別紙様式第２－１

医薬品　研究報告　調査報告書

識別番号・報告回数			報告日	第一報入手日 平成 18 年 9 月 12 日	新医薬品等の区分 該当なし	機構処理欄
一般的名称	ﾃｸﾈﾁｳﾑ人血清ｱﾙﾌﾞﾐﾝ (^{99m}Tc)		研究報告の公表状況	WHO sites Media centre: Notes for the Media 5 September 2006	公表国 南アフリカ他	
販売名（企業名）	テクネアルブミンキット （第一 RI）					

研究報告の概要	使用上の注意記載状況・その他参考事項等
WHO が病原性が強く、致死性の結核の全世界的な拡大防止の強化および措置を求めた。広範囲の薬剤耐性結核（XDR-TB）は世界の様々な地域で確認されており、とくに旧ソビエト連邦諸国やアジアにおいてもっとも多く確認されている。また南アフリカにおいて報告された XDR-TB 疑いとされた HIV 陽性患者群において極めて高い死亡率（53 例中 52 例死亡。ただし、HIV の合併や適切な医療行為の欠如や重症患者のみが確認されていることによる影響を受けている可能性あり。）が確認されている。	特になし 尚、後日（10/30）、追加情報として、南アフリカより報告された 53 例の患者は XDR-TB の現状の定義には合致していなかったとの報告を入手。 [WHO The Weekly Epidemiological Record, No41,2006,81,386-390 (13 October 2006)]

報告企業の意見	今後の対応
本報告は、既知で重大な感染症に関する報告であるが、高度に治療抵抗性であり、従来認められない非常に高い死亡率が確認されていることから、重大な感染症の発生傾向の変化を示す報告と考えられる。なお、結核は呼吸器疾患であり、くしゃみや咳により感染するものであり、血液を介して感染するものではないことから、ヒト血液を原料とする当該生物由来製品には直接関連しないと判断する。	本報告はヒト血液を原料とする血漿分画製剤とは直接関連するものではなく、現時点で特に自社の当該生物由来製品に関し、措置を行う必要はないと判断する。しかしながら、世界的に重要な問題として WHO 等でも取り上げていることもあるため、今後とも関連情報については、注目して、情報収集に努める。

MedDRA Version(9.1)

World Health Organization

Emergence of XDR-TB
WHO concern over extensive drug resistant TB strains that are virtually untreatable

5 SEPTEMBER 2006 | GENEVA -- The World Health Organization (WHO) has expressed concern over the emergence of virulent drug-resistant strains of tuberculosis (TB) and is calling for measures to be strengthened and implemented to prevent the global spread of the deadly TB strains. This follows research showing the extent of XDR-TB, a newly identified TB threat which leaves patients (including many people living with HIV) virtually untreatable using currently available anti-TB drugs.

Later this week, WHO will join other TB experts at a two-day meeting in South Africa (7-8 September) to assess the response required to critically address TB drug resistance, particularly in Africa, and will take part in a news conference scheduled for Thursday, 7 September in Johannesburg.

What is XDR-TB?

MDR-TB (Multidrug Resistant TB) describes strains of tuberculosis that are resistant to at least the two main first-line TB drugs - isoniazid and rifampicin. XDR-TB, or Extensive Drug Resistant TB (also referred to as Extreme Drug Resistance) is MDR-TB that is also resistant to three or more of the six classes of second-line drugs.

The description of XDR-TB was first used earlier in 2006, following a joint survey by WHO and the US Centers for Disease Control and Prevention (CDC).

Resistance to anti-TB drugs in populations is a phenomenon that occurs primarily due to poorly managed TB care. Problems include incorrect drug prescribing practices by providers, poor quality drugs or erratic supply of drugs, and also patient non-adherence.

What is the current evidence of XDR-TB?

Recent findings from a survey conducted by WHO and CDC on data from 2000-2004 found that XDR-TB has been identified in all regions of the world but is most frequent in the countries of the former Soviet Union and in Asia.

In the United States, 4% of MDR-TB cases met the criteria for XDR-TB.

In Latvia, a country with one of the highest rates of MDR-TB, 19% of MDR-TB cases met the XDR-TB criteria.

Separate data on a recent outbreak of XDR-TB in an HIV-positive population in Kwazulu-Natal in South Africa was characterized by alarmingly high mortality rates.

Of the 544 patients studied, 221 had MDR-TB. Of the 221 MDR-TB cases, 53 were defined as XDR-TB. Of the 53 patients, 44 had been tested for HIV and all were HIV-positive.

52 of 53 patients died, on average, within 25 days including those benefiting from antiretroviral drugs.

Scarce drug resistance data available from Africa indicate that while population prevalence of drug resistant TB appears to be low compared to Eastern Europe and Asia, drug resistance in the region is on the rise.

Given the underlying HIV epidemic, drug-resistant TB could have a severe impact on mortality in Africa and requires urgent preventative action.

What action is required to prevent XDR-TB?

XDR-TB poses a grave public health threat, especially in populations with high rates of HIV and where there are few health care resources. Recommendations outlined in the WHO Guidelines for the Programmatic Management of Drug Resistant Tuberculosis include:

- strengthen basic TB care to prevent the emergence of drug-resistance
- ensure prompt diagnosis and treatment of drug resistant cases to cure existing cases and prevent further transmission

- increase collaboration between HIV and TB control programmes to provide necessary prevention and care to co-infected patients
- increase investment in laboratory infrastructures to enable better detection and management of resistant cases.

The Expert Consultation on Drug Resistant TB, hosted by the South African Medical Research Council with support from WHO and CDC, takes place in Johannesburg, 7-8 September.

A news conference will be held at 12.30pm, Thursday, 7 September, at the conference venue: Sunnyside Park Hotel, Parktown, Johannesburg.

For more information contact:

Glenn Thomas
Stop TB Department
WHO
Mobile: +41 79 5 09 06 77
E-mail: thomasg@who.int

Dr Karin Weyer
Director, South African Medical Research Council
Unit for TB Operational and Policy Research
Tel: +27 12 3 39 85 50
Mobile: +27 82 4 60 88 36
E-mail: kweyer@mrc.ac.za

別紙様式第2

医薬品 研究報告 調査報告書

識別番号・報告回数		報告日	第一報入手日 2006年4月14日	新医薬品等の区分	厚生労働省処理欄
一般的名称	①乾燥濃縮人活性化プロテインC ②乾燥濃縮人血液凝固第IX因子	研究報告の公表状況	Pathogenicity of a highly pathogenic avian influenza virus, A/chicken/Yamaguchi/7/04 (H5N1) in different species of birds and mammals. Archives of Virology. 2006 Feb 26 [Epub ahead of print]	公表国 日本	
販売名(企業名)	①注射用アナクトC2,500単位 ②ノバクトM				

研究報告の概要

(問題点：高病原性トリインフルエンザウイルスH5N1は、マウスにも感染する。)

2004年初頭、H5N1ウイルスによる高病原性トリインフルエンザが日本の2つの農場とペットのニワトリの間で発生した。最初のアウトブレイク中に死んだニワトリから分離されたA/chicken/Yamaguchi/7/04及びその他のH5N1ウイルスをニワトリ、ウズラ、セキセイインコ、子ガモ、マウス、ミニブタに実験的に感染させてウイルスの病原性を評価した。結果、ウイルスはすべてのトリで高病原性を示した。マウスは感受性を示したが、死亡率は低かった。ミニブタには感染しなかった。

22匹のマウスに対して、H5N1ウイルスを経鼻で感染させた。22匹のうち、2匹が接種3日及び4日後に死亡した。死亡したマウスを死亡後に、また残りの生存した20匹のマウスを接種3日及び14日後に剖検し、ウイルスの組織分布を調べた。4日後に死亡したマウスは呼吸器官以外に腎臓と肝臓からウイルスが分離された。3日後に死亡したマウス及び3日後に剖検したマウスは呼吸器官でのみウイルスが分離され、14日後に剖検したマウスからはウイルスは分離されなかった。また、すべてのマウスにおいて、脳からのウイルスの分離はなかった。

報告企業の意見	今後の対応
別紙のとおり	今後とも情報収集に努め、本剤の安全性の確保を図っていきたい。

使用上の注意記載状況・その他参考事項等

記載なし

別紙

一般的名称	①乾燥濃縮人活性化プロテインC、②乾燥濃縮人血液凝固第IX因子
販売名（企業名）	①注射用アナクトC2,500単位、②ノバクトM
報告企業の意見	「注射用アナクトC2,500単位」及び「ノバクトM」は、有効成分である活性化プロテインCあるいは血液凝固第IX因子を精製するために、プロテインCあるいは血液凝固第IX因子に対するモノクローナル抗体をリガンドとするアフィニティクロマトグラフィを使用しており、それぞれのモノクローナル抗体の調製にマウスを用いている。これらのモノクローナル抗体を精製する際にウイルス除去膜ろ過を実施しており、一定のウイルス除去効果が期待される。 一方、当該製剤は、「血漿分画製剤のウイルスに対する安全性確保に関するガイドライン（医薬発第1047号、平成11年8月30日）」に従い、ウシウイルス性下痢ウイルス（BVDV）、仮性狂犬病ウイルス（PRV）、ブタパルボウイルス（PPV）、A型肝炎ウイルス（HAV）または脳心筋炎ウイルス（EMCV）をモデルウイルスとして、ウイルスプロセスバリデーションを実施した製造工程により製造されている。今回報告した高病原性トリインフルエンザH5N1は、エンベロープの有無、核酸の種類等からモデルウイルスとしてはBVDVが該当すると考えられるが、上記バリデーションの結果から、当該製剤の製造工程の内、上記のイムノアフィニティクロマトグラフィ工程の下流に導入されている加熱工程がウイルス不活化効果を有することを確認している。 また、これまでに当該製剤による高病原性トリインフルエンザH5N1感染の報告例は無い。 以上の点から、当該製剤は高病原性トリインフルエンザウイルスH5N1に対する安全性を確保していると考える。

Arch Virol (2006)
DOI 10.1007/s00705-005-0723-6

Archives of Virology
Printed in Austria

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

N. Isoda[1], Y. Sakoda[1], N. Kishida[1], G.-R. Bai[1], K. Matsuda[2], T. Umemura[2], and H. Kida[1]

[1]Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan
[2]Laboratory of Comparative Pathology, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan

Received September 20, 2005; accepted December 26, 2005
Published online February 26, 2006

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

Introduction

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

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

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

Materials and methods

Viruses

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

Animals

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

Animal experiments

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

Virus titration

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

Antibody detection

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

Histopathological examination

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

Results

Chickens

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

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 (H5N1)	5	2–4d	dead	5 (7.1)	5 (5.8)	5 (6.4)	5 (5.8)	5 (7.7)	ND	ND
	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) (H5N1)	3	3d	sacrificed	0	0	0	0	0	0	—
	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

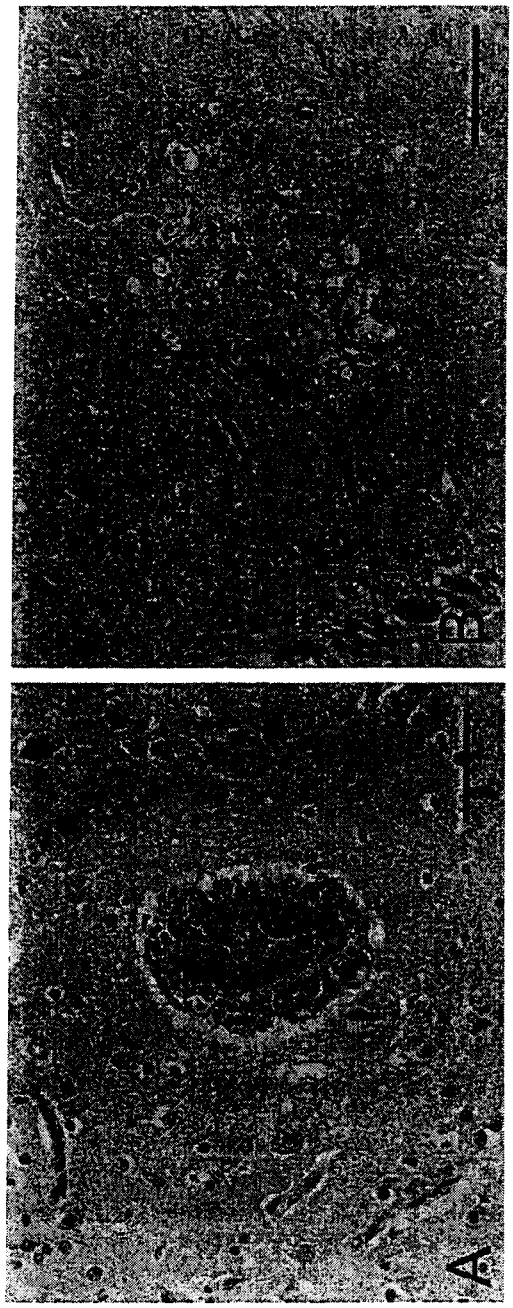

Fig. 1. Histopathological examination in chickens (**A**) and quails (**B**) inoculated with Dk/Yokohama/03. Photomicrographs of hematoxylin and eosin-stained tissue sections. **A**: Perivascular cuffing, swelling of endothelial cells, infiltration and proliferation of microglia in the brain (cerebrum) 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.

Acknowledgments

We extend special thanks to the National Institute of Animal Health (Ibaraki, Japan) and the Animal Quarantine Service (Kanagawa, Japan) for providing the A/chicken/Yamaguchi/7/04

(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.

References

1. Alexander DJ (2000) A review of avian influenza in different bird species. Vet Microbiol 74: 3–13
2. Alexander DJ (2004) Highly pathogenic avian influenza. In: Manual of diagnostic tests and vaccines for terrestial animals (mammals, birds and bees), 5th edn, vol. 1. OIE, Paris, pp 258–269
3. Bai GR, Sakoda Y, Mweene AS, Kishida N, Yamada T, Minakawa H, Kida H (2005) Evaluation of the ESPLINE® INFLUENZA A&B-N Kit for the diagnosis of avian and swine influenza. Microbiol Immunol 49: 1063–1067
4. Chen H, Smith GJD, Zhang SY, Qin K, Wang J, Li KS, Webster RG, Peris JSM, Guan Y (2005) H5N1 virus outbread in migratory waterfowl. Nature 436: 191–192
5. Dybing JK, Schultz-Cherry S, Swayne DE, Suarez DL, Perdue ML (2000) Distinct pathogenesis of hong kong-origin H5N1 viruses in mice compared to that of other highly pathogenic H5 avian influenza viruses. J Virol 74: 1443–1450
6. Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus AD (2005) Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 79: 2814–2822
7. Gao P, Watanabe S, Ito T, Goto H, Wells K, McGregor M, Cooley AJ, Kawaoka Y (1999) Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. J Virol 73: 3184–3189
8. Horimoto T, Nakayama K, Smeekens SP, Kawaoka Y (1994) Proprotein-processing endoproteases PC6 and furin both activate hemagglutinin of virulent avian influenza viruses. J Virol 68: 6074–6078
9. Ito T, Okazaki K, Kawaoka Y, Takada A, Webster RG, Kida H (1995) Perpetuation of influenza A viruses in Alaskan waterfowl reservoirs. Arch Virol 140: 1163–1172
10. Kida H, Brown LE, Webster RG (1982) Biological activity of monoclonal antibodies to operationally defined antigenic regions on the hemagglutinin molecule of A/seal/Massachusetts/1/80 (H7N7) influenza virus. Virology 122: 38–47
11. Kida H, Ito T, Yasuda J, Shimizu Y, Itakura C, Shortridge KF, Kawaoka Y, Webster RG (1994) Potential for transmission of avian influenza viruses to pigs. J Gen Virol 75: 2183–2188
12. Kida H, Yanagawa R, Matsuoka Y (1980) Duck influenza lacking evidence of disease signs and immune response. Infect Immunol 30: 547–553
13. Kishida N, Sakoda Y, Isoda N, Matsuda K, Eto M, Sunaga Y, Umemura T, Kida H (2005) Pathogenicity of H5 influenza viruses for ducks. Arch Virol 150: 1383–1392
14. Klenk HD, Garten W (1994) Host cell ptotease controlling virus pathogenicity. Trends Microbiol 2: 39–43
15. Li KS, Guan Y, Wang J, Smith GJ, Xu KM, Duan L, Rahardjo AP, Puthavathana P, Buranathai C, Nguyen TD, Estoepangestie AT, Chaisingh A, Auewarakul P, Long HT, Hanh NT, Webby RJ, Poon LL, Chen H, Shortridge KF, Yuen KY, Webster RG, Peiris JS (2004) Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature 430: 209–213
16. Liu J, Xiao H, Lei F, Zhu Q, Qin K, Zhang X-w, Zhang X-l, Zhao D, Wang G, Feng Y, Ma J, Liu W, Wang J, Gao GF (2005) Highly pathogenic H5N1 influenza virus infection in migratory birds. Science 309: 1206

17. Makarova NV, Ozaki H, Kida H, Webster RG, Perez DR (2003) Replication and transmission of influenza viruses in Japanese quail. Virology 310: 8–15
18. Mase M, Tsukamono K, Imada T, Imai K, Tanimura N, Nakamura K, Yamamoto Y, Hitomi T, Kira T, Nakai T, Kiso M, Horimoto T, Kawaoka Y, Yamaguchi S (2005) Characterization of H5N1 influenza A virus isolated during the 2003–2004 influenza outbreak in Japan. Virology 332: 167–176
19. Mase M, Eto M, Tanimura N, Imai K, Tsukamoto K, Horimoto T, Kawaoka Y, Yamaguchi S (2005) Isolation of a genotyopically unique H5N1 influenza virus from duck meat imported into Japan from China. Virology 339: 101–109
20. Nestorowicz A, Kawaoka Y, Bean WJ, Webster RG (1987) Molecular analysis of the hemagglutinin genes of Australian H7N7 influenza viruses: role of passerine birds in maintenance or transmission? Virology 160: 411–418
21. Perkins LE, Swayne DE (2002) Pathogenicity of a Hong Kong-origin H5N1 highly pathogenic avian influenza virus for emus, geese, ducks, and pigeons. Avian Dis 46: 53–63
22. Reed LJ, Muench H (1938) A simple method for estimating fifty percent endpoints. Am J Hyg 37: 493–497
23. Scholtissek C, Burger H, Kistner O, Shortridge KF (1985) The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. Virology 147: 187–294
24. Sims LD, Ellis TM, Liu KK, Dyrting K, Wong H, Peiris M, Guan Y, Shortridge KF (2003) Avian influenza in Hong Kong 1997–2002. Avian Dis 47: 832–838
25. Stieneke-Grover A, Vey M, Angliker H, Shaw E, Thomas G, Roberts C, Klenk HD, Garten W (1992) Influenza virus hemagglutinin with multibasic cleavage site is acticated by furin, a subtilisin like endoprotease. EMBO J 11: 2407–2414
26. Suarez DL, Perdue ML, Cox N, Rowe T, Bender C, Huang J, Swayne DE (1998) Comparisons of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Kong. J Virol 72: 6678–6688
27. Tumpey TM, Kapczynski DR, Swayne DE (2004) Comparative susceptibility of chickens and turkeys to avian influenza A H7N2 virus infection and protective efficacy of a commercial avian influenza H7N2 virus vaccine. Avian Dis 48: 167–176
28. Xu X, Subbarao K, Cox NJ, Guo Y (1999) Genetic Characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. Virology 261: 15–19

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