Arch Virol (2004) 149: 2095–2104
DOI 10.1007/s00705-004-0372-1

Archives of Virology
Printed in Austria

Co-infection of *Staphylococcus aureus* or *Haemophilus paragallinarum* exacerbates H9N2 influenza A virus infection in chickens

N. Kishida[1], **Y. Sakoda**[1], **M. Eto**[2], **Y. Sunaga**[2], and **H. Kida**[1]

[1]Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan
[2]Animal Quarantine Service, Ministry of Agriculture, Forestry and Fisheries, Isogo, Yokohama, Japan

Received March 1, 2004; accepted May 24, 2004
Published online July 15, 2004

Summary. H9N2 influenza viruses are frequently isolated from chicken meat and bone marrow imported from China to Japan since 2001. These isolates were experimentally inoculated into specific pathogen-free chickens intranasally. Viruses were recovered from the meat and bone marrow of birds showing no overt signs. On the other hand, chickens co-infected with H9N2 virus and either *Staphylococcus aureus* or *Haemophilus paragallinarum* showed clinical signs severer than those shown by birds infected only with the virus alone or each of the bacteria alone. In addition, H9N2 viruses were more efficiently recovered from the chickens co-infected with *S. aureus* or *H. paragallinarum* than those from the birds infected with only the virus. The present results indicate that co-infection of H9N2 influenza virus with *S. aureus* or *H. paragallinarum* enhances the replication of the virus in chickens, resulting in exacerbation of the H9N2 virus infection.

Introduction

Avian influenza virus is widely distributed in poultry as well as in wild waterfowl, gulls, and shorebirds. The chicken is not a natural host for this virus [6]. The pathogenicity of influenza viruses for chickens may range from asymptomatic to respiratory disease with low mortality to highly pathogenic with high mortality [7]. The influenza virus has two major surface glycoproteins – hemagglutinin (HA) and neuraminidase (NA), and 15 HA and 9 NA subtypes are recognized at present [10, 18, 23]. The HA plays a central role in the pathogenicity of avian influenza viruses [4, 11]. The HAs of virulent viruses differ from those of avirulent influenza

A viruses by virtue of possessing multiple basic amino acids at the carboxyl terminus of the HA1 subunit. This structural feature permits cellular proteases, such as ubiquitous furin and PC6, which recognize multiple basic amino acids, to cleave the HA, rendering the virus infectious, to spread to a variety of organs, leading to systemic infection. In contrast, the HAs of avirulent viruses do not possess multiple basic amino acid residues at the cleavage site and are cleaved only by trypsin-like proteases which are secreted from cells in the respiratory and intestinal tracts, resulting in producing only a limited local infection in host animals that do not show overt symptoms [11].

Since 1994, the H9N2 influenza A virus has caused outbreaks of disease in poultry with high morbidity in Korea and China [1, 9, 12]. These viruses are avirulent and none of them have multiple basic amino acids at the cleavage site of the HA that confer high pathogenicity to H5 and H7 avian influenza viruses [9, 13]. When specific pathogen-free (SPF) chickens were experimentally infected with H9N2 influenza virus isolates from the diseased chickens, none of the birds showed any disease signs [14]. Since 2001, H9N2 viruses have been frequently isolated from chicken meat and bone marrow imported from China at Yokohama Animal Quarantine Station in Japan. Since it is presumed that only virulent viruses cause systemic infection in chickens, it is not understood why H9N2 viruses are isolated from chicken carcasses from China.

To assess the pathogenicity of H9N2 viruses, SPF chickens were experimentally infected with the isolates from the chicken meat and bone marrow imported from China and examined for their tissue tropism. In addition, to obtain information on the H9N2 virus infection of chickens in a natural setting, we investigated the replication of H9N2 influenza viruses in chickens co-infected with either *S. aureus* or *H. paragallinarum*, which prevail in field chickens [2, 3, 19].

Materials and methods

Viruses

The H9N2 and H5N3 influenza A viruses used in this study were propagated in 10-day-old chicken embryos for 48 h at 35 °C. A/chicken/aq-Y-55/01 (H9N2) and A/chicken/aq-Y-135/01 (H9N2) were isolated from the chicken meat and bone marrow imported from China to Japan. A/chicken/Beijing/2/97 (H9N2) was isolated from a chicken showing clinical signs in China [13]. A/duck/Hokkaido/9/99 (H9N2) was isolated from a fecal sample of migratory ducks in our laboratory. A/tern/South Africa/61 (H5N3) was used as a reference virulent strain that causes systemic infection in chickens.

Bacteria

Staphylococcus aureus strain *Hyogo and Haemophilus paragallinarum* strain HK-1 were purchased from the Japanese Association of Veterinary Biologics (Tokyo, Japan). *S. aureus* was incubated on a mannitol salt agar plate (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) for 24 hr at 37 °C. Agar medium for *H. paragallinarum* was prepared according to Rimler [17]. The plates were incubated in the presence of 5% CO_2 for 48 hr at 37 °C.

Experimental infection of chickens with influenza viruses and bacteria

Four-week-old SPF white leghorn chickens were hatched and raised exclusively in our laboratory. Each of the three H9N2 influenza virus isolates from chicken was inoculated into four chickens. A/duck/Hokkaido/9/99 (H9N2) virus isolated from a migratory duck and A/tern/South Africa/61 (H5N3) virus were inoculated into two chickens each. These chickens were infected intranasally with 0.1 ml of allantoic fluid containing $10^{7.0}$ 50% egg infectious dose (EID_{50}) of viruses. The trachea, lung, colon, kidney, spleen, liver, bone marrow, muscle, and blood of the chickens were collected after 3, 7 and 14 post-infection (p.i.) days. The infectivity of the virus in these samples was titrated in embryonated chicken eggs by the method of Reed and Muench [16].

In experimental studies of co-infection with *S. aureus*, four-week-old SPF white leghorn chickens were infected intranasally with a 10 μl suspension containing 2.2×10^5 c.f.u. of *S. aureus* at 3 days prior to virus inoculation. In the studies of experimental co-infection with *H. paragallinarum*, four-week-old SPF white leghorn chickens were infected intranasally with a 10 μl suspension containing $3.0 \times 10^{3.0}$ c.f.u. of *H. paragallinarum* and H9N2 virus concurrently.

Experimental infection studies were performed under BL3+ biosafety conditions.

Results

Tissue tropism of the H9N2 influenza virus isolates from chicken meat and bone marrow in SPF chickens

H9N2 viruses isolated from chicken meat and bone marrow imported from China were experimentally inoculated into SPF chickens. None of the birds infected with any of the H9N2 virus strains showed any overt signs. Even the birds inoculated only with A/chicken/Beijing/2/97 (H9N2) that was isolated from diseased chicken in China did not show clinical signs. From the bone marrow and muscle of the birds, H9N2 viruses of limited infectivity titers were recovered (Table 1). Viruses were efficiently recovered from the respiratory organs of the birds, indicating that the H9N2 viruses replicated extensively in the respiratory tissues. By contrast, no virus recovery was made from any tissues, even from the respiratory tracts of the chickens inoculated with A/duck/Hokkaido/9/99 (H9N2). From the birds inoculated with the H9N2 isolates from chicken meat and/or bone marrow, the virus was recovered after 3 p.i. days, and not after 7 or 14 p.i. days. On the other hand, two chickens inoculated with the virulent control strain, A/tern/South Africa/61 (H5N3), died within 1 day and viruses were recovered from each of the tissues examined at high titers. The results, thus, demonstrate that H9N2 influenza virus isolates from chickens reach the muscle and bone marrow, although the titers were low.

Exacerbation of H9N2 virus infection by co-infection with S. aureus in chickens

For better understanding of the pathogenicity of the H9N2 influenza virus in the field, we compared the virus titers in the chickens experimentally infected with the H9N2 virus isolates from chicken meat and bone marrow to those in the chicken co-infected with *S. aureus* and the H9N2 viruses. All of the chickens co-infected with

Table 1. Virus recovery from chickens experimentally inoculated with H9N2 and H5N3 viruses

Virus	Virus titer ($\log_{10}$ EID_{50}/g)*,[a]									Clinical signs[c]
	Trachea	Lung	Liver	Kidney	Spleen	Colon	Bone marrow	Muscle	Blood	
Ck/Y-55/2001 (H9N2)	+++	+++	−	−	+	−	+	−	−	−
	+	++	−	+	+	−	+	−	−	−
	++	+++	NT[b]	NT	NT	−	−	+	−	−
	++	+++	NT	NT	NT	−	−	+	−	−
Ck/Y-135/2001 (H9N2)	++	−	NT	NT	NT	−	−	+	−	−
	++	−	NT	NT	NT	−	−	−	−	−
	+++	++	−	−	−	−	+	−	−	−
	−	−	−	−	−	+	−	−	−	−
Ck/Bj/2/97 (H9N2)	+++	++	−	−	+	++	+	−	−	−
	−	−	−	−	+	−	+	−	−	−
	−	−	+	−	−	−	+	−	−	−
	++	−	−	−	−	−	−	−	−	−
Dk/Hok/9/99 (H9N2)	−	−	NT	NT	NT	−	−	−	−	−
	−	−	NT	NT	NT	−	−	−	−	−
Tn/SA/1/61 (H5N3)	+++	+++	+++	NT	NT	+++	+++	+++	+++	†
	+++	+++	+++	NT	NT	+++	+++	++	+++	†

*Organs were collected at 3 days postinoculation from chickens infected with each virus for virus titeration. Organs were collected at 1 day postinoculation from chickens infected with H5N3 virus as the chickens died at 1 day postinoculation with the virus

[a] −: <1.6, +: 1.6–2, ++: 2–4, +++: >4 $\log_{10}$ EID_{50}/g tissue

[b] NT: not tested

[c] −: No clinical signs, †: Dead

Table 2. Virus recovery from chickens experimentally inoculated with *S. aureus* and H9N2 virus

Inoculation samples	Virus titer ($\log_{10}$ EID_{50}/g)*,[a]									Clinical signs[b]
	Trachea	Lung	Liver	Kidney	Spleen	Colon	Bone marrow	Muscle	Blood	
Ck/Y-55/2001 (H9N2)	++	−	−	−	−	−	−	−	−	−
	+++	+++	−	−	+	−	+	−	−	−
	−	−	−	−	−	−	−	−	−	−
	+	++	−	+	+	−	+	−	−	−
Ck/Bj/2/97 (H9N2)	+++	++	−	−	+	++	+	−	−	−
	−	−	−	−	+	−	+	−	−	−
	−	−	+	−	−	−	+	−	−	−
	++	−	−	−	−	−	−	−	−	−
S. aureus and Ck/Y-55/2001 (H9N2)	++	+	+	−	+	−	−	−	+	+
	+	+++	+	++	+	+	+	−	+	+
	++	−	−	−	−	−	−	−	+	+
	+++	+	−	+	+	−	+	−	+	+
S. aureus and Ck/Bj/2/97 (H9N2)	+++	+	−	−	−	−	+	−	−	+
	+++	+++	+	+	−	+	++	−	+	+
	++	−	−	−	−	−	−	−	+	+
	+++	+++	−	−	+	−	+	−	−	+

*Organs were collected at 3 days postinoculation from chickens infected with each virus for virus titeration

[a] −: <1.6, +: 1.6–2, ++: 2–4, +++: >4 $\log_{10}$ EID_{50}/g tissue

[b] −: No clinical signs, +: All of the chickens infected with *S. aureus* and H9N2 virus showed severer clinical signs of swelling of the face, depression, ruffled feathers than the chickens infected only with *S. aureus*

Table 3. Experimental co-infection of chickens with *H. paragallinarum*

Inoculation samples	Virus titer ($\log_{10}$ EID_{50}/g)*,[a]									Clinical signs[b]
	Trachea	Lung	Liver	Kidney	Spleen	Colon	Bone marrow	Muscle	Blood	
Ck/Y-55/2001 (H9N2)	++	+++	+	+	+	++	−	−	+	−
	−	−	−	−	+	−	−	−	−	−
	++	−	−	−	−	−	−	−	−	−
	+++	+++	−	−	+	−	+	−	−	−
H. paragallinarum and Ck/Y-55/2001 (H9N2)	+++	++	+	++	+	+	−	+	−	+
	+++	+++	+	++	+	−	+	−	−	+
	+++	+++	+	+	−	−	−	−	−	+
	+++	+++	++	−	−	−	+	−	−	+

*Organs were collected at 3 days postinoculation from chickens infected with each virus for virus titeration

[a] −: <1.6, +: 1.6–2, ++: 2–4, +++: >4 $\log_{10}$ EID_{50}/g tissue

[b] −: No clinical signs, +: Clinical signs were swelling of the face, depression, ruffled feathers, and dyspnea. None of the chickens inoculated with only *H. paragallinarum* showed clinical signs

S. aureus and Ck/Y-55/2001 (H9N2) virus or Ck/Bj/2/97 (H9N2) virus showed severer clinical signs such as swelling of the face, depression, paralysis, and ruffled feathers, compared to those infected only with *S. aureus*, but none of them died. On the other hand, none of the chickens infected only with Ck/Y-55/2001 (H9N2) virus or Ck/Bj/2/97 (H9N2) virus showed clinical signs. The present findings indicate that pathogenicity of H9N2 virus is affected by the co-infection with these bacteria. H9N2 viruses were recovered from the blood of all of the chickens co-infected with *S. aureus* and Ck/Y-55/2001 (H9N2) virus and the blood of two of the four chickens co-infected with *S. aureus* and Ck/Bj/2/97 (H9N2) virus while viruses were not recovered from the blood of the birds infected only with the virus. In the birds infected only with Ck/Y-55/2001 (H9N2) virus, virus recovery was made from the respiratory organs of three of the four birds. In the birds infected only with Ck/Bj/2/97 (H9N2) virus, the virus recovery was made from the respiratory organs of two of the four birds. On the contrary, from the birds co-infected with *S. aureus* and Ck/Y-55/2001 (H9N2) virus or Ck/Bj/2/97 (H9N2) virus, virus recovery was made from the respiratory organs of all of the chickens (Table 2). The present results indicate that co-infection with *S. aureus* exacerbates H9N2 virus infection in chickens.

Enhancement of replication of H9N2 influenza virus by co-infection with H. paragallinarum in chickens

We next investigated the effect of co-infection with *H. paragallinarum*, which is known to cause respiratory disease in chickens, on the outcome of H9N2 infection. All of the chickens co-infected with *H. paragallinarum* and H9N2 virus showed clinical signs of swelling of the face, depression, ruffled feathers, and dyspnea. None of the chickens inoculated only with H9N2 virus or *H. paragallinarum* showed clinical signs. H9N2 viruses were more efficiently recovered from the tissues of the chickens co-infected with *H. paragallinarum* than from those of the birds infected only with the virus. The virus infectivity titers in the trachea and lungs of the chickens inoculated with *H. paragallinarum* and H9N2 virus were significantly ($P < 0.05$) higher than those of the birds inoculated only with H9N2 virus (Table 3). The present results indicate that the replication of H9N2 virus was enhanced by co-infection with *H. paragallinarum* in chickens.

Discussion

H9N2 viruses were isolated from the meat and bone marrow of chickens imported from China. In the present study, SPF chickens were experimentally infected with these viruses to examine whether the viruses reach the muscle and bone marrow of chickens. It was confirmed that the H9N2 influenza virus was recovered from the muscle and/or bone marrow of the SPF chickens intranasally infected. In addition, the viruses were also recovered from the kidney, spleen, and liver (Table 1), indicating that H9N2 virus isolates from chickens disseminate throughout the body of the chicken. Extensive virus replication, however, was not observed in the bone marrow or muscle.

An outbreak of H9N2 influenza virus infection in chickens in Hong Kong (A/chicken/Hong Kong/739/94) was associated with coughing and respiratory distress in 75% of the birds, with 10% mortality. Treatment with antibiotics reduced the mortality rate, suggesting that bacteria may play some role in the exhibition of the clinical syndrome [8]. To obtain information on the exacerbation of H9N2 virus infection by co-infection with bacteria, we examined the infectivity of the H9N2 virus in chickens co-infected with either *S. aureus* or *H. paragallinarum*, which prevail in field chickens. It was revealed that co-infection with *S. aureus* or *H. paragallinarum* exacerbated H9N2 virus infection in chickens (Tables 2, 3). Although Ck/Bj/2/97 (H9N2) isolated from diseased chicken in China did not cause clinical signs in the SPF chickens, this virus caused disease signs in the chickens co-infected with *S. aureus*. The present findings indicate that pathogenicity of H9N2 virus is affected by the co-infection with these bacteria. Similar findings were obtained in the case of Ck/Y-55/2001 (H9N2) virus infection (Tables 2, 3). It is not known how these bacteria enhance the replication of H9N2 virus in chickens. It was demonstrated that the protease of *S. aureus* activated the HA of the influenza virus, allowing multiple cycles of virus replication in the lungs of mice [20]. Co-infection with *S. aureus* may confer a similar effect on H9N2 virus infection in chickens. An alternative explanation for the exacerbation of the pathogenicity of H9N2 influenza virus infection is that the stress of bacterial infection affects the immune system of chickens. H9N2 viruses were recovered from the blood of all of the chickens co-infected with *S. aureus* and Ck/Y-55/2001 (H9N2) virus and the blood of two of the four chickens co-infected with *S. aureus* and Ck/Bj/2/97 (H9N2) virus. On the contrary, H9N2 viruses were not recovered from the blood of the chickens co-infected with *H. paragallinarum* and Ck/Y-55/2001 (H9N2) virus. The results may indicate that virus recovery from the blood may be correlated to the tissue tropism of the bacteria co-infected.

In cases of co-infection with *S. aureus*, H9N2 viruses were recovered from the blood of the chickens (Table 2). H9N2 virus might be disseminated throughout the body of the chicken through the blood or lymph. When the viruses replicate extensively in the respiratory tissues, some of those viruses may leak from the damaged area to the blood or lymph vessels. Another possibility is that the lymphocytes infected with viruses may spread throughout the body as shown with virulent avian influenza virus [21].

Although it is perceived that influenza A viruses circulating in wild ducks are the origin of influenza A viruses of poultry including chickens, viruses were not recovered from any organs of the chickens inoculated intranasally with A/duck/Hokkaido/9/99 (H9N2) (Table 1). In that case, how does the H9N2 virus of wild ducks cross the interspecies barrier and acquire the ability to replicate in chickens? The mechanism of interspecies transmission is now under investigation.

In the present study, we demonstrate that the H9N2 viruses isolated from the chicken meat and bone marrow disseminate throughout the body of the chicken, and that the pathogenicity of the H9N2 virus for chickens is enhanced by co-infection with either *S. aureus* or *H. paragallinarum*. Avian H9N2 virus is directly transmitted to humans from chickens [15], as are H5N1 and H7N7 [5, 22]. These

cases may indicate that a virus which can replicate in chickens may acquire transmissibility crossing the host-range barrier. To clarify the molecular basis of interspecies transmission, it is important to continue the surveillance of avian influenza.

References

1. Alexander DJ (2000) A review of avian influenza in different bird species. Vet Microbiol 74: 3–13
2. Awan MA, Matsumoto M (1998) Heterogeneity of staphylococci and other bacteria isolated from six-week-old broiler chickens. Poult Sci 77: 944–949
3. Blackall PJ (1999) Infectious coryza: overview of the disease and new diagnostic options. Clin Microbiol Rev 12: 627–632
4. Bosch FX, Orlich M, Klenk HD, Rott R (1979) The structure of the hemagglutinin, a determinant for the pathogenicity of influenza viruses. Virology 95: 197–207
5. Claas EC, Osterhaus AD, van Beek R, De Jong JC, Rimmelzwaan GF, Senne DA, Krauss S, Shortridge KF, Webster RG (1998) Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 351: 472–477
6. Davidson WR, Yoder HW, Brugh M, Nettles VF (1988) Serological monitoring of eastern wild turkeys for antibodies to Mycoplasma spp. and avian influenza viruses. J Wildl Dis 24: 348–351
7. Easterday BC, Hinshaw VS, Halvorson DA (1997) Influenza. In: Calnec BW (ed) Diseases of poultry, 10th ed. Iowa State University Press, Ames, IA, pp 583–605
8. Guan Y (1997) H9 avian influenza virus: A candidate of next influenza pandemic strain? PhD thesis, The University of Hong Kong, Hong Kong, Chapter 6, pp 105–116
9. 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 lineages in Asia. Virology 267: 279–288
10. Kawaoka Y, Yamnikova S, Chambers TM, Lvov DK, Webster RG (1990) Molecular characterization of a new hemagglutinin, subtype H14, of influenza A virus. Virology 179: 759–767
11. Klenk HD, Garten W (1994) Host cell proteases controlling virus pathogenicity. Trends Microbiol 2: 39–43
12. Lee CW, Song CS, Lee YJ, Mo IP, Garcia M, Suarez DL, Kim SJ (2000) Sequence analysis of the hemagglutinin gene of H9N2 Korean avian influenza viruses and assessment of the pathogenic potential of isolate MS96. Avian Dis 44: 527–535
13. Liu J, Okazaki K, Ozaki H, Sakoda Y, Wu Q, Chen F, Kida H (2003) H9N2 influenza viruses prevalent in poultry in China are phylogenetically distinct from A/quail/Hong Kong/G1/97 presumed to be the donor of the internal protein genes of the H5N1 Hong Kong/97 virus. Avian Pathol 32: 551–560
14. Mo IP, Song CS, Kim KS, Rhee JC (1998) An occurrence of non-highly pathogenic avian influenza in Korea. In: Proc 4th International Symposium on Avian Influenza, Athens, GA, pp 379–383
15. 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
16. Reed LJ, Muench H (1938) A simple method for estimating fifty percent endpoints. Am J Hyg 37: 493–497
17. Rimler RB (1979) Studies of the pathogenic avian Haemophili. Avian Dis 23: 1006–1008
18. Rohm C, Zhou N, Suss J, Mackenzie J, Webster RG (1996) Characterization of a novel influenza hemagglutinin, H15: criteria for determination of influenza A subtypes. Virology 217: 508–516

19. Scanlan CM, Hargis BM (1989) A bacteriologic study of scabby-hip lesions from broiler chickens in Texas. J Vet Diagn Invest 1: 170–173
20. Tashiro M, Ciborowski P, Klenk HD, Pulverer G, Rott R (1987) Role of staphylococcus protease in the development of influenza pneumonia. Nature 325: 536–537
21. Van Campen H, Easterday BC, Hinshaw VS (1989) Destruction of lymphocytes by a virulent avian influenza A virus. J Gen Virol 70(Pt 2): 467–472
22. Van Kolfschooten F (2003) Dutch veterinarian becomes first victim of avian influenza. Lancet 361: 1444
23. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y (1992) Evolution and ecology of influenza A viruses. Microbiol Rev 56: 152–179

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