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

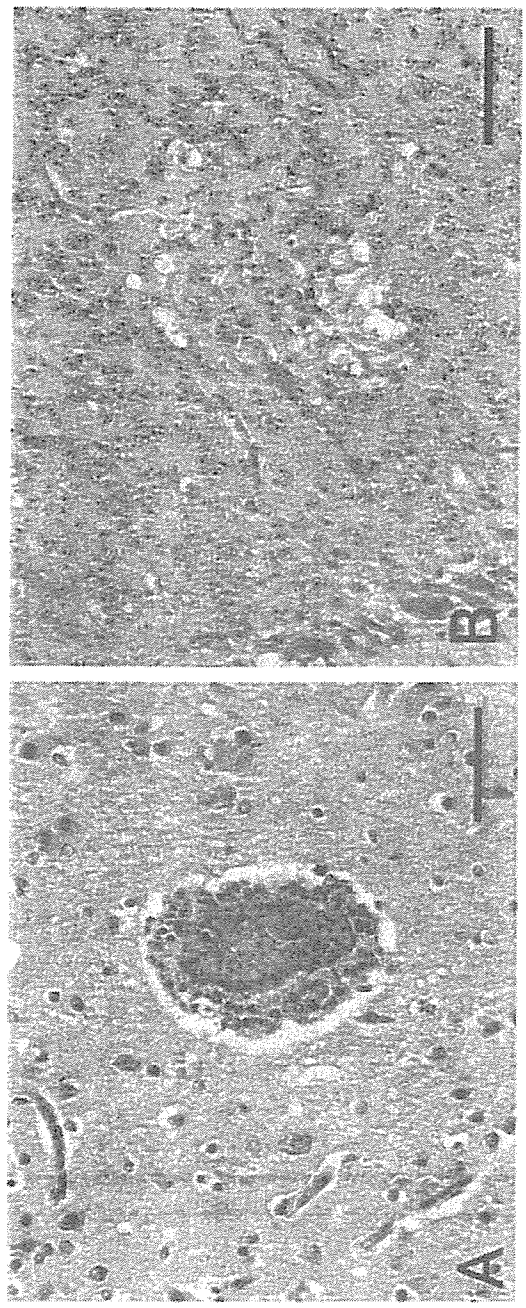

Fig. 1. Histopathological examination in chickens (A) and quails (B) inoculated with Dk/Yokohama/03. Photomicrographs of hematoxylin and eosin-stained tissue sections. A: Perivascular cuffing, swelling of endothelial cells, infiltration and proliferation of microglia in the brain (cerebrum) of the chickens 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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Author's address: Prof. Hiroshi Kida, Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan; e-mail: kida@vetmed.hokudai.ac.jp

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Outbreak of Highly Pathogenic Avian Influenza in Japan and Anti-Influenza Virus Activity of Povidone-Iodine Products

Hiroshi Ito[a] Toshihiro Ito[a] Muneo Hikida[c] Junko Yashiro[c] Akira Otsuka[c]
Hiroshi Kida[d] Koichi Otsuki[b]

Departments of [a]Veterinary Public Health and [b]Veterinary Microbiology, Faculty of Agriculture, Tottori University, Tottori, [c]Meiji Seika Co. Ltd., Tokyo, and [d]Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan

Key Words
Povidone-iodine · Highly pathogenic avian influenza · Virucidal activity · Antiseptics

Abstract
Objectives: On January 12, 2004, an outbreak of highly pathogenic avian influenza, caused by the H5N1 strain, occurred in a one-layer flock in Yamaguchi Prefecture, Japan. It had been 79 years since the last outbreak of avian influenza was confirmed in Japan. By February, 3 additional outbreaks had occurred (1 in Oita Prefecture and 2 in Kyoto Prefecture). Influenza viruses are enveloped viruses and are relatively sensitive to inactivation by lipid solvents, such as detergents. Infectivity is also rapidly destroyed by ether, sodium hypochlorite, povidone-iodine (PVP-I), peracetic acid and alcohol. However, these antiviral effects were only tested against human influenza A viruses. In the present study, the antiviral activity of PVP-I products against H5, H7 and H9 avian influenza A viruses, which had recently been transmitted to humans, were investigated. ***Methods:*** The in vitro antiviral activity of PVP-I products (2% PVP-I solution, 0.5% PVP-I scrub, 0.25% PVP-I palm, 0.23% PVP-I gargle, 0.23% PVP-I throat spray and 2% PVP-I solution for animals) against avian influenza A viruses [a highly pathogenic avian influenza virus, A/crow/Kyoto/T2/04 (H5N1; $10^{6.5}$ EID_{50}/0.1 ml), and 3 low pathogenic avian influenza A viruses, A/whistling swan/Shimane/499/838 (H5N3; $10^{4.8}$ EID_{50}/0.1 ml), A/whistling swan/Shimane/42/80 (H7N7; $10^{5.5}$ EID_{50}/0.1 ml) and A/duck/Hokkaido/26/99 (H9N2; $10^{4.8}$ EID_{50}/0.1 ml)] were investigated using embryonated hen's eggs. ***Results/Discussion:*** Viral infectious titers were reduced to levels below the detection limits by incubation for only 10 s with the PVP-I products used in this study. These results indicate that PVP-I products have virucidal activity against avian influenza A viruses. Therefore, the PVP-I products are useful in the prevention and control of human infection by avian influenza A viruses.

Introduction

In January 2004, an outbreak of highly pathogenic avian influenza occurred in a one-layer flock in Yamaguchi Prefecture, Japan. It had been 79 years since the last outbreak of avian influenza was confirmed in Japan. By February, 3 additional outbreaks had occurred, 1 in Oita Prefecture and 2 in Kyoto Prefecture.

Highly pathogenic avian influenza is an acute infectious disease that causes systemic symptoms in poultry [1]. Although the symptoms are varied, the mortality rate

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Toshihiro Ito
Department of Veterinary Public Health, Faculty of Agriculture
Tottori University, 4-101 Koyama-cho Minami
Tottori 680-8553 (Japan)
Tel./Fax +81 857 31 5437, E-Mail toshiito@muses.tottori-u.ac.jp

is high and the disease is highly contagious. Therefore, when it occurs, the poultry industry suffers devastating damage.

The virus contains two surface glycoprotein spikes, hemagglutinin and neuraminidase. Influenza A viruses are classified serologically based on their hemagglutinin and neuraminidase subtype combinations.

It was thought that avian influenza virus does not transmit directly from birds to humans. However, in 1997, highly pathogenic H5N1 avian influenza virus was transmitted from chickens to humans in Hong Kong [2, 3]. Thus far, 18 cases have been identified and of those, 6 died. In addition, that low pathogenic H9N2 avian influenza virus infected 2 people in Hong Kong was reported in 1999 [4]. During 2003, an avian influenza outbreak caused by the highly pathogenic H7N7 virus occurred in the Netherlands and human infection with this virus was also reported [5].

Therefore, avian influenza is now thought to be an important zoonosis, not a simple avian disease [6–8].

Summary of the Outbreaks of Highly Pathogenic Avian Influenza in Japan

The First Case

In an egg production farm in Ato-Cho, Yamaguchi Prefecture, Japan, on December 28, 2003, dead chickens were confirmed in the poultry house No. 1. Since the number of chicken deaths subsequently increased and spread to other houses, the egg farm requested the local Livestock Hygiene Service Center to make a disease diagnosis. On January 11, Yamaguchi Prefecture sent test samples to the National Institute of Animal Health, confirming infection by subtype H5 avian influenza virus. Thus, the chickens were found to be suffering from highly pathogenic avian influenza infection.

After confirmation of the outbreak, Yamaguchi Prefecture devised the necessary epidemic prevention measures including the culling of all chickens at the farm, disinfection, restrictions on movement at other farms in the vicinity and implementation of epidemiological surveys.

The movement of chickens and other domestic fowl as well as objects that could potentially cause the spread of the virus was prohibited within a zone with a radius of 30 km centered around the infected farm.

In virus-free confirmation tests conducted by Yamaguchi Prefecture on February 14, no other infections were confirmed in any of the flocks.

The Second Case

On February 14, 2004, three bantams died at the home of a fancier of pet bantams in Kokonoe-machi, Oita Prefecture of Kyushu Island. Therefore, the breeder reported the event to the local Livestock Hygiene Service Center.

Oita Prefecture sent test samples to the National Institute of Animal Health, and the chickens were confirmed to be infected with the same highly pathogenic avian influenza virus.

As in the case of Yamaguchi Prefecture, as initial epidemic prevention measure, entry into the infected location by outsiders was restricted and the poultry house was disinfected, etc. At the same time, a zone with a radius of 30 km was designated centered around the infected location.

The Third Case

There was an increase in dead chickens in the poultry house No. 8 of an egg production facility in Tanba-cho, Kyoto Prefecture, on February 17, 2004. The infection spread to virtually all of the houses within a few days.

The breeder did not report this to the Livestock Hygiene Service Center while this was occurring. The Service Center, which had received an anonymous telephone call reporting large-scale deaths at the farm, conducted an on-site inspection before dawn on February 27. The virus was also confirmed to be H5.

Kyoto Prefecture also devised the necessary epidemic prevention measures, such as restricted entry to the farm by outsiders, constraints on egg shipments, the culling of the chickens at the farm, disinfection of poultry houses, and restrictions on movement at other farms in the vicinity.

Slaked lime was used to disinfect poultry houses.

However, it became clear meanwhile that infected poultry had already been shipped to chicken processing plants in Hyogo and Aichi Prefectures.

H5 avian influenza viruses were also isolated from a total of 9 jungle crows between May 7 and April 9 in the restricted movement zone of the third outbreak. No other infection of wild birds was confirmed.

The Fourth Case

Since there was a rapid increase in dead chickens on March 3 at a broiler farm in Kyoto Prefecture located about 4 km northeast of the infected farm of the third outbreak, the farm manager notified the Livestock Hygiene Service Center. Kyoto Prefecture sent samples of the dead chickens to the National Institute of Animal Health on March 5 and a disease diagnostic investigation

Table 1. Virucidal activity of PVP-I against influenza viruses

PVP-I product	PVP-I concentration %	Mean titer of remaining viruses (EID_{50}/0.1 ml)			
		HPAI	LPAI		
		H5 (T2)	H5 (499)	H7 (42)	H9 (26)
PVP-I solution	2	–	–	–	–
PVP-I scrub	0.5	–	–	–	–
PVP-I palm	0.25	–	–	–	–
PVP-I gargle	0.23	–	–	–	–
PVP-I throat spray	0.23	–	–	–	–
PVP-I solution for animals	2	–	–	–	–
Control (PBS)	0	$10^{6.5}$	$10^{4.8}$	$10^{5.5}$	$10^{4.8}$

Concentrations indicate the final concentration of each PVP-I product. HPAI = Highly pathogenic avian influenza; LPAI = low pathogenic avian influenza; – = below the detection limit.

was conducted. The result showed that the isolated virus was the same H5 highly pathogenic avian influenza virus.

Since this outbreak was related to the third case in the restricted movement zone, a response was implemented concomitant with the third outbreak without setting a new restricted movement zone.

Thus, a total of 4 poultry-raising facilities had to sacrifice about 275,000 domestic fowl.

Although it was fortunately possible to keep the damage to a minimum in Japan, large-scale outbreaks of the disease were also confirmed in Southeast Asia, and in some countries, for example Thailand and Vietnam, human infections increased. Therefore, those outbreaks not only involved livestock hygiene, but also became an issue of nationwide interest as a public health problem.

Study on Antiviral Activity of Povidone-Iodine Products against Avian Influenza Viruses

Introduction

Influenza viruses are enveloped viruses and are relatively sensitive to inactivation by lipid solvents, such as detergents. Infectivity is also rapidly destroyed by ether, sodium hypochlorite, povidone-iodine (PVP-I), peracetic acid and alcohol. Wutzler et al. [9] demonstrated that PVP-I products exhibited an antiviral effect against human influenza viruses. However, no avian influenza viruses have been tested.

Therefore, in the present study, the antiviral activity of povidone-iodine products against avian influenza viruses was investigated.

Materials and Method

A total of 3 low pathogenic avian influenza strains, A/whistling swan/Shimane/499/83 (H5N3), A/whistling swan/Shimane/42/80 (H7N7) and A/duck/Hokkaido/26/99 (H9N2) viruses, were used. Furthermore, a highly pathogenic avian strain, A/crow/Kyoto/T2/04 (H5N1) which was isolated from a crow during the Kyoto outbreak in 2004, was also used.

These viruses were grown in 10-day-old embryonated chicken eggs at 35°C for 2 days.

The following 6 kinds of PVP-I products were used in this study: 2% PVP-I solution, 0.5% PVP-I scrub, 0.25% PVP-I palm, 0.23% PVP-I gargle, 0.23% PVP-I throat spray and 2% PVP-I solution for animals. Each PVP-I product was diluted with sterilized distilled water just before the examination. The concentration of each PVP-I product which was used for this study was set mainly as that prescribed for usage.

The examination was performed as described by Kawana et al. [10], namely 0.25 ml of each PVP-I product was mixed with 0.25 ml of tested virus and incubated at 25°C for 10 s. The reaction was stopped promptly by adding phosphate-buffered saline containing 0.5% sodium thiosulfate.

Serial 10-fold dilutions of each reaction fluid were inoculated into allantoic cavities of 10-day-old embryonated eggs and incubated for 2 days at 35°C. Virus titers were determined by the hemagglutination test and the method of Reed and Muench [11].

The examination of the highly pathogenic strain A/crow/ Kyoto/T2/04 was carried out in BL3 containments in Hokkaido University.

Results

As shown in table 1, viral infectious titers were reduced to levels below the detection limits by incubation for only 10 s with all PVP-I products used in this study.

Conclusion

These results indicate that PVP-I products have virucidal activity against avian influenza A viruses. Therefore, the PVP-I products are probably useful in the prevention and control of human infection by avian influenza A viruses.

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Library of Influenza Virus Strains for Vaccine and Diagnostic Use Against Highly Pathogenic Avian Influenza and Human Pandemics

H. Kida, Y. Sakoda

Department of Disease Control, Hokkaido University Graduate School of Veterinary Medicine, Sapporo, Japan

Abstract: To prepare for the emergence of pandemic influenza in birds and mammals including humans, we have carried out global surveillance of avian influenza. Influenza A viruses of 48 combinations of 15 HA and 9 NA subtypes out of 135 theoretical combinations have been isolated from faecal samples of ducks in Alaska, Siberia, Mongolia, Taiwan, China and Japan. So far, viruses of 73 other combinations have been generated by genetic reassortment in chicken embryos. Thus, avian influenza viruses of 121 combinations of HA and NA subtypes have been stocked for use in vaccine and diagnosis. Their pathogenicity, antigenicity, genetic information, and yield in chicken embryo have been analysed and registered in the database.

Background

Although many infectious diseases have been eradicated or controlled in the last century, influenza remains to be overcome, since influenza A viruses cause occasional catastrophic pandemics in humans, poultry and mammals. In addition, recent outbreaks of highly pathogenic avian influenza (HPAI) in the world have sent alarm signals as we realise that there is no border for infections. These outbreaks have given rise to concern for human health as well as the livestock industry. H5N1 avian influenza viruses have jumped the species barrier and caused severe disease with high mortality in humans in Hong Kong in 1997, and Vietnam, Thailand and Cambodia in 2004-2005; eight deaths of 18 cases, and 29 of 37, 11 of 16, and one of one, respectively. It is now obvious that live-bird markets play an important role in the generation of highly pathogenic strains.

To elucidate the origin and evolution of pandemic influenza viruses, we have conducted global surveillance of influenza in birds, animals and humans. On the basis of antigenic and genetic analyses of influenza virus isolates from migratory ducks, domestic ducks, pigs and humans, and experimental infection studies of

birds and animals with those viruses, we proposed that the haemagglutinin (HA) gene of A/Hong Kong/68 (H3N2) strain was introduced into the precedent human H2N2 Asian influenza virus by genetic reassortment in pigs through domestic ducks from an H3 influenza virus circulating in migratory ducks in southern China [2-5].

Ecological studies have revealed that a vast influenza virus gene pool for avian and mammalian influenza exists in migratory ducks. Each of the known subtypes of influenza A virus perpetuates among migratory ducks and their nesting lake water in nature [6,9]. Experimental infection studies established that influenza viruses preferentially replicate in the columnar epithelial cells in the colon, causing no disease signs and are excreted in high concentration in ducks [1]. Influenza viruses have been isolated from freshly deposited faecal materials and from lake water, indicating that migratory ducks have an efficient way to transmit viruses, i.e. via faecal material in the water supply.

In comparison with influenza viruses of humans, those in ducks are in evolutionary stasis [2]. The remarkable lack of amino acid changes among influenza viruses isolated from migratory ducks suggests that the virus genes in this host are subject to stringent stabilising selection. The genetic and antigenic conservation of influenza viruses in ducks suggests that the virus and host have reached a long established adaptive optimum. Such a stasis in migratory ducks is further evidence that the duck population is the natural reservoir of influenza viruses.

In experimental infection of pigs, most tested strains of each of the known HA subtypes of avian influenza virus replicated in the upper respiratory tract of the animals. Co-infection of pigs with a swine virus and with an avian virus unable to replicate in this animal generated reassortants, whose polymerase and HA genes were entirely of avian origin; these could be passaged in pigs [5]. The results indicate that avian viruses of any subtype can contribute genes in the generation of reassortants, so that none of the 15 HA and nine neuraminidase (NA) subtypes can be ruled out as potential candidates for future pandemics in humans and poultry. The direct transmission of H5N1 influenza viruses from domestic poultry to humans in Asia and Europe in recent years further emphasised the need to have information on all influenza A subtypes circulating in avian species in the world.

To provide information on the ecology of influenza viruses in the natural host, virological surveillance of avian influenza was carried out in Alaska and Siberia during the breeding season in summer. Influenza viruses of different subtypes were isolated from faecal samples of ducks and water samples of the lakes where they nest. Even in autumn, when the ducks had left for migration to the south, viruses were still isolated from the lake water. This indicated that influenza viruses are maintained in the duck population by water-borne transmission in nature and revealed the mechanism of year-by-year perpetuation of the viruses in the frozen lake water while ducks are absent. Phylogenetic analysis of the isolates from Alaska indicates that ducks carrying these viruses migrate to the south through the continent of North America and not to Asia [6].

Influenza virus isolates from faecal samples of ducks in their nesting areas in Siberia phylogenetically belonged to Eurasian lineage and were closely related to those from birds, pigs and horses in Asia. It was noted that these isolates closely correlated to the H5N1 influenza virus isolates from chickens and humans [9]. Antigenic and phylogenetic analysis of the HA of H5 influenza virus isolates from ducks in Japan revealed a close relationship with those of H5N1 influenza viruses from Hong Kong, southern China, Thailand, Vietnam and Japan, indicating that the

H5HA of these viruses originates from influenza viruses maintained in migratory ducks nesting in Siberia. These results indicate that the precursor genes of pandemic influenza viruses are perpetuated in water in the lakes where ducks nest in their northern territory.

Vaccine strains should be antigenically closely related to the pandemic strain, avirulent for humans and chicken embryos, of high yield in chicken embryos, and immediately prepared when the causative virus is characterised. We tested immunogenicity in mice to inactivated avirulent H5N4 influenza viruses isolated from reservoir host ducks. The results showed that they were potent enough to protect animals from challenge with a lethal dose of the highly pathogenic H5N1 virus [8].

RESULTS

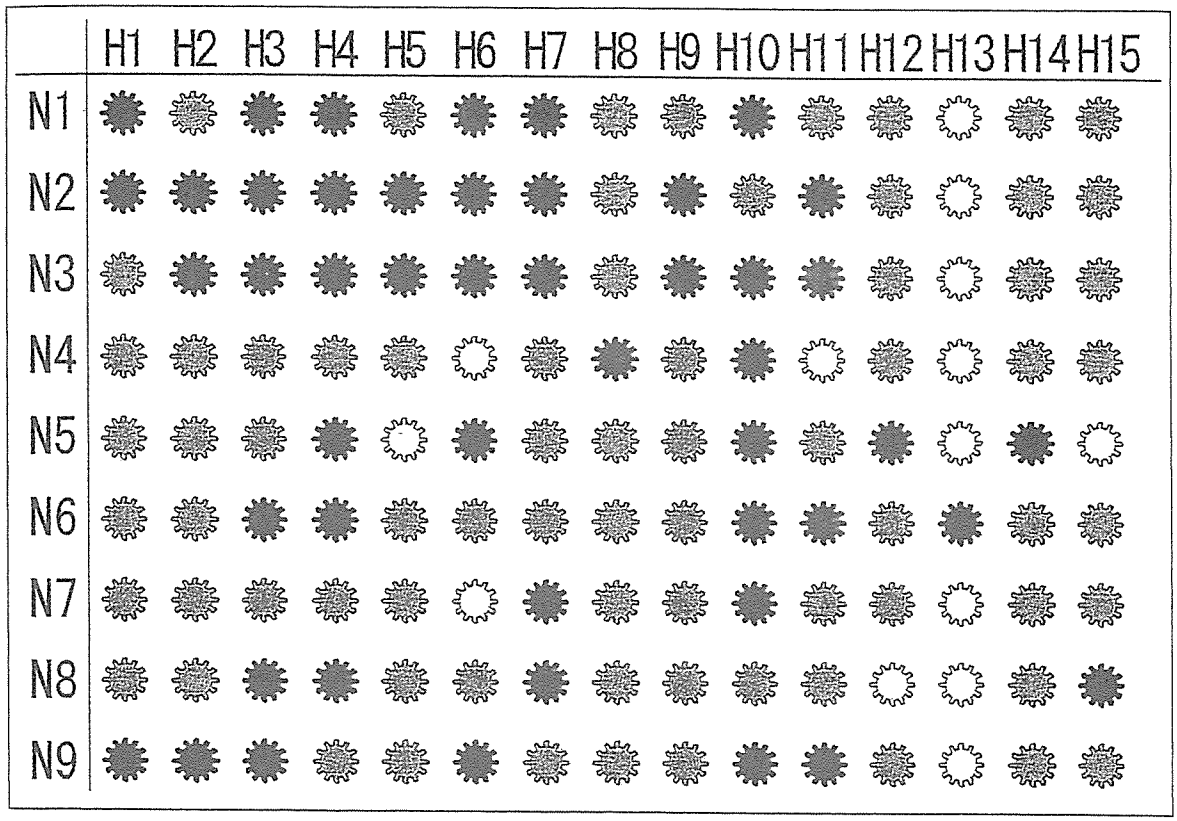

Fig. 1: Present status of the library of influenza virus strains for vaccine and diagnostic use.
Influenza viruses of 48 combinations of HA and NA subtypes have been isolated from faecal samples of ducks in Alaska, Siberia, Mongolia, Taiwan, China and Japan (shown in black). So far, 73 other combinations have been generated by genetic reassortment procedure in the laboratory (shown in grey). Thus, avian influenza viruses of 121 combinations of HA and NA subtypes have been stocked as vaccine strain candidates. Their pathogenicity, antigenicity, genetic information and yield in chicken embryo have been analysed.

For the prediction of possible HA and NA subtypes of pandemic strains emerging in poultry and humans, the surveillance study of avian influenza has been carried out since 1976. Influenza virus isolates of 48 combinations of HA and NA subtypes have been isolated from faecal samples of ducks in Alaska, Siberia, Mongolia, Taiwan, China and Japan. So far, viruses of more than 73 other combinations have been

generated by genetic reassortment in chicken embryos. Thus, avian influenza viruses of 121 combinations of HA and NA subtypes have been stocked as vaccine strain candidates and diagnostic use (Fig. 1). Their pathogenicity, antigenicity, genetic information and yield in chicken embryo have been analysed and registered in the database.

Discussion

Since influenza A viruses of all the known subtypes are perpetuated among migratory ducks and their nesting lakes, we must accept that influenza is not an eradicable disease. At present, prevention and control should be the only realistic goals. Since it is now clear that humans, pigs, poultry and migratory ducks are associated with the interspecies transmission of influenza virus and the emergence of pandemics in birds and mammals including humans, it is essential to expand global surveillance in these species. Live-poultry markets provide a critical environment for genetic mixing and spreading of influenza viruses. Continued surveillance and epidemiological monitoring and analysis should serve to reduce the possibility of the emergence of highly pathogenic avian influenza virus strains in these markets.

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Dr. H. Kida, Research Center for Zoonosis Control, Department of Disease Control, Hokkaido University Graduate School of Veterinary Medicine, Kita 18, Nishi-9, Kita-ku, Sapporo, 060-0818 Japan.
E-mail: kida@vetmed.hokudai.ac.jp

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Swine Influenza in China

H. Chen, H. Li, H. Yang, C. Qiao, K. Yu

Harbin Veterinary Research Institute, Chinese Academy of Agriculture Sciences, Harbin, P. R. China

Abstract: Swine influenza is a common infectious respiratory disease of pigs that caused by influenza A viruses, and H1N1 and H3N2 are currently the most popular subtypes of influenza viruses circulated in pig population in China. H9N2 virus was detected from pigs that imported from Mainland China in Hong Kong in 1998, and extensive viral surveillance conduced since then indicated that H9N2 viruses are widely distributed in pig population in China. Phylogenetic analysis indicated that H9N2 viruses from pigs are highly related, implying they may establish a stable lineage in pigs in China. During the routine surveillance, we also isolated 2 H5N1 viruses from pigs in Fujian province in 2001 and 2003, respectively. Molecular analysis indicated that the two viruses are origin from duck H5N1 viruses. Their replication in chickens, mice and pigs has also been investigated.

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Hierarchy among Viral RNA (vRNA) Segments in Their Role in vRNA Incorporation into Influenza A Virions

Yukiko Muramoto,[1,2,3] Ayato Takada,[2,3] Ken Fujii,[2,3] Takeshi Noda,[3,4] Kiyoko Iwatsuki-Horimoto,[2,3] Shinji Watanabe,[5] Taisuke Horimoto,[2,3] Hiroshi Kida,[1] and Yoshihiro Kawaoka[2,3,4,5]*

Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818,[1] Division of Virology, Department of Microbiology and Immunology,[2] and International Research Center for Infectious Diseases,[4] Institute of Medical Science, University of Tokyo, Tokyo 108-8639, and Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama 332-0012,[3] Japan, and Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin—Madison, Madison, Wisconsin 53706[5]

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The genome of influenza A viruses comprises eight negative-strand RNA segments. Although all eight segments must be present in cells for efficient viral replication, the mechanism(s) by which these viral RNA (vRNA) segments are incorporated into virions is not fully understood. We recently found that sequences at both ends of the coding regions of the HA, NA, and NS vRNA segments of A/WSN/33 play important roles in the incorporation of these vRNAs into virions. In order to similarly identify the regions of the PB2, PB1, and PA vRNAs of this strain that are critical for their incorporation, we generated a series of mutant vRNAs that possessed the green fluorescent protein gene flanked by portions of the coding and noncoding regions of the respective segments. For all three polymerase segments, deletions at the ends of their coding regions decreased their virion incorporation efficiencies. More importantly, these regions not only affected the incorporation of the segment in which they reside, but were also important for the incorporation of other segments. This effect was most prominent with the PB2 vRNA. These findings suggest a hierarchy among vRNA segments for virion incorporation and may imply intersegment association of vRNAs during virus assembly.

Viruses must retain their genomes during repeated cycles of replication. At a late stage of the infectious cycle, progeny viruses begin assembly into infectious virions by selecting their genomes from a large pool of viral and cellular nucleic acids. The regions important for the incorporation of a viral genome into virions are generally called packaging signals (10, 18, 29, 34). For example, the packaging signals of retroviruses, which are located in the 5′ end of the genome, interact with the Gag precursor, leading to the incorporation of the genome into virions (reviewed in reference 1). However, for many other viruses, the genomic packaging signals and the underlying packaging mechanisms remain unknown.

Influenza A virus is an enveloped negative-strand RNA virus. Its genome, which encodes up to 11 proteins, comprises eight viral RNA (vRNA) segments (PB2, PB1, PA, HA, NP, NA, M, and NS) (3, 17). The noncoding regions at the 3′ and 5′ termini of the eight vRNA segments contain highly conserved sequences that consist of 12 and 13 nucleotides, respectively. These sequences are partially complementary to allow the formation of a corkscrew structure (2, 5, 9, 14, 30) that constitutes the viral promoter for replication and transcription (28). Each vRNA segment interacts with viral nucleoprotein (NP) and three polymerase subunit proteins, PB2, PB1, and PA, to form viral ribonucleoprotein (vRNP) complexes, the minimal unit for transcription and replication of vRNAs. Newly synthesized vRNPs are exported from the nuclei of host cells to the plasma membrane, where they are incorporated into progeny virions. For influenza A viruses, only virions that possess all eight RNA segments can successfully complete viral replication. Hence, signals that ensure the incorporation of the viral genomic segments into virions must be present in the genome; however, the mechanism that drives vRNA virion incorporation is unknown.

When influenza viruses are passaged at a high multiplicity of infection, defective interfering virus particles, which contain defective RNAs with deletions in their coding regions, are produced (reviewed in reference 20). Defective RNAs are generated during vRNA replication, incorporated into virions, and maintained by coinfection with infectious viruses (8). Defective RNAs have been described for all eight influenza A virus segments (15, 24), although most are derived from the polymerase segments (i.e., PA, PB1, and PB2). These defective RNAs do not encode functional viral proteins, but they are retained in virus populations, indicating that they contain the structural features required for their incorporation into virions.

We recently found that, in addition to the noncoding regions (11), coding regions at both ends of the HA, NA, and NS vRNA segments play important roles in the incorporation of these segments into virions (11, 12, 33). These findings suggested that all influenza A virus segments possess segment-specific packaging signals for their efficient virion incorporation. We also found that the exclusion of a vRNA segment decreased the efficiency of infectious virion formation, even if the protein encoded by the omitted vRNA was supplied by a protein expression plasmid (12). These results suggested that

* Corresponding author. Mailing address: Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. Phone: 81-3-5449-5310. Fax: 81-3-5449-5408. E-mail: kawaoka@ims.u-tokyo.ac.jp.