is closely related to the amplification of DNA (12).

Experimental guidelines. The study design was approved by ethical committee of the Kitasato Institute for Life Sciences, and informed consent was obtained

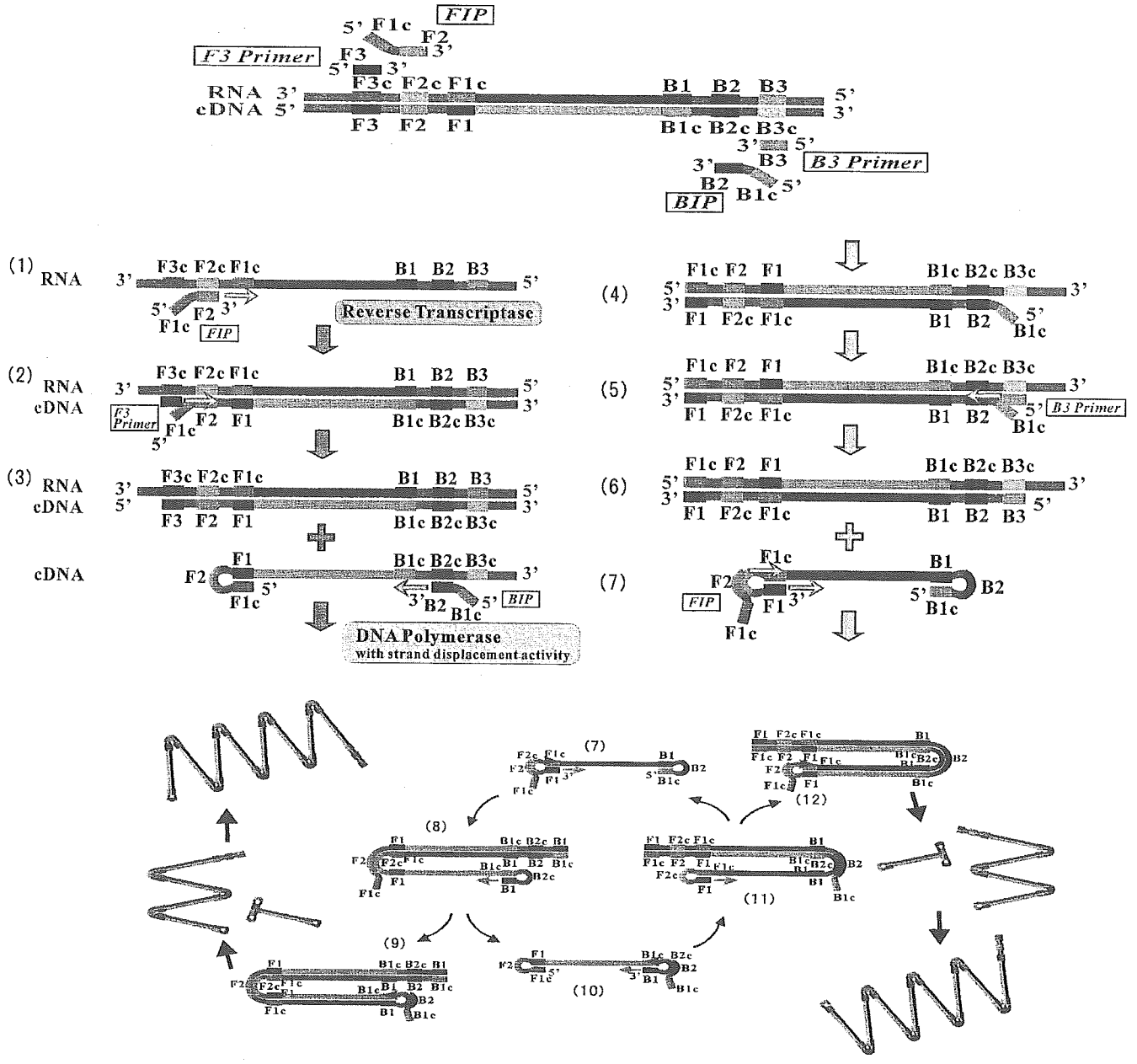

FIG. 2. Diagram of LAMP reaction. (Top) Schema of the primer location. The F loop primer is located from F1c to F2c and the B loop primer is located from B1c to B2c. The RT process is shown from panels 1 to 3 and follows the DNA polymerase reaction (panels 4 to 7). Panel 7 shows the characteristic dumbbell structure of the LAMP reaction. Panels 7, 8, 10, and 11 demonstrate a cycling reaction; this reaction occurs repeatedly and automatically. Multibranched loop structures are started from structures shown in panels 9 and 12.

from the parents of mumps patients consulting Okafuji Pediatric Clinic and Department of Pediatrics, Mie National Hospital.

RESULTS

Sensitivity of LAMP. The Takamatsu 1/JPN.2000 strain (genotype G) was used for analysis of the sensitivity of LAMP. Viral RNA was extracted from culture fluid containing 6×10^4 PFU/200 μl and serially diluted at a ratio of 1:10. In preliminary examinations, the LAMP reaction was performed at different temperatures of 60, 63, and 65°C, and the reaction at 63°C was the most productive (data not shown). Thereafter, LAMP was carried out at 63°C for 60 min. RT-PCR and LAMP were performed for serial 10-fold dilutions, and the results of LAMP are shown in Fig. 3. Mumps virus genome was detected at a 10^{-5} dilution by both RT-PCR and LAMP, which contained 0.12 PFU/5 μl of RNA out of 25 μl of RNA extracted from 200 μl of samples. The threshold of LAMP positive for the spectrophotometric measurement was defined as 0.1 (12), and we analyzed the correlation between the time (in seconds) to reach threshold and infectivity (number of PFU). A linear correlation was obtained: $y = -0.0056x + 10.03$, where y is the number of PFU and x is the number of seconds.

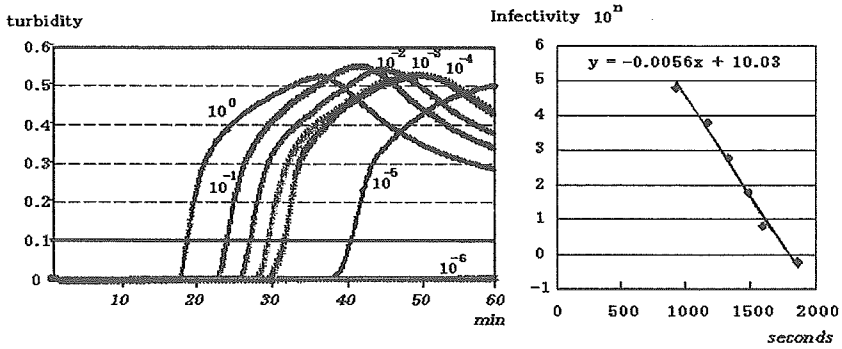

FIG. 3. Sensitivity of LAMP for the detection of mumps virus genome and quantitative LAMP reaction. Approximately 6×10^4 PFU/200 μl of virus culture medium (genotype G) was used. Serial 10-fold dilutions were subjected to LAMP, and the results of real-time LAMP are shown (left). LAMP reaction times were monitored to reach to the threshold of 0.1, and the correlation between the logarithmic value of the virus titer and the LAMP reaction time is shown (right).

Using the equation, we calculated the virus genome quantity related to the infectivity of the samples by real-time LAMP. We examined measles virus, respiratory syncytial virus serotypes A and B, and influenza virus types A and B, but mumps LAMP was negative (data not shown).

We compared the sensitivity of LAMP for different mumps virus genotypes. Recently, four different genotypes (B, G, K, and L) were isolated in Japan (5). Our genotype K was registered as genotype J according to the worldwide nomenclature for mumps virus genotyping (L. Jin, Centre for Infections Health Protection Agency, London, United Kingdom, personal communication). The infectivity was adjusted to 2×10^2 PFU, and genomic RNA was amplified with similar sensitivity (Fig. 4). LAMP products exhibited a typical ladder pattern, and after digestion with Alw44I they became a single band (Fig. 3). The Alw44I site was located at genome positions 8121 to 8126. When the mumps virus genome is correctly amplified, multibranched structures should become a single DNA band after digestion with Alw44I.

Virus isolation and LAMP during the course of mumps infection. In the preliminary examination, we obtained 75 salivary samples from the patients clinically diagnosed with mumps infection who had no past history of mumps vaccination and natural infection. The samples were obtained within 2 days after the onset of parotid swelling, and we compared the sensitivity of three virological examinations. Mumps virus was isolated in 30 samples, and mumps genome was detected in virus isolation positives. Mumps virus was also detected in 18 samples by LAMP and in 17 samples by RT-nested PCR in the remaining 45 samples that were negative by virus isolation. In samples from 27 patients with virus isolation-negative and LAMP-negative results, 18 samples showed no serological response in enzyme immunoassay between acute-phase and convalescent-phase sera. We suppose that the sensitivity of LAMP is similar to that of RT-nested PCR and higher than that of virus isolation.

We obtained a series of salivary swabs from 34 patients with natural mumps infection to investigate the change in the quantity of mumps virus genome during the course of illness. The results of virus isolation and LAMP are shown in Table 1. We designated the day when the parotid swelling was noticed as day 0. Within day 2 of the illness, mumps virus was isolated in 30 of 33 samples, and its genome was amplified by LAMP in 32 samples. From day 3 of the illness onward, mumps virus was isolated in nine samples, and six samples were positive for LAMP. Sixteen samples were negative for virus isolation, but mumps genome was amplified by LAMP in 7 of 16 virus isolation-negative samples.

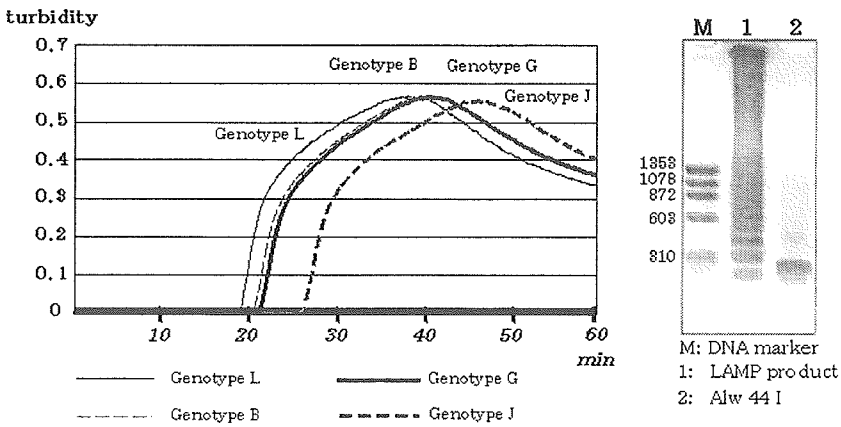

FIG. 4. Detection of mumps virus genome for different genotypes and electrophoresis of LAMP products. (Left) The results of real-time LAMP of four genotypes. (Right) The results of electrophoresis; the Alw44I site in the LAMP target region is shown.

TABLE 1. Positive rate of virus isolation and LAMP during the course of illness

Day of illness (no. of patients)	No. of patients			
	Virus isolation		LAMP	
	+	−	+	−
Day 0–2 (33)	30	3	32	1
Day 3–7 (25)	9	16	13	12

Virus load for different pathophysiology of mumps infection. We obtained the samples from different categories of mumps infection; 60 samples from 34 patients with natural primary mumps infection, 10 samples from patients with vaccine-associated parotitis, and 5 samples from patients with SVF. RNA was extracted from 200 μl of each sample, and the reaction time was monitored when the spectrophotometric values reached to a threshold of 0.1 of the turbidity. The virus titers (in PFU) are shown in Fig. 5 for different days of illness among different categories of illness. The virus load seemed to decrease after day 4 of the illness during the course of natural infection; in the case of reinfection (SVF), a lower virus load was observed. But scattering virus load was demonstrated in the cases of vaccine-associated parotitis. The virus quantities calculated for each day of illness are shown in Table 2. In natural infection, the mean virus load of 18 LAMP positives was $10^{0.89 \pm 0.81}$ PFU on day 0, $10^{1.54 \pm 1.01}$ PFU on days 1 or 2, and decreased to $10^{0.49 \pm 0.77}$ PFU on day 3 and later ($P = 0.0275$). The virus load was $10^{0.26 \pm 0.52}$ PFU on day 1 in patients with acute parotitis who had a previous history of mumps vaccination, with a significantly lower load ($P = 0.0351$). The virus load was $10^{2.00 \pm 1.46}$ PFU on day 0 or 1 in eight patients with vaccine-associated parotitis. Statistical significance was analyzed by Student's t test, and there was no significant difference in viral load between natural infection and vaccine-associated illness.

DISCUSSION

Annual mumps outbreaks still occur with fluctuating magnitude in Japan (5, 8, 16, 24). Acute clinical parotitis is mainly caused by mumps virus infection, but for some patients it is caused by other pathogens such as parainfluenza virus or enterovirus infection, bacterial infection, and Stenson's duct obstruction (1). Mumps is a highly contagious disease among kindergarten or primary school children. Once it is introduced into a closed community, a second or third transmission cycle occurs with typical incubation periods (16, 20). To prevent transmission, an index case should be correctly diagnosed with a reliable virological rapid diagnostic tool, and immunization should be recommended for susceptible individuals. It is difficult to accurately diagnose mumps infection only from clinical observations. The principal of virological examination is virus isolation, but the results are not applicable to clinical practice because of its tardiness. Mumps virus can usually be isolated in 40 to 60% of patients, and virus isolation is time consuming for more than 1 to 2 weeks. Recently, a more sensitive shell vial culture method has been employed (21, 22). In a clinical setting, rapid virological diagnosis is required to prevent further extension of the outbreak. We developed nested RT-PCR in the HN region to detect the genome directly from clinical samples (5, 7, 24), but it takes nearly 8 h with skillful experience to prevent cross-contamination. It was applied mainly for the retrospective laboratory studies, not as a clinical diagnostic tool. Recently, real-time PCR was introduced for the detection of several pathogens and is now being used for RNA viruses in single-tube real-time RT-PCR for the detection of RNA viruses such as respiratory syncytial virus (3, 28). A special apparatus is also required for real-time PCR, but this is beneficial for obtaining virus copies from the samples within a few hours.

TABLE 2. Comparison of virus load in natural mumps infection, secondary vaccine failure, and vaccine-related acute parotitis

Condition	Day of illness (no. of patients)	Virus load (PFU)	P value
Natural infection	0 (18)	0.89 ± 0.81	0.0741
	1–2 (10)	1.54 ± 1.01	
	3–5 (8)	0.49 ± 0.77	0.0275
SVF	1 (4)	0.26 ± 0.52	0.0351
Vaccine-related acute parotitis	0–1 (8)	2.00 ± 1.46	

LAMP was developed to amplify the target DNA without any temperature shifts, normally required for denaturing, annealing, and extension. LAMP has been used to detect many kinds of virus infection, mainly for DNA virus or bacterial genome infection (4, 6, 10, 11). Recently, RT-LAMP was used to detect West Nile virus (17). We developed RT-LAMP to detect the mumps virus genome and compared the sensitivity of RT-LAMP, nested RT-PCR, and virus isolation. In a preliminary study, the sensitivity of LAMP was the same as that of nested RT-PCR, but LAMP is a simple and timesaving procedure, allowing the results to be obtained within 1 h after extraction of the viral genome. LAMP for the detection of genomes of pathogenic agents is a useful tool for hospital-based rapid diagnosis and will contribute to laboratory-based surveillance studies.

We obtained a linear correlation between the genome quantity and the reaction time when the spectrophotometric values reached to a threshold of 0.1 and calculated the virus load in a sample by monitoring the spectrophotometric value. We found a difference in virus load for different mumps conditions. We detected $10^{1.54 \pm 1.01}$ PFU on days 1 or 2 of natural infection, $10^{0.26 \pm 0.52}$ PFU in patients who had a past history of mumps vaccination (likely due to SVF), and $10^{2.00 \pm 1.46}$ PFU in those who developed vaccine-associated acute parotitis. A lower virus load was noted for those who had been immunized before, and we speculated that the remaining immunity modified virus growth even though SVF occurred. Among recipients with the Hoshino mumps vaccine strain, approximately 2 to 3% developed parotitis 2 to 3 weeks after vaccination (9). Although the symptoms for vaccine-associated parotitis were mild without febrile illness in comparison with natural primary infection, no significant difference in viral load was observed in patients with vaccine-associated acute parotitis or natural primary infection. Vaccine strains are attenuated, and we believe that even though a similar quantity of the genome was detected, the symptoms were mild. As is the nature of live attenuated virus vaccines, mumps vaccine strains have characteristics similar to those of the wild strains. After immunization with mumps

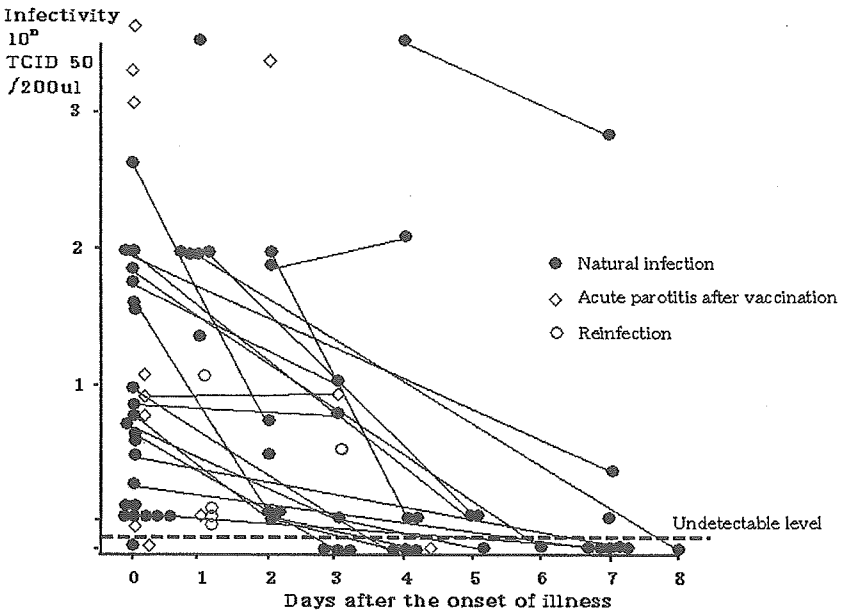

FIG. 5. Virus load for different conditions of mumps infection. Natural mumps infection (●), reinfection (○), and acute parotitis after vaccination (◇) are shown. The day when the patients noticed the parotid swelling was designated day 0.

vaccine, the vaccine strain of genome was detected 1 to 2 weeks after vaccination among the recipients without any symptoms (13). However, in this study, the number of patients was limited, and we plan to investigate a larger number of the cases to confirm the observation by LAMP.

We reported a simple, sensitive, reliable, rapid diagnostic method for the detection of mumps virus based on genome amplification, results of which can be obtained within 60 min after RNA extraction. No rapid diagnostic tool is available for detecting mumps virus at present, and a mumps LAMP system is a useful, reliable diagnostic tool for hospital- or clinic-based infection control in a clinical setting.

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A Phylogenetic Study of Human Respiratory Syncytial Viruses Group A and B Strains Isolated in Two Cities in Japan from 1980–2002

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The circulation pattern and genetic evolution of respiratory syncytial virus (RSV) in Japan were examined based on 109 RSV field strains isolated over 20 seasons (1980–2002) in two cities, Sapporo and Tokyo. The second hypervariable region of the large glycoprotein (G) gene was amplified by RT-PCR and the products sequenced directly. The nucleotide sequences were compared to those representatives of RSV genotypes identified previously. Japanese group A and B isolates clustered into five and four genotypes defined previously, respectively. Another one group A and one group B genotypes, which could not be assigned to previous genotypes, were also identified. Although different genotypes usually co-circulated in each season, the isolates in proximate seasons from two communities were usually located in the same branches. Moreover, the strains with genotypes defined previously were usually isolated at the same time as each reference strain of Western countries. Several mutant group B strains with 1–20 longer amino acid G proteins were newly identified in Sapporo. These findings suggest that Japanese RSV strains underwent geographical and also temporal clustering while participating in RSV genetic evolution in a global setting. In addition, Japanese strains, especially group B, might have evolved individually in each community, sometimes changing the length of the G protein. ***J. Med. Virol. 76:241–247, 2005.***

KEY WORDS: molecular epidemiology; respiratory syncytial virus; respiratory syncytial virus group A; respiratory syncytial virus group B; large glycoprotein

INTRODUCTION

Human respiratory syncytial virus (RSV) is the most important viral cause of lower respiratory tract infections in infants and young children, and also infects immunocompromised individuals and the elderly [Falsey et al., 1995; Whimbey et al., 1996]. RSV isolates can be divided into two subgroups, designated A and B, on the basis of their reaction with monoclonal antibodies [Andersen et al., 1985; Mufson et al., 1985; Tsutsumi et al., 1988]. In addition, the genetic variability within each subgroup of RSV has been studied extensively worldwide. The significance of strain differences in relation to clinical features and for vaccine development has not yet been elucidated fully [Brandenburg et al., 2001; Cane, 2001; Martinello et al., 2002].

RSV consists of 11 viral proteins [Sullender, 2000; Hall, 2001], and the RSV large glycoprotein (G protein) gene is thought to be the most variable viral membrane protein gene [Cane et al., 1991; Sullender et al., 1991; Cane and Pringle, 1995]. G protein variability is concentrated in the ectodomain, which contains two hypervariable regions separated by a conserved 11 amino acid motif [Johnson et al., 1987]. Analysis of the RSV G gene revealed that each epidemic with group A and B consisted of several genotypes and predominant genotypes gave way frequently to others [Cane et al., 1991; Choi and Lee, 2000; Seki et al., 2001]. The second variable region has been used in phylogenetic analysis for molecular epidemiological studies and to assign

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