

Avian influenza vaccination: the experience in China

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Summary

Highly pathogenic H5N1 avian influenza virus was first detected in the People's Republic of China (China) in 1996 and has caused over 100 outbreaks of disease in poultry since 2004. The Chinese Government has pursued a vaccination strategy to control avian influenza infection in poultry. A series of vaccines including whole-virus inactivated vaccine, recombinant fowlpox vaccine and recombinant Newcastle disease virus vaccine have been developed and billions of doses of the vaccines are produced every year. The Government has also developed strategies to fund vaccine production and to offer financial compensation for the slaughter of infected poultry. The vaccination strategy has been effective and has played an important role in reducing the incidence of H5N1 infection in poultry and in markedly reducing the number of cases of human infection. Despite the successes obtained with the vaccination strategy, China still faces challenges in its efforts to eliminate H5N1 virus circulation in poultry.

Keywords

China – H5N1 avian influenza – Vaccination.

Introduction

The People's Republic of China (hereafter referred to as China) is one of the largest poultry producing countries in the world, accounting for over 70% of the world's domestic waterfowl production. The majority of domestic poultry is bred in backyards or in small-scale farms that lack biosecurity practices. Therefore, in China it is a great challenge to completely control or eradicate an infectious disease such as avian influenza in poultry.

Multiple subtypes of avian influenza viruses have been detected in chickens and ducks in China since 1994. These subtypes include H9N2 (17), H7N2 (19), H14N5, H6N1, H6N2, and H4N2. H5N1 influenza viruses were first

detected in geese from Guangdong Province in 1996, and have been repeatedly detected since 1999 in apparently healthy ducks in southern China (3, 14). Since 2004, over 100 outbreaks in 23 provinces have occurred in domestic poultry and in wild birds (4, 20, 32). A total of over 35 million poultry have been culled to control the spread of H5N1 infection and disease. Therefore, H5N1 influenza virus outbreaks have caused severe economic damage for the poultry industry in China.

Vaccination has been an important component in the strategy used for the control of H5N1 influenza infection in domestic poultry in China since 2004. This review will discuss the vaccine development experience in China as well as the challenges that the country faces in vaccine application for the control of H5N1 avian influenza.

Vaccines developed in China

Inactivated vaccines

H5N2 inactivated vaccine

An inactivated, oil-emulsified vaccine was developed using an H5N2 low pathogenic virus, A/turkey/England/N-28/73 (kindly provided by Dr Dennis Alexander), as a seed virus. The vaccine was first approved in August of 2003 for use in those chickens in Guangdong Province that were to be exported to Hong Kong and Macao. This vaccine was fully evaluated by the Chinese Veterinary Drug Evaluation Committee and received certification by the end of 2003. After the H5N1 outbreak in 2004, this vaccine was licensed to nine companies with good manufacturing practice facilities and experience in producing egg-cultured vaccines. In total, 2.5 billion doses of H5N2 inactivated vaccine were used in the districts where H5N1 outbreaks occurred in 2004 (Table I).

H5N1 inactivated vaccine

Although the H5N2 vaccine played an important role in the rapid control of the H5N1 outbreaks in China in 2004, the vaccine was not ideal. The vaccine seed virus exhibited antigenic diversity with the prevalent H5N1 strains circulating in China at the time. Also, the seed virus could not grow to high titres in egg, which severely impaired vaccine production. To solve these problems, using plasmid-based reverse genetics (8, 12, 22), the author and her colleagues at the Harbin Veterinary

Research Institute generated a series of reassortant viruses that contained the internal genes from the high growth A/Puerto Rico/8/34 (PR8) virus, and the haemagglutinin (HA) and neuraminidase (NA) genes from several H5N1 viruses including GS/GD/1/96 (Re-1), A/bar-headed goose/Qinghai/3/2005 (Re-3) and A/duck/Anhui/1/2006 (Re-5) (Table I). To reduce the virulence of the reassortant viruses, the multiple basic amino acid motif present in the cleavage site of the HA protein that is associated with high pathogenicity in H5 avian influenza viruses (-RRRKKR-) was changed into a sequence (-RETR-) that is characteristic of avian influenza viruses with low pathogenicity (23, 25), as reported previously (18, 28).

Reassortant virus Re-1 was created bearing the HA and NA genes from GS/GD/1/96. The virus was completely attenuated in chicken embryos and chickens (30), but grew to high titres. Most importantly, the presence of the HA and NA genes of GS/GD/1/96 allowed Re-1 to antigenically match the H5N1 viruses that were circulating in China (3). Re-1 induced higher haemagglutination inhibition (HI) antibody responses and longer-lasting protective immunity in chickens compared to the H5N2 vaccines. It was also shown to be effective in ducks and geese (30). The Re-1 vaccine was approved for use in the field at the end of 2004, and over 20 billion doses of the Re-1 vaccine have been used since then in China (Table I), Vietnam, Mongolia and Egypt.

In February 2006, an H5N1 avian influenza virus was isolated from a chicken flock in Shanxi Province in

Table I
Vaccines developed and used for H5N1 avian influenza control in China from 2004 to 2008

| Vaccine | Seed virus generated | | Doses used in the year ^(a) (billions) | | | | | Total |
|----------------------------------|--|-----------------------------------|--|-------|------|------|------|-------|
| | Seed name | HA and/or NA gene donor virus | 2004 | 2005 | 2006 | 2007 | 2008 | |
| Inactivated vaccine | | | | | | | | |
| H5N2 subtype | A/turkey/England/N-28/73 (H5N2) (N-28) | NA | 2.5 | 4.08 | 3.6 | / | / | 10.18 |
| H5N1 subtype | H5N1/PR8 (H5N1) (Re-1) | A/goose/Guangdong/1/1996 | 0.57 | 3.3 | 4.5 | 9.6 | 4.6 | 22.64 |
| | H5N1/PR8 (H5N1) (Re-3) | A/bar-headed goose/Qinghai/3/2005 | / | / | / | / | / | / |
| | H5N1/PR8 (H5N1) (Re-4) | A/chicken/Shanxi/2/2006 | / | / | 0.84 | 0.42 | / | 1.26 |
| | H5N1/PR8 (H5N1) (Re-5) | A/duck/Anhui/1/2006 | / | / | / | / | 4.4 | 4.4 |
| | H5N1/PR8-5B19 | A/goose/Guangdong/1/1996 | / | / | / | / | / | / |
| | Re-1/Re-4 | NA | / | / | / | 2.2 | 1.5 | 3.7 |
| Re-4/Re-5 | NA | / | / | / | / | 1.5 | 1.5 | |
| Live virus vector vaccine | | | | | | | | |
| Recombinant fowlpox vaccine | rFPF-HA-NA | A/goose/Guangdong/1/1996 | / | 0.615 | / | / | / | 0.615 |
| Recombinant NDV vaccine | rLH5-1 | A/goose/Guangdong/1/1996 | / | / | 2.6 | 1.3 | / | 3.9 |
| | rLH5-3 | A/bar-headed goose/Qinghai/3/2005 | / | / | / | / | / | / |
| | rLH5-4 | A/chicken/Shanxi/2/2006 | / | / | / | / | / | / |
| | rLH5-5 | A/duck/Anhui/1/2006 | / | / | / | / | 1.2 | 1.2 |
| | pCAGGoptiHA | A/goose/Guangdong/1/1996 | / | / | / | / | / | / |

a) / a forward slash indicates that the vaccine was not used in that particular year
HA: haemagglutinin

NA: neuraminidase
NDV: Newcastle disease virus

northern China that had been vaccinated with the H5N2 inactivated vaccines. The disease in those flocks was recorded as a decrease in egg production and mortality in the range of 10% to 20%. These viruses, represented by A/chicken/Shanxi/2/06(CK/SX/06), exhibited significant antigenic drift from the viruses that were previously isolated in China. Though 187,000 poultry were depopulated to control the spread of this new virus after its first detection, it was re-isolated in June in Shanxi and Ningxia Provinces. The researchers at the Harbin Veterinary Research Institute determined that the inactivated H5 vaccines used in China, including the H5N2 and H5N1(Re-1) vaccines, only provided 80% protection to the novel variant strain in a laboratory challenge study in specific-pathogen-free (SPF) chickens (unpublished data). This result was quite different from the protective efficacy to other H5N1 influenza viruses, which could reach 100% protection (30). A new reassortant virus was therefore developed at the Institute, designated as Re-4. Re-4 contained the NA gene and the cleavage site modified HA gene from CK/SX/06, and six internal genes from PR8. This new vaccine was approved in August 2006 for use in the Shanxi and Ningxia Provinces and in several of the neighbouring provinces in northern China. A total of 1.26 billion doses were used in 2006 and 2007 (Table I).

Co-circulation of both GS/GD/96-like viruses and CK/SX/06-like viruses was detected in some areas in 2006. A new H5N1 vaccine was then produced from the combined antigens of Re-1 and Re-4. This vaccine was approved in 2007 for use in a limited area in northern China, and a total of 2.2 billion doses of this vaccine were administered in that year (Table I).

In May 2008, the Re-1 reassortant virus was replaced as the seed virus used for vaccine production. The new H5N1/PR8 reassortant virus, which derives its HA and NA genes from A/duck/Anhui/1/06 (clade 2.3), was designated as Re-5 (Table I).

H5N1 inactivated marker vaccine

The current commercially used H5N1 inactivated vaccines are safe and effective, providing solid protection from H5N1 highly pathogenic influenza viruses (30). However, these inactivated vaccines do not allow for serological distinction between vaccination and field infection. This poses an obstacle to serological surveillance of influenza viruses circulating in the wild. Recently, intensive vaccination with marker vaccines and stamping-out strategies have been gaining popularity in veterinary medicine for eradication of specific diseases of national or international interest. A marker vaccine can be used in conjunction with a diagnostic test to differentiate a vaccinated animal from a carrier animal (1). A genetically marked H5N1 influenza vaccine that could readily be

distinguished from wild-type strains would therefore be of great value in an eradication plan.

The author and her colleagues recently generated an attenuated H5N1 influenza marker vaccine seed virus, denoted H5N1/PR8-5B19, which derived its internal genes from PR8 virus and its HA and NA genes from the GS/GD/1/96 virus (16). This new reassortant virus, H5N1/PR8-5B19, encodes an HA molecule with a monobasic cleavage site for low pathogenicity in poultry, and an NA molecule bearing the 5B19 epitope of the S2 glycoprotein of murine hepatitis virus. H5N1/PR8-5B19 grew to high titres in embryonated eggs and in chickens without leading to disease, and vaccinated chickens were completely protected against lethal H5N1 challenge. Chickens that received two doses of the H5N1/PR8-5B19 inactivated vaccine were 100% positive for anti-5B19 antibody. In contrast, sera obtained from chickens vaccinated with the H5N1/PR8 inactivated vaccine or infected with the H5N1/PR8 virus showed no reactivity against the 5B19 epitope in a peptide-based enzyme-linked immunosorbent assay (ELISA) test. Although additional experiments are necessary, these results suggest that H5N1/PR8-5B19 may be a potential candidate for an H5N1 influenza marker vaccine that could be used in the field to control H5N1 influenza virus infection and disease in poultry.

Live virus-vectored vaccine

Recombinant fowlpox vaccine

Whole-virus inactivated vaccines and fowlpox virus-based recombinant vaccines have been used in the laboratory and in poultry farms across the world to control highly pathogenic avian influenza (HPAI) (2, 7, 29, 31). The author's colleagues developed recombinant live virus-vectored vaccines using fowlpox virus and Newcastle disease virus (NDV) as vectors (11). Development of a recombinant fowlpox (rFP) virus expressing the HA and NA genes of H5N1 began after detection of GS/GD/96 in 1999. The efficacy of the rFPF-HA-NA recombinant virus was proven in both laboratory and field tests (24). Approximately 615 million doses of rFPF-HA-NA vaccine have been used in poultry in China since 2005.

Recombinant Newcastle disease virus vaccine

An NDV live virus-vectored vaccine against influenza has several advantages, including high yields of vaccine production and the ease with which the vaccine can be produced and administered to animals in the field. In addition, it may serve as a bivalent vaccine against two viruses that can decimate bird populations. The use of NDV as the vaccine backbone should prevent confusion between vaccinated and infected birds during surveillance, which is a problem with the use of whole-virus influenza

vaccines. Highly pathogenic Newcastle disease has been endemic in China and more than 30 billion doses of live vaccines are used in chickens every year.

In 2005, the author and her colleagues established a reverse genetics system of NDV (LaSota) and generated recombinant NDV vaccines expressing the HA genes from several H5N1 viruses representing different phylogenetic lineages isolated in China (Table I, unpublished data). The recombinant NDV vaccines contained influenza virus HA sequences from GS/GD/96 (rLH5-1), A/bar-headed goose/Qinghai/3/05 (rLH5-3) and CK/SX/06 virus (rLH5-4). The recombinant NDVs expressing the various HA genes induced strong HI antibody responses to NDV and to H5 avian influenza viruses in chickens (11). Chickens vaccinated with recombinant NDV-vector vaccine were protected from disease and death after challenge with highly pathogenic NDV. Most importantly, the vaccinated chickens were completely protected from homologous and heterologous H5N1 virus challenges with no virus shedding, signs of disease, or death (11).

In the beginning of 2006, rLH5-1 was approved for use in chickens as a bivalent, live attenuated vaccine for the control of H5N1 avian influenza and highly pathogenic Newcastle disease. By the end of 2007, a total of 4 billion doses of this vaccine had been applied in chickens (Table I). In May 2008, a recombinant NDV virus rLH5-5 with the HA gene derived from A/duck/Anhui/1/06 (clade 2.3) replaced rLH5-1 as the seed virus strain for vaccine production (Table I).

DNA vaccine

Though whole-virus inactivated vaccines (30), recombinant fowlpox vaccines (24) and the recombinant NDV vaccine (11) have been used in China and some other countries, DNA vaccines may offer yet additional advantages for the control of avian influenza virus. First, DNA immunisation can induce both humoral and cell-mediated immune responses, similar to an attenuated live virus vaccine, with the safety of a killed or subunit vaccine (6, 10, 21, 33). Second, DNA vaccines are easier to manufacture and store than inactivated whole-virus vaccines. Third, immune responses are generated against the expressed gene product and not the DNA vaccine vector. Although previous studies (5, 9, 26, 27) have confirmed the efficacy of the HA gene-based DNA vaccine against HPAI virus (HPAIV) challenge in chickens, the high dosage (200 µg to 400 µg DNA) needed is a major obstacle for field application of such vaccines. The author and her colleagues therefore explored a strategy to decrease the dosage of DNA vaccine required by improving the expression of the target gene.

They constructed the plasmid pCAGGoptiHA that expressed a synthetic H5 HA gene, optiHA, under the

control of the chicken beta-actin promoter (13). optiHA contained the HA amino acid sequence of HPAIV H5N1 GS/GD/96 modified for codon bias in chickens. The vaccine efficacy of pCAGGoptiHA was evaluated by intramuscular injection with different amounts (100 µg, 10 µg or 1 µg) of the plasmid. All of the vaccinated chickens developed detectable levels of HI and neutralising antibodies, with antibody titres correlating with the inoculated amount of plasmid. After challenge with a lethal dose of HPAIV H5N1, all of the chickens inoculated with 100 µg and 10 µg of plasmid were completely protected from disease and death, while the chickens inoculated with 1 µg of plasmid were partially protected. The vaccine efficacy of the lowest dosage of plasmid inoculation (1 µg) could be improved by a second immunisation (13). Further investigations demonstrated that two doses of 10 µg of pCAGGoptiHA inoculation could induce protection lasting more than a year (13), covering the entire period that layer chickens were kept on the farm and eliminating the need for costly serial vaccinations.

Vaccination implementation against H5N1 avian influenza in poultry in China

Vaccines have been used for the control of H5N1 avian influenza in China since 2004. In the very beginning, only the birds in the buffer zone or in the areas where outbreaks occurred were vaccinated. In 2005, however, epidemiological studies indicated that all of the prior outbreaks had occurred in farms that did not vaccinate or vaccinated with unqualified vaccines. Thus, by the end of 2005, the Government required vaccinations to be applied to all domestic poultry. To ensure the implementation of this control strategy, eight companies were allowed to produce the vaccines with government aid, and the Government provided financial compensation for slaughtered poultry.

Challenges of the control of H5N1 avian influenza in China

Using advanced biotechnology techniques, the author and her colleagues have developed several types of avian influenza vaccines that have been administered to poultry in China and in other countries. Though the vaccination strategy for H5N1 influenza control has been questioned, the experiences in China have shown that vaccination can play an important role in protecting poultry from H5N1 virus infection, in reducing the virus load in the

environment, and in preventing the transmission of H5N1 virus from poultry to human.

However, there are still several challenges to overcome before H5N1 avian influenza can be completely controlled and eradicated. First, the vaccination coverage varies in different poultry species and in different types of poultry farms. The vaccines are relatively easy to apply in chickens on large farms, but are difficult to administer to chickens raised in backyard and small-scale farms. China also has a large number of domestic waterfowl. Though inactivated vaccines have proven to be very effective in ducks and geese (15, 30), the vaccination rates are relatively low in these species. Layer and breeder ducks are vaccinated when they are young, but adult ducks may not exhibit clinical symptoms after infection and may become asymptomatic carriers of H5N1 avian influenza viruses (3). Therefore, the required booster vaccination is not actually administered to waterfowl on most farms, when the levels of protective antibody have declined. Waterfowl raised for meat consumption are usually not vaccinated because of their short life span (four to six weeks). Thus, practical implementation of vaccination may be impaired because vaccination coverage may never reach all of the birds susceptible to avian influenza virus infection.

A second challenge to avian influenza virus control is that biosecurity measures in the backyard or small-scale farms are often not practised. Thus, virus can easily infect the birds on these types of farms. To complicate matters further, it is also difficult to perform extensive surveillance in poultry on these farms.

A third challenge to avian influenza virus control is the live bird trade and the important role it plays in the spread of infection and disease. There are no regulations for live bird trade amongst different provinces in China. Poultry can be transported for thousands of miles to the live poultry markets that are very popular in southern China.

No disinfection and cleaning procedures are applied in most of these live bird markets, and avian influenza viruses will actively circulate and infect poultry or other animals that do not have immunity to this pathogen. The majority of the human H5N1 cases in China have a history of exposure in the live bird markets, which is a strong argument for the improved management of the poultry trade system in China.

Conclusion

This review has briefly summarised the development and application of vaccines for the control of HPAI in China. A series of vaccines have been developed and over 50 billion doses of these vaccines have been used in China and other countries. Most importantly, the vaccine seed virus has been updated to ensure the best protective efficacy to the prevalent circulating strain. The vaccination strategy has been effective and has played an important role in reducing the incidence of H5N1 in poultry and markedly reducing the number of human cases. Although progress has been made in the control of avian influenza viruses, circulation of these viruses has not been eliminated from poultry. It is worth noting that complete control and eradication of H5N1 HPAIV viruses can only be ultimately achieved by a combination of vaccination, improved biosecurity, extensive surveillance and an effective monitoring programme.

Acknowledgements

The author is supported by the Animal Infectious Disease Control Program of the Ministry of Agriculture, the Chinese National Science and Technology Development Plan, Grants 2004BA519A-57 and 2006BAD06A05.



La vaccination contre l'influenza aviaire : l'expérience de la Chine

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Résumé

Le sous-type H5N1 du virus de l'influenza aviaire hautement pathogène a été détecté pour la première fois en Chine en 1996 ; depuis 2004, il est à l'origine de plus de cent foyers affectant les volailles. Le gouvernement chinois a mis en œuvre une stratégie de vaccination visant à lutter contre l'infection chez les volailles domestiques. Plusieurs vaccins ont ainsi été mis au point, y compris un vaccin à virus entier inactivé, un vaccin recombinant utilisant le virus de la variole aviaire et un vaccin recombinant utilisant le virus de la maladie de Newcastle ; plusieurs et plusieurs milliards de doses de vaccin sont produits chaque année. Le gouvernement a également élaboré des stratégies pour financer la production de vaccin et indemniser les éleveurs confrontés à l'abattage des volailles infectées. La stratégie de vaccination s'est avérée efficace et a permis de réduire l'incidence de l'infection due au virus H5N1 chez les volailles ; le nombre de cas humains a également diminué de manière notable. Malgré le succès de cette stratégie de vaccination, la Chine n'a pas encore réussi à éliminer le virus H5N1 de l'influenza aviaire, qui continue de circuler parmi les populations de volailles.

Mots-clés

Chine – Influenza aviaire due au sous-type H5N1 – Vaccination.



La experiencia china de vacunación contra la influenza aviar

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Resumen

En 1996 se detectó por primera vez en China el virus H5N1 de la influenza aviar altamente patógena, que desde 2004 ha causado más de 100 brotes infecciosos en aves de corral. Para luchar contra esa infección en las aves de corral, el Gobierno de China ha aplicado una estrategia de vacunación. Se han elaborado una serie de vacunas (vacuna con virus entero inactivado y vacunas de virus recombinante de viruela aviar y de la enfermedad de Newcastle), de las que ahora mismo se están fabricando miles de millones de dosis al año. El Gobierno también ha definido estrategias para financiar la producción de vacunas y ofrecer indemnizaciones económicas por el sacrificio de las aves infectadas. La estrategia de vacunación ha resultado eficaz y constituido un factor importante para reducir la incidencia de la infección por virus H5N1 en aves de corral y para disminuir sensiblemente el número de casos de infección humana. Pese a los éxitos obtenidos con la estrategia de vacunación, China sigue afrontando dificultades en su lucha por acabar con la circulación de virus H5N1 en aves de corral.

Palabras clave

China – Influenza aviar por H5N1 – Vacunación.



References

- Babiuk L.A. (1999). – Broadening the approaches to developing more effective vaccines. *Vaccine*, **17**, 1587-1595.
- Capua I., Marangon S., dalla Pozza M., Terregino C. & Cattoli G. (2003). – Avian influenza in Italy 1997-2001. *Avian Dis*, **47**, 839-843.
- Chen H., Deng G., Li Z., Tian G., Li Y., Jiao P., Zhang L., Liu Z. *et al.* (2004). – The evolution of H5N1 influenza viruses in ducks in southern China. *Proc. natl Acad. Sci. USA*, **101**, 10452-10457.
- Chen H., Li Y., Li Z., Shi J., Shinya K., Deng G., Qi Q., Tian G. *et al.* (2006). – Properties and dissemination of H5N1 viruses isolated during an influenza outbreak in migratory waterfowl in western China. *J. Virol.*, **80**, 5976-5983.
- Chen H., Yu K., Jiang Y. & Tang X. (2001). – DNA immunization elicits high HI antibody and protects chicken from AIV challenge. Options for the control of influenza IV. International Congress Series, Vol. 1219, 917-921.
- Donnelly J.J., Liu M.A. & Ulmer J.B. (2000). – Antigen presentation and DNA vaccines. *Am. J. respir. crit. Care Med.*, **162**, S190-193.
- Ellis T.M., Leung C.Y., Chow M.K., Bissett L.A., Wong W., Guan Y. & Malik Peiris J.S. (2004). – Vaccination of chickens against H5N1 avian influenza in the face of an outbreak interrupts virus transmission. *Avian Pathol.*, **33**, 405-412.
- Fodor E., Devenish L., Engelhardt O.G., Palese P., Brownlee G.G. & Garcia-Sastre A. (1999). – Rescue of influenza A virus from recombinant DNA. *J. Virol.*, **73**, 9679-9682.
- Fynan E.F., Webster R.G., Fuller D.H., Haynes J.R., Santoro J.C. & Robinson H.L. (1993). – DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proc. natl Acad. Sci. USA*, **90**, 11478-11482.
- Garmory H.S., Brown K.A. & Titball R.W. (2003). – DNA vaccines: improving expression of antigens. *Genet. Vaccines Ther.*, **1** (1), 2.
- Ge J., Deng G., Wen Z., Tian G., Wang Y., Shi J., Wang X., Li Y. *et al.* (2007). – Newcastle disease virus-based live attenuated vaccine completely protects chickens and mice from lethal challenge of homologous and heterologous H5N1 avian influenza viruses. *J. Virol.*, **81**, 150-158.
- Hoffmann E., Neumann G., Kawaoka Y., Hobom G. & Webster R.G. (2000). – A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc. natl Acad. Sci. USA*, **97**, 6108-6113.
- Jiang Y., Yu K., Zhang H., Zhang P., Li C., Tian G., Li Y., Wang X. *et al.* (2007). – Enhanced protective efficacy of H5 subtype avian influenza DNA vaccine with codon optimized HA gene in a pCAGGS plasmid vector. *Antiviral Res.*, **75**, 234-241.
- Jiao P., Tian G., Li Y., Deng G., Jiang Y., Liu C., Liu W., Bu Z. *et al.* (2008). – A single amino acid substitution in the NS1 protein changes the pathogenicity of H5N1 avian influenza viruses in mice. *J. Virol.*, **82** (3), 1146-1154.
- Kim J.K., Seiler P., Forrest H.L., Khalenkov A.M., Franks J., Kumar M., Karesh W.B., Gilbert M. *et al.* (2008). – Pathogenicity and vaccine efficacy of different clades of Asian H5N1 avian influenza A viruses in domestic ducks. *J. Virol.*, **82**, 11374-11382.
- Li C., Ping J., Jing B., Deng G., Jiang Y., Li Y., Tian G., Yu K. *et al.* (2008). – H5N1 influenza marker vaccine for serological differentiation between vaccinated and infected chickens. *Biochem. biophys. Res. Commun.*, **372** (2), 293-397.
- Li C., Yu K., Tian G., Yu D., Liu L., Jing B., Ping J. & Chen H. (2005). – Evolution of H9N2 influenza viruses from domestic poultry in Mainland China. *Virology*, **340**, 70-83.
- Li S., Liu C., Klimov A., Subbarao K., Perdue M.L., Mo D., Ji Y., Woods L. *et al.* (1999). – Recombinant influenza A virus vaccines for the pathogenic human A/Hong Kong/97 (H5N1) viruses. *J. infect. Dis.*, **179**, 1132-1138.
- Li Y., Li C., Liu L., Wang H., Wang C., Tian G., Webster R., Yu K. & Chen H. (2006). – Characterization of an avian influenza virus of subtype H7N2 isolated from chickens in northern China. *Virus Genes*, **33**, 117-122.
- Liu J., Xiao H., Lei F., Zhu Q., Qin K., Zhang X.W., Zhang X.L., Zhao D. *et al.* (2005). – Highly pathogenic H5N1 influenza virus infection in migratory birds. *Science*, **309**, 1206.
- Liu M.A., Fu T.M., Donnelly J.J., Caulfield M.J. & Ulmer J.B. (1998). – DNA vaccines. Mechanisms for generation of immune responses. *Adv. exp. Med. Biol.*, **452**, 187-191.
- Neumann G., Watanabe T., Ito H., Watanabe S., Goto H., Gao P., Hughes M., Perez D.R. *et al.* (1999). – Generation of influenza A viruses entirely from cloned cDNAs. *Proc. natl Acad. Sci. USA*, **96**, 9345-9350.
- Perdue M.L., Garcia M., Senne D. & Fraire M. (1997). – Virulence-associated sequence duplication at the hemagglutinin cleavage site of avian influenza viruses. *Virus Res.*, **49**, 173-186.
- Qiao C.L., Yu K.Z., Jiang Y.P., Jia Y.Q., Tian G.B., Liu M., Deng G.H., Wang X.R. *et al.* (2003). – Protection of chickens against highly lethal H5N1 and H7N1 avian influenza viruses with a recombinant fowlpox virus co-expressing H5 haemagglutinin and N1 neuraminidase genes. *Avian Pathol.*, **32**, 25-32.
- Senne D.A., Panigrahy B., Kawaoka Y., Pearson J.E., Suss J., Lipkind M., Kida H. & Webster R.G. (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.

26. Sharma S., Mamane Y., Grandvaux N., Bartlett J., Petropoulos L., Lin R. & Hiscott J. (2000). – Activation and regulation of interferon regulatory factor 4 in HTLV type 1-infected T lymphocytes. *AIDS Res. Hum. Retroviruses*, **16**, 1613-1622.
 27. Suarez D.L. & Schultz-Cherry S. (2000). – The effect of eukaryotic expression vectors and adjuvants on DNA vaccines in chickens using an avian influenza model. *Avian Dis.*, **44**, 861-868.
 28. Subbarao K., Chen H., Swayne D., Mingay L., Fodor E., Brownlee G., Xu X., Lu X. *et al.* (2003). – Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics. *Virology*, **305**, 192-200.
 29. Swayne D.E., Garcia M., Beck J.R., Kinney N. & Suarez D.L. (2000). – Protection against diverse highly pathogenic H5 avian influenza viruses in chickens immunized with a recombinant fowlpox vaccine containing an H5 avian influenza hemagglutinin gene insert. *Vaccine*, **18**, 1088-1095.
 30. Tian G., Zhang S., Li Y., Bu Z., Liu P., Zhou J., Li C. Shi J. *et al.* (2005). – Protective efficacy in chickens, geese and ducks of an H5N1-inactivated vaccine developed by reverse genetics. *Virology*, **341**, 153-162.
 31. Van der Goot J.A., Koch G., de Jong M.C. & van Boven M. (2005). – Quantification of the effect of vaccination on transmission of avian influenza (H7N7) in chickens. *Proc. natl Acad. Sci. USA*, **102**, 18141-18146.
 32. Wan X.F., Ren T., Luo K.J., Liao M., Zhang G.H, Chen J.D, Cao W.S., Li Y. *et al.* (2005). – Genetic characterization of H5N1 avian influenza viruses isolated in southern China during the 2003-04 avian influenza outbreaks. *Arch. Virol.*, **150**, 1257-1266.
 33. Webster R.G. (1999). – Potential advantages of DNA immunization for influenza epidemic and pandemic planning. *Clin. infect. Dis.*, **28**, 225-229.
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