REASSORTMENT AND MODIFICATION OF HEMAGGLUTININ CLEAVAGE MOTIF OF AVIAN/WSN INFLUENZA VIRUSES GENERATED BY REVERSE GENETICS THAT CORRELATE WITH ATTENUATION

J.-H. LU[1,2], J.-X. LONG[1], L.-J. JIA[1], Y.-L. LIU[1], W.-X. SHAO[1], Y.-M. ZHANG[1], X.-F. LIU[1*]

[1]Animal Infectious Disease Laboratory, School of Veterinary Medicine, Yangzhou University, Yangzhou, Jiangsu 225009, Jiangsu Province, P.R. China; [2]Cancer Research Institute, Xiangya School of Medicine, Central South University, Changsha, Hunan Province, P.R. China

Received April 12, 2006; accepted October 23, 2006

Summary. – Avian influenza associated with H9N2 and H5N1 subtypes of avian influenza viruses (AIVs) has raised great concerns in China. To study this problem, reverse genetics has been employed. Three reassortants, rgH9N2, rgH5N1 and rgH5N2, were prepared and compared. Their hemagglutinin (HA) and neuraminidase (NA) genes originated from Chinese AIV isolates of H9N2 or H5N1 subtype, while the rest of their genes were derived from A/WSN/33(H1N1) virus (WSN). In the H5 HA reassortants, the multibasic cleavage site was converted to a monobasic one. The results demonstrated that the reassortants did not produce CPE on MDCK cells in the absence of trypsin, showed egg-adaptation phenotype and stability of HA and NA during consecutive egg passages, and were not lethal to chickens and mice. However, the rgH5N1 reassortant exhibited a "residual" virulence in terms of lethality to chick embryos and pathogenesis in chickens. It can be concluded that (i) the genetic modification of H5 HA attenuated the H5 reassortants, (ii) the presence of internal WSN proteins contributed to the attenuated properties of the reassortants independently on H5 HA, and (iii) also the overall genome composition contributed to virulence differences. This report provides further contribution of reverse genetics to the knowledge of virulence of influenza viruses.

Key words: avian influenza; hemagglutinin; neuraminidase; Influenza A virus; reverse genetics; reassortment; cleavage motif; phenotype; WSN strain

Introduction

All known HN subtypes of Influenza A virus have been isolated from birds (Alexander, 2000; Fouchier *et al*., 2005). Due to segmented genome, its reassortment is a common and constant event occurring between different influenza viruses. In the human disease history, the introduction of novel HA and NA has contributed to the formation of an influenza pandemic strain which used to be of partially avian origin (Kawaoka *et al*., 1989; Webster *et al*., 1992). Avian influenza associated with H9N2 and H5N1 subtypes in Southeast Asia has raised great concerns for its economic importance to poultry industry and potential threat to the public health (Peiris *et al*., 1999; Guo *et al*., 2000; Webster, 2001). Since the Hong Kong bird flu incident in 1997, the ongoing surveillance in southern China region has demonstrated a continuous circulation of H5N1 and H9N2 viruses which possessing part of genes of the viruses which had occurred earlier (Li *et al*., 2003; Chen *et al*., 2004; Lu *et al*., 2005a). Studies mainly on

*Corresponding author. E-mail: xfliu@yzu.edu.cn; fax: +86514-7972591.

Abbreviations: AIV = avian influenza virus; HA = hemagglutinin, hemagglutination; HAI = hemagglutination-inhibition; NA = neuraminidase; p.i. = post infection; PR8 = A/Puerto Rico/8/1934(H1N1); WSN = A/WSN/33(H1N1); wtH9N2 = A/Chicken/Shanghai/F/98 (H9N2); wtH5N1 = A/Goose/Huadong/1/2000 (H5N1)

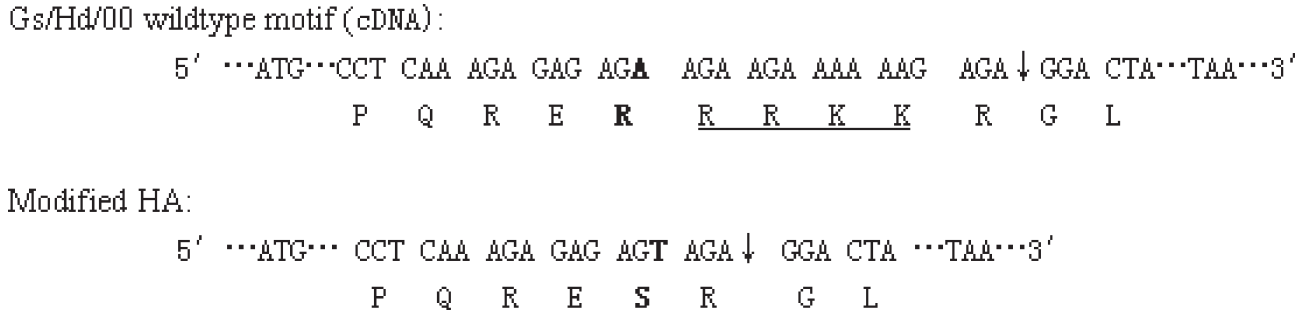

Fig. 1

Modification of the HA cleavage motif of wtH5N1

The deleted basic amino acids are underlined. The arrow indicates the cleavage site. Basic R and its neutral substitute S are in bold.

H9N2 viruses have revealed a two-way transmission between terrestrial and aquatic birds resulting in increased diversity of genotype and chance of species transmission (Li *et al*., 2003; Lu *et al*., 2005a). Moreover, recent isolates of highly pathogenic H5N1 viruses from healthy aquatic birds in China exhibit progressively increasing pathogenicity for mammals, and are characteristic by multiple basic amino acids adjacent to the cleavage site of HA (Chen *et al*., 2004); the latter fact was linked to virulence for chickens and mice (Senne *et al*., 1996; Hatta *et al*., 2001).

In response to emerging influenza threats, technical capability of the virus research appears essential. For a long time the research of influenza viruses has been hampered due to the lack of efficient manipulation system until entirely plasmid-based reverse genetics was developed (Neumann *et al*., 1999; Fodor *et al*., 1999; Hoffmann *et al*., 2000). This approach allowed recombinant influenza virus to be produced upon transfection of eukaryotic cells with a set of transcription/expression plasmids. In the eight-plasmid system, the negative single-stranded virion RNA and the positive single-stranded viral mRNA could be synthesized from the same template, and virus titers up to 2×10^7 PFU/ml could be obtained (Hoffmann *et al*., 2000). Several reports have shown that the plasmid-based systems are useful tools for rapid generation of influenza vaccine candidates on the background of a laboratory virus, A/Puerto Rico/8/1934(H1N1) (PR8) (Hoffmann *et al*., 2002; Liu *et al*., 2003; Subbarao *et al*., 2003; Webby *et al*., 2004).

To study the virulence of AIVs by using reverse genetics, two Chinese isolates of subtypes H9N2 and H5N1, respectively, were used for generation of three reassortants on the background of laboratory WSN strain. In the H5 HA of the reassortants, the multibasic amino acids in the cleavage motif were removed. Characterization of these reassortants demonstrated their phenotype differences in comparison to each other or to parental viruses.

Materials and Methods

Viruses and cells. Two Chinese AIV isolates were employed: A/Chicken/Shanghai/F/98 (H9N2) (wtH9N2), isolated from an outbreak in mainland China (all its gene sequences are available under Acc. Nos. AY253750-AY253756 and AY743216), and A/Goose/Huadong/1/2000 (H5N1) (wtH5N1), isolated from a healthy goose in coastal China (its HA and NA gene sequences are available under Acc. Nos. DQ201829 and DQ201830, respectively). They were isolated and biologically cloned by three rounds of limiting dilution cloned in 10-day-old SPF embryonated chicken eggs, and the obtained allantoic fluids served as stock virus (Guan *et al*., 1999; Chen *et al*., 2004). These isolates contain HA and NA genes that are closely related to those of the prototype Chinese strains A/Chicken/Beijing/1/94(H9N2) (Lu *et al*., 2005a) and A/Goose/Guandong/1/96(H5N1), respectively. MDCK and COS-1 cells were subcultured in DMEM (Sigma) supplemented with 10% of FCS.

Generation of reassortant viruses. Three reassortants and a WSN recombinant (rg WSN) were generated by using the eight-plasmid reverse genetics system (Hoffmann *et al*., 2000, 2001, and 2002). The reassortants rg H5N1 and rg H5N2 were already described (Lu *et al*., 2005b). First, the HA gene from wt H9N2 and the NA genes from wtH5N1 and wtH9N2, respectively, were amplified and cloned into the vector pHW2000 (Hoffmann *et al*., 2000). In cloning the H5 HA gene from wtH5N1, the multibasic amino acid cleavage motif PQRERRRKKR/GL was converted to PQRESR/GL (Fig. 1), which is similar to an H2 cleavage motif. To ensure that the cloned genes were free of unexpected mutations, they were sequenced. Eight plasmids carrying individual viral gene segments were then mixed according to the designed genotype and transfected into COS-1 cells with aid of Lipofectin (Invitrogen). At 72 hrs

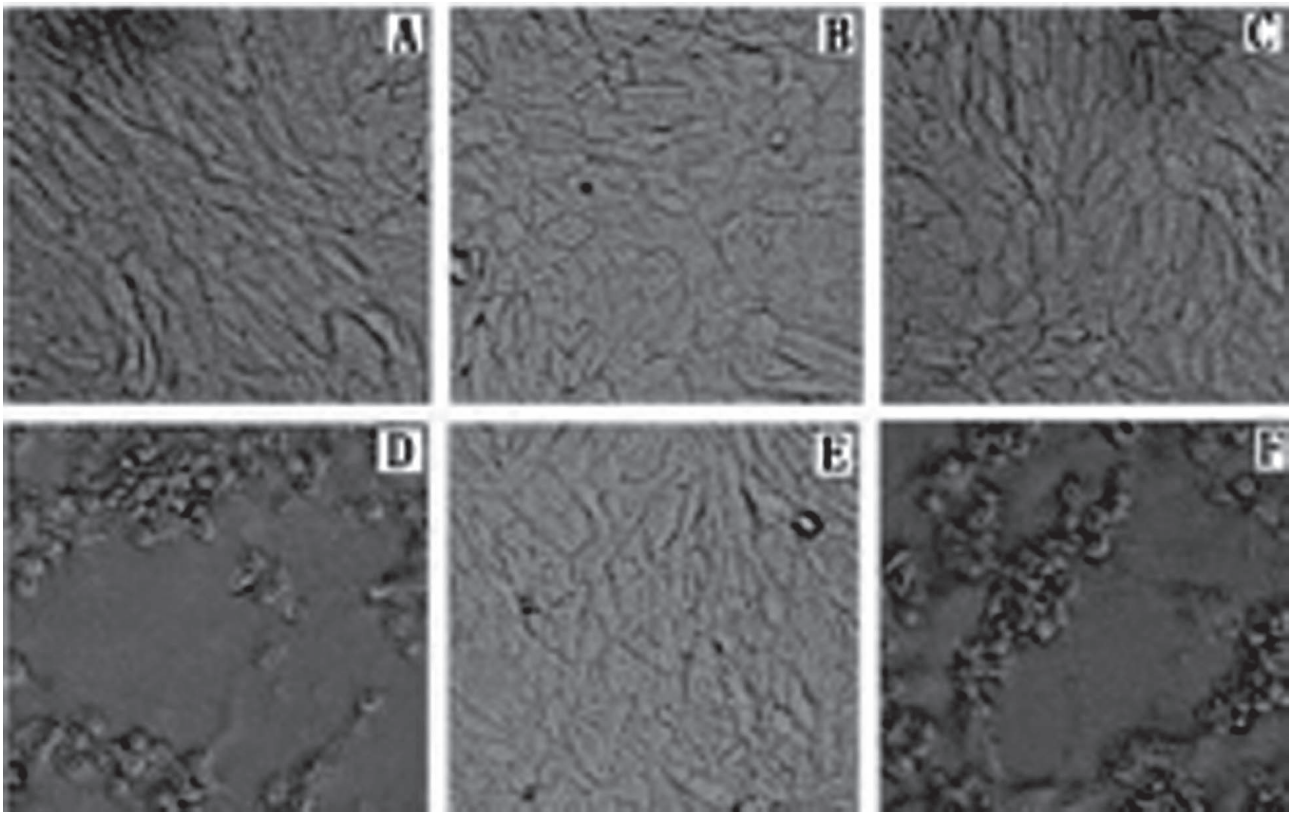

Fig. 2

Morphology of MDCK cells infected with the viruses

The cells were infected with the viruses diluted 10^{-3} in the absence of trypsin. Morphology was observed at 72 hrs p.i. rgH5N1 (A), rgH5N2 (B), rgH9N2 (C), wtH5N1 (D), wtH9N2 (E), and rgWSN (F).

post transfection, a 100 µl aliquot of supernatant was injected into 10-day-old SPF embryonated eggs. Each reassortant was identified in the allantoic fluid by HA and hemagglutination-inhibition (HAI) assays with wtH9N2- or wtH5N1-specific antisera prepared in our laboratory. The antiserum specific to H1 was obtained from Animal Influenza Laboratory of China Ministry of Agriculture. To confirm that each reassortant originated from the plasmids used in the system, all the eight viral genes were amplified after two additional egg passages and were entirely (NA and NS genes) or partially (the remaining six genes) sequenced. The reassortants were designated rg H5N1, rg H5N2, and rg H5N1, rgWSN, a recombinant made by the same approach, contained all the genes of parental WSN.

Cell infection. Dilutions of 10^{-2} to 10^{-5} of the three reassortants (rgH5N1, rgH5N2, and rgH5N1), the recombinant (rgWSN), and two wt virus isolates (wtH9N2 and wt H5N1) were used to infect MDCK monolayers in the absence of trypsin. These viruses showed HA titers of 64 (rgH5N1, rgH5N2, rgWSN, and wtH5N1) and 1024 (rgH9N2 and wtH9N2). CPE was monitored within 96 hrs p.i.

Passaging of viruses in embryonated eggs. Each virus was injected into four SPF embryonated chicken eggs for 14 passages. After 72 hrs of incubation at 35°C or earlier in case of death of embryos, the HA titer of the allantoic fluid of each egg was assessed using 0.8% chicken erythrocytes. The material with the highest titer was used for the next passage. To test the stability of the viruses during passaging, their HA and NA genes were PCR-amplified and sequenced after 14 passages. During the passaging, total egg mortality for each virus was assessed.

Virulence of viruses for animals. The virulence for chickens was assessed by the standard test of intravenous pathogenicity index (IVPI) according to the recommendations of OIE (Alexander and Spackman, 1981; OIE, 2000). Only fresh and bacteria-free allantoic fluids with HA titers ≥16 were tested. Five groups of 10 six-week-old SPF White Leghorn chickens were inoculated intravenously with 0.2 ml of 10^{-1} dilution of allantoic fluid.

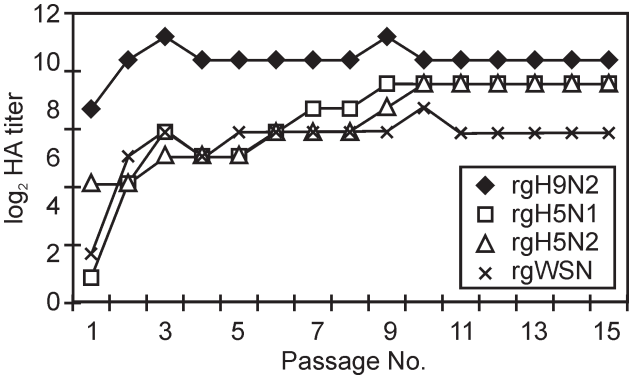

Fig. 3

HA titers of the viruses passaged in embryonated eggs

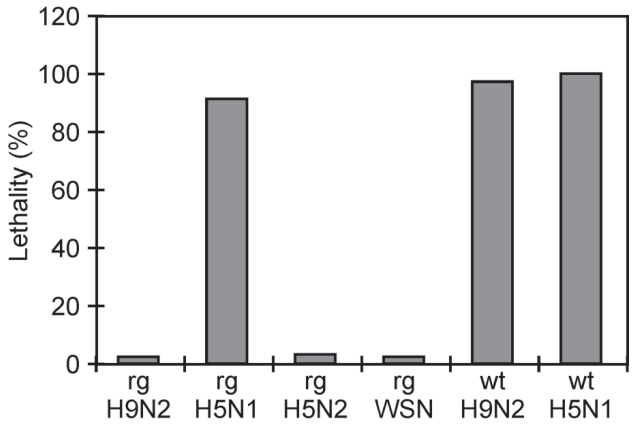

Fig. 4

Lethality of the viruses for chicken embryos

The chickens were daily monitored for disease signs and death for 10 days p.i. The IVPI (from 0 to 3) characterized both illness and survival time. IVPI >1.2 indicated a highly pathogenic virus. The virulence for mice was assessed in 8 six-week-old female BALB/c mice. The mice were inoculated intranasally with 10^5 EID_{50} of virus and were daily monitored for disease signs and weight loss for 15 days p.i. Control mice were injected with saline.

Results and Discussion

Growth of the viruses in MDCK cell cultures

To determine growth differences between the three reassortants, the backbone virus (the recombinant rgWSN), and the parental AIVs, 10^{-2}–10^{-5} dilutions of each virus were inoculated into MDCK cell cultures in the absence of trypsin and CPE was read at 72 hrs p.i. (Fig. 2). The results showed that none of the reassortants or wtH9N2 produced CPE. Only wtH5N1 and rgWSN were cytopathogenic. Such a behavior of rgWSN was expected as the genome composition of this recombinant corresponded to that of original, parental WSN; the latter is a human virus strain that can replicate in a variety of cultured cells with CPE in the absence of trypsin (Castrucci and Kawaoka, 1993; Goto and Kawaoka, 1998). The mechanism put forward to explain the growth of this virus in cell culture involves the binding of a host serum serine protease by N1 NA that allows cleavage activation of H1 HA (Goto and Kawaoka, 1998). In addition, most influenza viruses lacking the polybasic cleavage motif are unable to produce CPE on MDCK cells in the absence of typsin (Subbarao *et al*., 2003; Webby *et al*., 2004). Hence, the reassortants which possessed an NA entirely different from that of WSN and the H5 HA cleavage motif converted to a monobasic one, showed different growth properties.

Growth and stability of the viruses in embryonated eggs

HA titers of the viruses were followed during 15 passages in embryonated eggs (Fig. 3). After adaptation lasting several passages, all three reassortants as well as the recombinant rgWSN grew to a relatively stable titer. This titer was equal to that of the wtAIV containing the respective HA gene (data not shown). The egg-adaptation of the H5 reassortants was hampered in the first 8–9 passages probably by modification of the HA gene (Hoffmann *et al*., 2000). We have experience with a number of reassortants inclusive of rgH9N2 (Fig. 3) and an H5N1/WSN reassortant with unmodified HA and NA genes from wtH5N1 (data not shown), which shows that all of them have adapted smoothly to embryonated eggs during first two passages (data not shown). After the passaging, the HA proteins of the reassortants were antigenically indistinguishable from parental ones (H9, H5 or H1) in HAI assay, showing a titer of 256. It seems that the yield of reassortants in embryonated eggs is determined by HA as well as by internal proteins. That is why PR8, a virus strain with a phenotype of high-growth and egg-adaptation, is conventionally used as a backbone for generation of vaccine seed viruses (Hoffmann *et al*., 2002).

During the passaging, we observed among the tested viruses differences in lethality to embryonated eggs (Fig. 4). The reassortants rgH9N2 and rgH5N2 practically did not kill embryos while rgH5N1 did most of them within 72 hrs p.i. Since the H5 HA cleavage site was converted to a low-pathogenic one, the H5 reasortants were expected to lose fully their virulence for embryonated eggs, chickens and mice (Subbarao *et al*., 2003; Webby *et al*., 2004). Therefore we regard the virulence of rgH5N1 as “residual”. We think that

Table 1. Virulence of the viruses for chickens

Viruses	IVPI	No. of dead/ inoculated	Disease signs
rgH9N2	0	0/10	None
rgH5N1	0.12	0/10	One chicken showed paralysis of one leg on day 5
rgH5N2	0	0/10	None
wtH9N2	0.14	2/10	Two chickens died on days 8 and 10, respectively, others did not show any signs
wtH5N1	2.69	10/10	All chickens died within 4 days

Each virus was inoculated into 8 chickens. The birds were observed for 10 days.

the multibasic amino acid cleavage motif is not the only determinant of the virulence of H5 subtype viruses for embryonated eggs. The comparison of rgH9N2 (the reassortant with WSN backbone) and wtH9N2 (the virus with AIV backbone) showed that it was the internal virion proteins of wtH9N2, which were essential for the virulence for embryonated eggs (Fig. 4) as well as for chickens (Table 1).

To confirm that the tested viruses remained stable during passaging in embryonated eggs, we sequenced their HA and NA genes in consecutive passages and found no changes (data not shown).

Virulence of the viruses for animals

The viruses under study were tested for their virulence for chickens (lethality and disease signs, Table 1) and mice (lethality, disease signs and weight loss, Table 2). All the reassortants were not lethal to chickens; according to IVPI of 0–1 they were classified as non-pathogenic. Regarding the disease signs in chickens and weight loss in mice, rgH5N1 again showed a "residual" virulence. Also rgH5N2 caused a weight loss in mice. It seems that the genetic alteration of H5 HA cleavage motif could attenuate the H5 reassortants but not fully abolish their virulence inherited from parental H5 virus. The result also showed that wtH5N1 was found to be highly pathogenic to chickens but only lowly pathogenic to mice. Therefore it could be categorized within the group of viruses isolated from healthy aquatic birds in mainland China before 2001 (Chen *et al*., 2004). Further, rgH9N2 did not exhibit the low pathogenicity of wtH9N2 to chickens apparently due to the change in backbone genes.

Table 2. Virulence of the viruses for mice

Viruses	No. of dead/ inoculated	Disease signs	Mean weight loss/gain (–/+)
rgH9N2	0/8	None	–0.01%
rgH5N1	0/8	None	–9.30%
rgH5N2	0/8	None	–9.32%
wtH9N2	0/8	None	–0.02%
wtH5N1	1/8	Two mice showed signs; one died on day 8, the other survived with 30% weight losson day 15	–16.80%
Control (saline)	0/8	None	+0.35%

Each virus was inoculated into 8 mice. The animals were observed for 15 days.

In line with the embryo-lethality, rg H5N1 showed "residual" virulence for chickens and mice. This was not consistent with the results of previous studies in which H5N1/PR8 reassortants were considered preferable vaccine candidates (Subbarao *et al*., 2003; Webby *et al*., 2004); this inconsistency could be caused by entirely different backbone genes and slightly different HA and NA genes. Thereby, this added to the knowledge that gene constellation is an important factor of the virulence of avian HN/WSN reassortants. As suggested by others, balanced HA and NA activities are critical for efficient replication of influenza viruses (Kaverin *et al*., 1998; Mitnaul *et al*., 2000; Wagner *et al*., 2000). Extensive studies of avian-human reassortants in mammalian hosts have demonstrated the attenuation of phenotype as a multi-gene trait and neither HA nor NA as exclusive determinant of virulence (Mayer *et al*., 1973; Steinhauer, 1999). In contrast, an H5N1/WSN ressortant with unmodified H5 HA showed similar growth properties to wtH5N1 in MDCK cells and eggs but a much lower pathogenicity to chickens (IVPI of 0.4) than wtH5N1 (data not shown). As mentioned above, rgH9N2 in contrast to wtH9N2 did not exhibit any pathogenicity to chickens. Taken together, it can be seen that the WSN backbone genes were able to attenuate avian HN/WSN reassortants to some extent.

It is particularly of interest that rgH5N2 showed a lower pathogenicity to embryonated eggs and chickens compared to rgH5N1. It appears likely that the N1 NA gene contributed more to the virulence than the N2 NA gene did within a specific gene constellation. In order to obtain further evidence and understand how the N1 NA contributes to the virulence of H5 influenza viruses, further experiments have been started. Their preliminary results so far showed that N1 NA, particularly the length of its stalk, and the NS protein could cause marked changes in virulence.

In conclusion, we have shown some detailed biological properties of influenza viruses generated by reverse genetics, and provided additional information to that so far published on PR8-based reassortants. In general, both the modification of multibasic H5 HA cleavage site to monobasic one and the WSN backbone genes/proteins contributed to the attenuation of the H5/WSN reassortants.

Our data support the concept that the virulence is a multifactorial property dependent on the multibasic HA

cleavage motif, surface glycoproteins, internal proteins, genome composition, and host. Although we do not intend to use the reassortants made in this study for vaccine purposes, the generation of egg-adaptable, antigen-stable and attenuated viruses represents, in general, a strategy for development of a vaccine to potential pandemic of avian influenza. Besides, the reverse genetics technique has become a useful tool to facilitate the understanding of influenza virus virulence.

Acknowledgements. This study was supported by the grant No. 2001-83 from the China Ministry of Agriculture. Thanks are due to Drs R. Webster and E. Hoffmann, St. Jude Children's Research Hospital, Memphis, USA, for kind providing of the eight-plasmid system, and Mr H.M. Liu for technical assistance.

References

Alexander DJ, Spackman D (1981): Characterisation of influenza A viruses isolated from turkeys in England during March-May 1979. *Avian Pathol.* **10**, 281–293.

Alexander DJ (2000): A review of avian influenza in different bird species. *Vet. Microbiol.* **74**, 3–13.

Castrucci MR, Kawaoka Y (1993): Biologic importance of neuraminidase stalk length in influenza A virus. *J. Virol.* **67**, 759–764.

Chen H, Deng G, Li Z, Tian G, Li Y, Jiao P, Zhang L, Liu Z, Webster RG, Yu K (2004): The evolution of H5N1 influenza viruses in ducks in southern China. *Proc. Natl. Acad. Sci. USA* **101**, 10452–10457.

Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG, Garcia-Sastre A (1999): Rescue of influenza A virus from recombinant DNA. *J. Virol.* **73**, 9679–9682.

Fouchier RAM, Munster V, Wallensten A, Bestebroer T M, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus ADME (2005): Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J. Virol.* **79**, 2814–2822.

Goto H, Kawaoka Y (1998): A novel mechanism for the acquisition of virulence by a human influenza A virus. *Proc. Natl. Acad. Sci. USA* **95**, 10224–10228.

Guan Y, Shortridge KF, Krauss S, Webster RG (1999): Molecular characterization of H9N2 influenza viruses: Were they the donors of the "internal" genes of H5N1 viruses in Hong Kong. *Proc. Natl. Acad. Sci. USA* **96**, 9363–9367.

Guo YJ, Krauss S, Senne DA, Mo IP, Lo KS, Xiong XP, Norwood M, Shortridge KF, Webster RG, Guan Y (2000): Characterization of the pathogenicity of members of the newly established H9N2 influenza virus lineage in Asia. *Virology* **267**, 279–288.

Hatta M, Gao P, Halfmann P, Kawaoka Y (2001): Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. *Science* **293**, 1840–1842.

Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG (2000): A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc. Natl. Acad. Sci. USA* **97**, 6108–6113.

Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR (2001): Universal primer set for the full-length amplification of all influenza A viruses. *Arch. Virol.* **146**, 2275–2289.

Hoffmann E, Krauss S, Perez D, Webby R, Webster RG (2002): Eight-plasmid system for rapid generation of influenza virus vaccines. *Vaccine* **20**, 3165–3170.

Kaverin NV, Gambaryan AS, Bovin NV, Rudneva IA, Shilov AA, Khodova OM, Varich NL, Sinitsin BV, Makarova NV, Kropotkina EA (1998): Postreassortment changes in influenza A virus hemagglutinin restoring HA-NA functional match. *Virology* **244**, 315–321.

Kawaoka Y, Krauss S, Webster RG (1989): Avian-to-human transmission of the PB1 gene of influenza A virus in the 1957 and 1968 pandemics. *J. Virol.* **63**, 4603–4608.

Li KS, Xu KM, Peiris JSM, Poon LLM, Yu KZ, Yuen KY, Shortridge KF, Webster RG, Guan Y (2003): Characterization of H9 subtype influenza viruses from the ducks of southern China: a candidate for the next influenza pandemic in humans. *J. Virol.* **77**, 6988–6994.

Liu M, Wood JM, Ellis T, Krauss S, Seiler P, Johnson C, Hoffmann E, Humberd J, Hulse D, Zhang Y, Webster RG, Perez DR (2003): Preparation of a standardized, efficacious agricultural H5N3 vaccine by reverse genetics. *Virology* **314**, 580–590.

Lu JH, Liu XF, Shao WX, Liu YL, Wei DP, Liu HQ (2005a): Phylogenetic analysis of eight genes of H9N2 subtype influenza virus: a mainland China strain possessing early isolates' genes that have been circulating. *Virus Genes* **31**, 163–169.

Lu JH, Long JX, Shao WX, Wei DP, Liu XF (2005b): Generation of attenuated H5N1 and H5N2 subtype of influenza virus recombinants by reverse genetics. *Wei Sheng Wu Xue Bao* **45**, 53–57.

Mayer V, Schulman JL, Kilbourne ED (1973): Nonlinkage of neurovirulence exclusively to viral hemagglutinin or neuraminidase in genetic recombinants of A/NWS (H0N1) influenza virus. *J. Virol.* **11**, 272–278.

Mitnaul LJ, Matrosovich MN, Castrucci MR, Tuzikov AB, Bovin NV, Kobasa D, Kawaoka Y (2000): Balanced hemagglutinin and neuraminidase activities are critical for efficient replication of influenza A virus. *J. Virol.* **74**, 6015–6020.

Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, Hughes M, Perez DR, Donis R, Hoffmann E, Hobom G, Kawaoka Y (1999): Generation of influenza A viruses entirely from cloned cDNAs. *Proc. Natl. Acad. Sci. USA* **96**, 9345–9350.

Office International des Epizooties (2000): Highly Pathogenic Avian Influenza. In *Manual of Standards: Diagnostic tests and Vaccines*. Paris, Office International des Epizooties, pp. 212–220.

Peiris M, Yuen KY, Leung CW, Chan KH, Ip PL, Lai RW, Orr WK, Shortridge KF (1999): Human infection with influenza H9N2. *Lancet* **354**, 916–917.

Senne DA, Panigrahy B, Kawaoka Y, Pearson JE, Suss J, Lipkind M, Kida H, Webster RG (1996): Survey of the hemagglutinin (HA) cleavage site sequence of H5 and

H7 avian influenza viruses: Amino acid sequence at the HA cleavage site as a marker of pathogenicity potential. *Avian Dis.* **40**, 425–437.

Steinhauer DA (1999) Role of hemagglutinin cleavage for the pathogenicity of influenza virus. *Virology* **258**, 1–20.

Subbarao K, Chen H, Swayne D, Mingay L, Fodor E, Brownlee G, Xu X, Lu X, Katz, J, Cox N, Matsuoka Y (2003): Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics. *Virology* **305**, 192–200.

Wagner R, Wolff T, Herwig A, Pleschka S, Klenk HD (2000): Interdependence of hemagglutinin glycosylation and neuraminidase as regulators of influenzavirus growth: A study by reverse genetics. *J. Virol.* **74**, 6316–6323.

Webby RJ, Perez DR, Coleman JS, Guan Y, Knight J, Govorkova EA, McClain-Moss LR, Peiris JS, Reng JE, Tuomanen EI, Webster RG (2004): Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines. *Lancet* **363**, 1099–1103.

Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y (1992): Evolution and ecology of influenza A viruses. *Microbiol. Rev.* **56**, 152–179.

Webster RG (2001): A molecular whodunit. *Science* **293**, 1773–1775.