Contacto CONAMER GLS-TULS-AMMDC-B00073201?

De:	Lilia CASTELLANOS <lilia.castellanos@ceva.com>
Enviado el:	lunes, 4 de septiembre de 2023 06:21 p. m.
Para:	Contacto CONAMER
CC:	Rocio FERNANDEZ; Francisco ROJO; Ricardo FRANCO; Nicolas COUSSEAU; Poultry MEXICO
Asunto:	COMENTARIOS AL PROYECTO DE ACUERDO POR EL QUE SE DECLARA AL TERRITORIO D ELOS ESTADOS UNIDOS MEICANOS, COMO LIBRE DE INFLUENZA AVIAR TIPO A SURTIPO H5N1.
Datos adjuntos:	COMENTARIOS CEVA - ACUERDO LIBRE DE INFLUENZA AVIAR H5N1 FINAL.pdf; Bibliografia - Vacunar vs IA.zip

Por medio del presente, pongo a su consideración los comentarios del Servicio Técnico de CEVA Salud Animal correspondientes al Proyecto de "Acuerdo por el que se declara al territorio de los Estados Unidos Mexicanos, como libre de Influenza Aviar Tipo A Subtipo H5N1", publicado en el portal de anteproyectos de la Comisión Nacional de Mejora Regulatoria, el 29 de agosto del 2022.
Se anexa documento en PDF y Bibliografía de referencia.
Sin mas por el momento quedo a sus órdenes agradeciendo su amable atención a la presente.
Saludos

COMISIÓN NACIONAL DE MEJORA REGULATORIA
CONTROL DE GESTIÓN
05 SEP 2023
RECIBIDO
RÚBRICA 9:00

ANTENTAMENTE:
MVZ. Lilia Castellanos Novoa.
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Tel: 55 54049457

Ciudad de México a 4 de septiembre del 2023

Comisión Nacional de Mejora Regulatoria.
P r e s e n t e

El área técnica del laboratorio CEVA Salud Animal, con base en nuestro enfoque de Responsabilidad Social como líder mundial en salud animal, tenemos como objetivo primordial contribuir al bienestar animal y humano participando en la prevención y control de enfermedades que afecten la producción de proteína animal para la nutrición humana; por lo cual ponemos para su consideración los siguientes comentarios al Proyecto de "Acuerdo por el que se declara al territorio de los Estados Unidos Mexicanos, como libre de Influenza Aviar Tipo A Subtipo H5N1", publicado en portal de anteproyectos de la Comisión Nacional de Mejora Regulatoria, el día 29 de agosto del 2023.

Según la evidencia científica anexa a la presente, se demuestra que la vacunación es un elemento primordial en la estrategia de prevención y control de enfermedades, considerándose de gran importancia en enfermedades de alto impacto sanitario como la Influenza Aviar. Este hecho, ha sido constatado en México en la atención de brotes de este virus con la vacunación como parte estratégica para su prevención y control, lo que ha permitido que la Industria Avícola en México tenga una parvada Nacional de más de 500 millones de aves, considerada dentro de los 10 primeros países productores de huevo y pollo en el mundo y el primer lugar en consumo per-cápita de huevo fresco.

A la fecha y de acuerdo a la información oficial respecto a la afectación por la presencia de Influenza Aviar de Alta patogenicidad subtipo H5N1 en México, entre el 2022 y 2023; registró la pérdida de un total de 7,400,273 de aves en producción, generando un impacto negativo para la avicultura Nacional.

Se considera que un importante factor de riesgo para la presentación de esta enfermedad es la localización geográfica de México, debido a que es territorio de arribo y permanencia estacional de aves silvestres migratorias que se han identificado como elementos que participan epidemiológicamente en el contagio y diseminación de la influenza aviar en la población avícola Nacional.

Para mitigar lo anterior, en la estrategia de prevención de esta enfermedad, la vacunación oportuna constituye una herramienta crítica en las empresas avícolas

de países como México que se encuentra entre los diez principales productores avícolas a nivel mundial y provee cerca del 65% de la proteína de origen animal en México.

En mayo de 2023, en la Sesión General de la Organización Mundial de Sanidad Animal (OMSA), los Miembros de esta organización, entre ellos México, adoptaron una resolución que servirá de base para configurar las futuras actividades de lucha contra la influenza aviar y proteger la fauna silvestre, sin dejar de brindar asistencia a la industria avícola y garantizar la continuidad de las actividades comerciales.

En particular, esta resolución destaca la importancia de que los Miembros respeten e implementen las normas internacionales de la OMSA, con miras a combatir eficazmente la influenza aviar. En esta Resolución la No. 28 “Desafíos estratégicos para el control mundial de la influenza aviar de alta patogenicidad” en el inciso 4, se enuncia que “La vacunación con vacunas registradas de alta calidad que sean eficaces contra las cepas de campo en circulación puede proporcionar protección adicional y reducir las cantidades del virus y el riesgo de una mayor propagación. La vacunación requiere la adaptación de la vigilancia para la detección precoz, la demostración de la ausencia de influenza aviar de alta patogenicidad y el seguimiento de los cambios en las cepas en circulación. De acuerdo con las normas internacionales de la OMSA, el uso de la vacunación no afectará al estatus de un país o zona libre de influenza aviar de alta patogenicidad si su vigilancia respalda la ausencia de infección”.

Actualmente en México, contamos con vacunas producidas con un alto nivel de tecnología, aprobadas por la autoridad sanitaria nacional al haber comprobado su eficacia y su valor epidemiológico que permiten diferenciar aves vacunadas de aves infectadas por cepas de campo; sistema DIVA (*Diferentiating Infected from Vaccinated Animals*); esta herramienta es importante en el plan de prevención y control de enfermedades, siempre supervisada por las autoridades sanitarias.

Por lo anterior, se considera importante considerar la vacunación contra la Influenza Aviar, teniendo como objetivos críticos:

1. Prevenir la enfermedad en las aves vacunadas, dándoles resistencia ante un desafío.
2. Evitar signos clínicos y mortalidad que repercute en las pérdidas para los avicultores, así como en el abasto de proteína para la población.
3. Reducir la excreción viral lo que conlleva al control de la enfermedad con un medioambiente libre de enfermedad contribuyendo al bienestar de todos.

Agradecemos de antemano su amable atención quedando a sus órdenes
Atentamente.

Servicio Técnico CEVA Salud Animal

LITERATURA:

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3.**-Review : A Decade of Avian Influenza in Bangladesh: Where Are We Now?** Nadia A. Rimi [1,*], Md. Zakiul Hassan [1], Sukanta Chowdhury [1], Mahmudur Rahman [1], Rebeca Sultana [1], Paritosh K. Biswas [2], Nitish C. Debnath [2], SK Shaheenur Islam [3] and Allen G. Ross[1]. Trop. Med. Infect. Dis. **2019**, 4, 119;doi:10.3390/tropicalmed4030119

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5.- **Review Status and Challenges for Vaccination against Avian H9N2 Influenza Virus in China** Jinze Dong †, Yong Zhou †, Juan Pu and Litao Liu* *Life 2022, 12, 1326. https://doi.org/10.3390/life12091326*

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7.- **Session Keynote Address— Avian Influenza in Italy 1997–2001** I. Capua,[A] S. Marangon,[B] M. dalla Pozza,[B] C. Terregino,[A] and G. Cattoli[A] . AVIAN DISEASES 47:839–843, 2003

8.- **Vaccination of chickens against H5N1 avian influenza in the face of an outbreak interrupts virus transmission**. Trevor M. Ellis[1 *], Connie Y. H. C. Leung[2], Mary K. W. Chow[3], Lucy A. Bissett[1], William Wong1, Yi Guan[2] and J. S. Malik Peiris[2] Avian Pathology (August 2004) 33(4), 405 - 412

Avian Influenza in Italy 1997–2001

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AVIAN DISEASES 47:839–843, 2003

Session Keynote Address—

Avian Influenza in Italy 1997–2001

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SUMMARY. From 1997 to 2001, Italy has been affected by two epidemics of high-pathogenicity avian influenza. The first epidemic was caused by a virus of the H5N2 subtype and was limited to eight premises in backyard and semi-intensive flocks. The prompt identification of the disease was followed by the implementation of European Union (EU) directive 92/40/EEC and resulted in the eradication of infection without serious consequences to the poultry industry. The 1999–00 epidemic was caused by a virus of the H7N1 subtype that originated from the mutation of a low pathogenic virus and resulted instead in a devastating epidemic that affected industrially reared poultry, culminating in the infection of 413 flocks. The description of the epidemics and the result of the control policies are reported.

RESUMEN. Influenza Aviar en Italia 1997–2001.

Durante el periodo comprendido entre los años 1997 y 2001, Italia ha sido afectada por dos epidemias causadas por virus de influenza aviar altamente patógenos. La primera epidemia fue causada por un virus del tipo H5N2, la cual se limitó a 8 parvadas de traspatio y a explotaciones semi-intensivas. La rápida identificación de la enfermedad fue seguida por la implementación de la directiva de la Unión Europea 92/40/EEC, resultando en la erradicación de la infección sin que se presentaran consecuencias serias para la industria avícola del país. La epidemia de los años 1999–00, causada por un virus de influenza del tipo H7N1 originado por mutaciones ocurridas en un virus de baja patogenicidad, resultó en una epidemia devastadora que afectó la industria avícola y culminó en la infección de 413 parvadas. En este trabajo se reportan las descripciones de las epidemias y los resultados de las políticas de control adoptadas.

Key words: avian influenza, Italy, epidemic, H5N2, H7N1, H7N3, control, vaccine

Abbreviations: AI = avian influenza; DIVA = differentiating infected from vaccinated animals; DPPA = densely populated poultry areas; EU = European Union; HPAI = high-pathogenicity avian influenza; IVPI = intravenous pathogenicity index; LPAI = low-pathogenicity avian influenza; OIE = Office International des Epizooties

Avian influenza (AI) is a viral disease of poultry, and high-pathogenicity avian influenza is included as an Office International des Epizooties (OIE) List A disease. In the European Union the regulations for its control are imposed by European Union (EU) directive 92/40/EEC (8). The disease may have devastating effects on the poultry industry, particularly when it affects intensive poultry rearing systems and its presence in a given territory results in restrictions on animal movements, marketing, and trade of poultry and poultry products.

Italy has been affected by two different epidemics of high-pathogenicity avian influenza (HPAI), in 1997–98 and in 1999–2000, caused by two different viruses of the H5N2 and of the H7N1 subtypes, respectively.

Although both epidemics occurred in the north-

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eastern part of Italy, they had different impacts on the poultry industry, primarily due to the production circuits that were affected. The characteristics, consequences, and control policies applied in the two outbreaks are reported below.

1997–98 H5N2 HPAI EPIDEMIC

The epidemic consisted of a total of eight premises (3), in backyard or semi-intensive flocks, located in the Veneto, Friuli, Venezia, and Giulia regions. Although the origin of the epidemic was not established, the epidemiological investigation allowed the identification of risk factors in the affected farms, primarily the marketing of infected birds, presence of mixed species, and rearing of birds in the open. The disease was eradicated by the prompt implementation of directive 92/40/EEC. A total of 7731 birds were depopulated, and no further isolations of the H5N2 virus have been made to date.

1999–00 H7N1 EPIDEMIC

During 1999 and 2000 northeastern Italy has been affected by a devastating epidemic of HPAI, caused by a type A influenza virus of the H7N1 subtype that originated from the mutation of a low-pathogenicity avian influenza (LPAI) virus of the same subtype (1). The LPAI epidemic and the subsequent HPAI epidemic occurred in the Veneto and Lombardia regions, which raise 65% of Italy's intensively reared poultry. In addition, some areas affected by the epidemic (particularly south of Verona province) are densely populated poultry areas (DPPA), with some municipalities of Verona province having a density of 70,000 birds raised per square kilometer.

The HPAI epidemic caused directly or indirectly the death or culling of over 13 million birds, which resulted in a serious disruption of the marketing system, great economic losses to the poultry industry, and distress in the social community. Following depopulation and restocking of the HPAI infected areas, LPAI re-emerged twice, thus leading to the request from the poultry industry to vaccinate against H7 avian influenza.

First epidemic wave of low pathogenicity avian influenza. On the March 29, 1999, the first isolation of a type A, H7 avian influenza virus was officially announced. The virus was further characterized, in accordance with EU directive 92/40/EEC (8), as a LPAI virus of the H7N1 subtype. The intravenous pathogenicity index (IVPI) of the isolate in 6-week-old SPF chickens was of 0.0, and the deduced amino acid sequence of the cleavage site of the hemagglutinin protein was typical of LPAI viruses since it did not contain multiple basic amino acids (5).

Following the first official notification, a total of 199 flocks were diagnosed with H7 influenza infection. Most of the infected flocks were meat turkeys (164), with only a limited number of turkey breeder, chicken (twelve in layers, eleven in broiler breeders, and four in broilers), and guinea-fowl (two) flocks affected. From the epidemiological inquiry it appeared that from the time of the first submission, over 70 turkey farms had already been infected. The disease was particularly severe in the turkey industry, causing severe losses to farmers (2).

Nevertheless, this virus did not meet the definition of the controllable disease, "avian influenza," since it did not have the characteristics listed in EU directive 92/40/EEC. Therefore a compulsory stamping out policy could not be implemented, and it was not possible at the time to stamp out such a large number of flocks on a voluntary basis. In addition, controlled marketing and quarantine practices could not be enforced at the time. Moreover, since LPAI is not considered in Italian veterinary legislation, there were no legislative tools to prevent its spread. However, the regional authorities of the two affected regions implemented restriction orders with the aim of reducing the number of new outbreaks. The main strategies of these orders were to avoid movement of viremic birds and to avoid movement of dead birds and infected litter, which were identified as being among the primary sources of infection. These policies, aided by the oncoming warm season, led to a decrease in the number of newly infected flocks during the summer.

Emergence of highly pathogenic avian influenza. On the December 13, 1999, a private practitioner submitted pathological samples from a meat turkey flock exhibiting high mortality rates. The outbreak was confirmed as HPAI on December 17 with the characterization of an H7N1 isolate with an IVPI index of 3.0 and a deduced amino acid sequence containing multiple basic amino acids, typical of highly pathogenic avian influenza viruses (15).

Owing to the complex field situation (isolation of an H7 virus was not unusual at the time), it was not possible to determine immediately the presence of HPAI virus and to promptly implement eradication measures, thus aiding in the spread of infection. Furthermore, the holiday season was approaching and high slaughter levels resulted in a further spread

of the virus with complete loss of control of infection. Four hundred thirteen outbreaks were diagnosed involving 177 meat turkey flocks, 121 table-egg layer flocks, 39 broiler flocks, 29 broiler breeder flocks (6), 25 backyard flocks (4), 9 guinea-fowl flocks, 6 turkey breeder flocks, 3 ostrich farms (5), 2 pheasant flocks, 1 Pekin duck flock, 1 quail flock, and death of over 13,000,000 birds. The last outbreak was confirmed on April 5, 2000.

As a result of the mass mortality (stamping out policy and pre-emptive slaughter), several establishments such as hatcheries, feed mills, abattoirs, processing plants, and other connected activities were forced to interrupt their activity, causing unemployment and heavy economic losses to the poultry industry and to the community, due to disruption of the marketing system. Further economic losses also resulted from the export bans imposed on the infected regions and by the depopulation of the infected area.

Eradication of HPAI. Following the implementation of directive 92/40/EEC (8) infected flocks were stamped out, and cleaning and disinfection of infected premises was carried out. To improve eradication procedures, depopulation of intensively reared poultry in the infected area was imposed. With the exclusion of a few regularly tested breeder and game farms (that were kept for repopulation purposes) an area of 5,500 square kilometers was depopulated, including intensive and semi-intensive flocks that remained empty for a minimum period of 60 days. Restocking began on June 15, 2000.

Second and third LPAI epidemic waves. On August 14, 2000, a clinical suspicion of LPAI was detected in a turkey flock located in the DPPA and was confirmed by the laboratory on August 20, 2000. The Italian Ministry of Health ordered the eradication of infection with a stamping out policy imposed by an extraordinary act. Fifty-five outbreaks were diagnosed and eliminated through stamping out or controlled marketing.

A vaccination policy against avian influenza was, at this point, strongly requested by the farmers and by the poultry industry, and a vaccination program was drawn up and approved by the European Commission.

The third epidemic wave of LPAI was detected on December 22, 2000, and involved 23 flocks. Among these only one was vaccinated. The epidemic was controlled by stamping-out and controlled marketing.

Vaccination policy. The vaccination program began on November 15, 2000, and will last until May 2002. Six million birds [only meat type birds and table-egg layers (that apply the all-in all-out system)] raised in a restricted zone (1,156 km^2) south of Verona were involved in the vaccination program. No vaccinated live birds or poultry products that originate from the vaccination zones were authorized for intracommunity trade.

In order to aid the official control of the infection and to develop a novel control strategy, the vaccine that was used did not contain a homologous H7N1 virus but has been prepared from an inactivated H7N3 virus (A/CK/Pakistan/95/H7N3). This allowed the possibility of a natural "marker" vaccine, or more correctly a differentiating infected from vaccinated animals (DIVA) vaccine. In fact, the presence in the vaccine of an H7 antigen ensures protection against clinical signs and the reduction of virus shedding, since it is well known that neutralizing antibodies to influenza A viruses are induced primarily by the hemagglutinin protein (14). The presence of a different neuraminidase (N) subtype, which induces specific antibodies (against N3 rather than N1), has enabled, with the aid of an *ad hoc* diagnostic test (7), discrimination between infected and vaccinated flocks, and has allowed continued monitoring of the outbreak.

DISCUSSION

A few considerations can be made from retrospectively analyzing the experience gained in the past 5 years with avian influenza in Italy. Firstly, northeastern Italy can be considered as an area at risk for avian influenza infections. This is also supported by AI epidemics that have occurred in the past (9,10,11,12,13) caused by viruses of the H6 and H9 subtypes. This could probably be linked both to the large numbers of wild birds that fly through the area during their migration, the large numbers of live birds imported into the area, and the high poultry density. For these reasons, it is imperative that surveillance programs are implemented to diagnose AI infections promptly.

The comparison between the 1997–98 and 1999–00 epidemics points out that if HPAI is diagnosed promptly and is not preceded by extensive circulation of the LPAI progenitor, the application of the measures imposed by directive 92/40/EEC is efficacious in disease eradication. The devastating impact of the HPAI H7N1 epidemic in 1999–00 was linked to loss of control of infection, primarily due to the previous circulation of the LPAI virus, which caused difficulties in identifying

infected flocks promptly. Clearly, spread of infection was also aided by the penetration of infection in the industrial circuits of intensively reared poultry.

The Italian 1999–00 AI epidemic also emphasized that farmers and private companies should bear well in mind that within the current European legislation there is no financial aid from local or national governments or from the European Union in case of LPAI. Therefore, voluntary and permanent surveillance programs should be implemented in order to allow the prompt diagnosis of infection by H5 and H7 LPAI viruses, to allow the enforcement of restriction and eradication policies until this is economically feasible. In the spring of 1999 we were faced with more then 70 infected flocks, and it was not possible to enforce restriction policies, including stamping out of infected flocks without compensation.

The spread of infection was also a result of the structure and organization of the local poultry industry. In several areas worldwide, the poultry industry has substantially grown in an often irrational way, particularly where a semivertical integration system has developed. This system (i.e., house owned by the farmer and day-old chicks and feed supplied by private company) has the disadvantage that there is no planning behind the spatial distribution of the units that are involved in the system, and, furthermore, there are a sensible number of contacts between establishments. In fact, feed trucks and other vehicles (e.g., abattoir delivery) frequently visit a number of farms daily, regardless of the species reared and of the type of production, and basic biosecurity measures are rarely respected. The concentration of poultry houses, hatcheries, abattoirs, litter processing plants, and other establishments in a restricted area is definitely convenient from an organizational point of view, but has a series of drawbacks from the sanitary point of view that dramatically emerge when an epidemic of a highly contagious disease occurs.

The disruption of the marketing system determined social consequences, forcing farmers out of business and in some instances favoring the use of illegal vaccines. This practice could have led to the re-emergence of LPAI, through the movement of infected birds or litter collected from farms containing clinically healthy carriers.

With reference to control, the strategy implemented in Italy indicates that the association between strict biosecurity, animal movement restrictions, and a DIVA vaccination program can be effective in controlling AI. However, in our opinion it is imperative that the results obtained from the territorial control strategy are made available to support decision making, and this can only be achieved if there is extensive collaboration between farmers, official and field veterinarians, the poultry industry, the diagnostic laboratories, the epidemiology units, and the central and local governments. Only in this way will it be possible to establish a network of collaboration able to make the best of the data and tools available in the effort to control avian influenza infections in poultry.

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Vaccination of chickens against H5N1 avian influenza in the face of an outbreak interrupts virus transmission

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Vaccination of chickens with a commercially available killed H5N2 vaccine was being evaluated as an additional tool to enhanced biosecurity measures and intensive surveillance for control of highly pathogenic avian influenza subtype H5N1 disease in Hong Kong in 2002. In December 2002 to January 2003, there were outbreaks of H5N1 disease in waterfowl in two recreational parks, wild water birds, several poultry markets and five chicken farms. In addition to quarantine, depopulation of the affected sheds and increased biosecurity, vaccination of the unaffected sheds and surrounding unvaccinated farms was undertaken on three farms. In at least two farms, infection spread to the recently vaccinated sheds with low rates of H5N1 mortality in sheds when the chickens were between 9 and 18 days post-vaccination. However, after 18 days post-vaccination no more deaths from H5N1 avian influenza occurred and intensive monitoring by virus culture on these farms showed no evidence of asymptomatic shedding of the virus. This provides evidence that H5 vaccine can interrupt virus transmission in a field setting.

Introduction

Outbreaks of H5N1 highly pathogenic avian influenza (HPAI) have occurred in Hong Kong in chickens and other gallinaceous poultry in 1997, 2001, 2002 and 2003 (Sims *et al*., 2003; Ellis *et al*., 2004a). High mortality rates were seen in gallinaceous birds on farms (1997, 2002 and 2003) and/or in poultry markets (1997, 2001, 2002, 2003) in all outbreaks and in wild or captive waterfowl (geese, ducks and swans) in outbreaks in two bird parks during December 2002 to January 2003. Deaths also occurred in other wild or captive water birds (Little Egrets, *Egretta garzetta*; Greater Flamingo, *Phoenicopterus ruber*; Grey Heron, *Ardea cinerea*; Black-headed Gull, *Larus ridibundus*) during these outbreaks (Ellis *et al*., 2004a). Outbreaks of H5N1 HPAI were also detected in five chicken farms in Hong Kong in late December 2002 and January 2003. These were detected after the outbreaks were detected in water birds in the two bird parks and the detection of H5N1 HPAI in the two wild Grey Herons.

The 1997, 2001 and early 2002 H5N1 outbreaks had substantial economic impacts in Hong Kong due to costs of partial or total depopulation of poultry, closure of live poultry markets, reductions in tourism and the costs of a comprehensive H5N1 testing and surveillance system for local and imported poultry. After the outbreak in 2001, the poultry farm and market biosecurity measures and monitoring systems in place since 1998 were enhanced. Following a detailed epidemiological study of the February to April 2002 H5N1 HPAI

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outbreak, further measures were introduced to improve farm and market biosecurity. However, due to the large daily movement of poultry into retail live poultry markets of Hong Kong from farms in Hong Kong and elsewhere in southern China, together with the possibility of H5N1 virus infections occurring in the wider region, the Hong Kong SAR Government investigated the use of H5 avian influenza vaccination as an additional control measure for H5N1 avian influenza.

A field evaluation trial using Nobilis® Influenza H5, an inactivated avian influenza Type A H5N2 virus (A/Chicken/Mexico/232-CPA/94) water-in-oil emulsion vaccine (Intervet International, Boxmeer, The Netherlands), on chicken farms was commenced in April 2002 in the district where the last four farm outbreaks of H5N1 had occurred, and evaluation continued until March 2003. The evaluation trial showed that acceptable flock H5 antibody responses were generated by the vaccine and vaccinated chickens were protected from high-dose laboratory challenge with H5N1 HPAI viruses from the February to April 2002 outbreaks (Ellis *et al*., 2004b). In December 2002, the H5N1 outbreaks in waterfowl and wild birds and the detection of viruses in several retail markets, together with the encouraging vaccine trial results, led to the decision to broaden the chicken farm vaccination area to cover those areas most exposed to wild bird movements. H5N1 outbreaks occurred in five unvaccinated chicken farms in late December 2002 and January 2003. Strict quarantine and movement controls were initiated immediately, followed on two farms by total depopulation with ring vaccination of surrounding farms. In three other affected farms only sheds showing high or rising mortality rates were depopulated together with vaccination of unaffected sheds and surrounding farms. The effect of killed H5N2 vaccination in the face of these outbreaks was monitored in terms of protection from disease and ability to interrupt transmission of H5N1 virus.

Materials and Methods

Outbreak history. The three chicken farms referred to in this report rear chickens in semi-enclosed sheds in A-frame cage banks, as do most of the 154 chicken farms in Hong Kong. Farm sizes in Hong Kong vary between approximately 20 000 and 100 000 chickens and they rear slow-growing yellow–brown broiler chickens, which are imported as one-day-old chickens from Mainland China and are usually marketed at between 80 and 100 days of age.

Vaccinations on the affected farms were given using a single standard 0.5 ml dose of the Nobilis® Influenza H5 vaccine using the subcutaneous route at the base of the neck. Separate farm staff members were used to vaccinate the different sheds and all staff wore their standard working uniform. Disinfectant footbaths containing Virkon™ were located at the entrance of each shed and hand washing facilities were available and were used by staff. Experienced teams of Agriculture, Fisheries and Conservation Department (AFCD) staff were involved in depopulation of chicken sheds and they wore impervious protective overalls with built-in hoods, boots, gloves and masks and were not permitted into other sheds on the farms.

Farm 1. A moderate sized farm (51 000 chickens) in Tai Kong Po (see farm plan in Figure 1) reported increased mortalities (about 35 chickens) on 6 January 2003, and follow-up inspection by AFCD staff detected further deaths in three sheds (shed 7 had 107 dead/6000 in 98-day-old birds, shed 8 had 44 dead/1500 in 65-day-old birds and shed 11 had eight dead/2600 in 77-day-old birds) by that afternoon. Rapid testing (real-time reverse transcriptase polymerase chain reaction [RRT-PCR] for H5) detected HPAI H5 virus that evening. Movement control and strict attention to biosecurity was initiated immediately and the farm was officially quarantined on 7 January 2003. However, unresolved issues with compensation resulted in culling of chickens in the affected sheds being delayed. Vaccination with the killed H5N2 vaccine was started in the six unaffected sheds on the farm on 7 January 2003. Mortalities commenced in sheds 3, 6 and 10, which were adjacent to the three initially affected sheds, on 8 January 2003. Subsequently mortalities started to rise rapidly in sheds 7, 8 and 11, then in sheds 3, 6 and 10 and by 15 January 2003 in sheds 4 and 5 (Table 1). The 8000 remaining chickens in sheds 7, 8 and 11 were killed on 10 January 2003 and the 12 000 remaining chickens in sheds 3, 4, 5, 6 and 10 were killed on 15 January 2003.

On 16 January 2003 (day 9 post-vaccination) deaths, confirmed as H5N1 HPAI after postmortem examination, H5 RRT-PCR and virus culture in chicken embryos, were detected in the remaining large shed on the farm (shed 2 containing 19 000 chickens). This shed was physically well isolated from the other sheds on the farm and staffed by a separate group of workers. Mortalities confirmed as H5-positive continued at a low level until 25 January (day 18 post-vaccination) (Table 1). Sequential measurements of haemagglutination inhibition (HI) test antibody levels to H5 avian influenza were conducted for chickens from this shed (days 15, 22, 28, 34, 38 and 42 post-vaccination). Cloacal swabs were collected from 60 randomly selected chickens in this shed for virus culture on days 15, 22 and 28 post-vaccination by AFCD. A larger cross-sectional sampling of chickens in the shed for virus culture (300 throat and 300 cloacal swabs each time) was conducted by the Department of Microbiology, University of Hong Kong on days 28, 33 and 38 post-vaccination.

Farm 2. A second farm (35 000 chickens) in Tai Kong Po reported increased mortalities (about 150 chickens in two batches of chickens) in two adjacent sheds (shed 1 with 46-day-old chickens and shed 3 with 39-day-old chickens) on 20 January 2003. Rapid testing (postmortem examination and H5 RRT-PCR) diagnosed H5 HPAI. Movement controls and strict adherence to biosecurity procedures were instigated immediately. The farm was placed in quarantine and the 5300 chickens in those sheds were killed on 21 January 2003. This farm was near the first outbreak farm at Tai Kong Po and had been included in the ring vaccination programme around that farm. Shed 3 was vaccinated on 8 January 2003 and Shed 1 on the 14 January 2003. Other sheds were vaccinated on 9 to 15 January 2003.

The remaining chickens on the farm were checked daily for mortalities that were investigated to determine the cause of death. On 22 to 23 January 2003 AFCD staff collected 180 chicken cloacal swabs and 120 fresh faecal droppings the trays under the cages from sheds 2, 4 and 5 for virus culture. On 11 February 2003 60 cloacal swabs were randomly collected from the market-aged chickens (about 100 days old) and on 20 February 2003 100 swabs of fresh faecal droppings from the cage trays were also collected and tested by virus culture from the market aged birds. Subsequently, the seven batches of market-aged chickens between February and April 2003 all had 60 cloacal swabs randomly collected and tested by H5 RRT-PCR and virus culture.

Serum antibody levels were measured by H5 HI tests on 14 birds from shed 4 at 10 days post-vaccination and 14 birds from shed 2 at 13 days post-vaccination on 22 January 2003, then 30 birds each from shed 2 and 4 (30 to 33 days post-vaccination) on 11 February 2003.

Farm 3. A third farm (20 700 chickens) in Shek Kong Tsuen reported increased mortalities (about 100 chickens of 38 days old) in a single shed on 20 January 2003. Rapid testing (post-mortem examination and H5 RRT-PCR) detected HPAI H5 virus that day. This farm was in a separate valley 1.5 km away from the Tai Kong Po farms. The farm was immediately placed in quarantine and the 5600 chickens in the affected shed were killed on 21 January 2003. All remaining chickens on the farm between 8 and 55 days old were vaccinated and ring vaccination

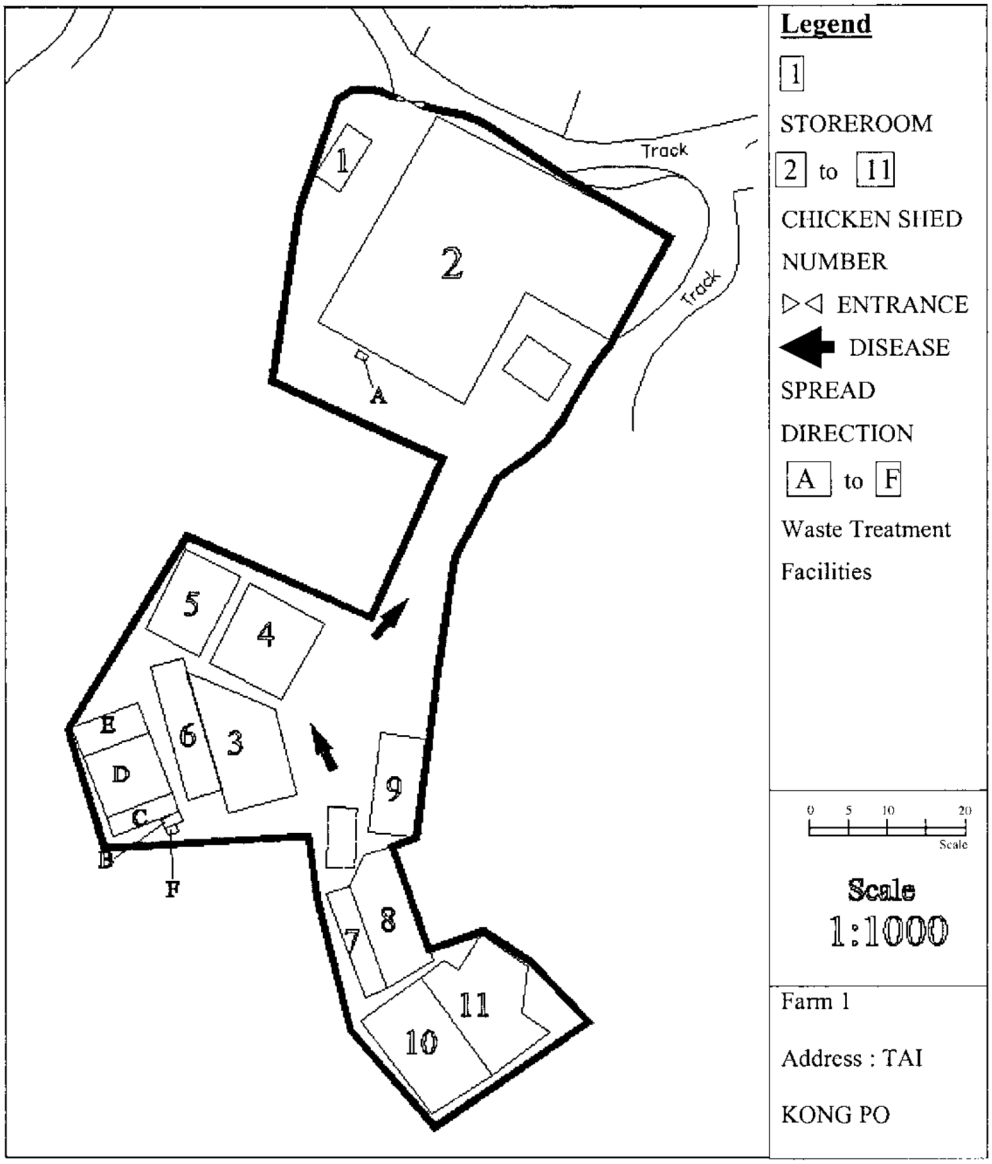

Figure 1. *Location plan of chicken sheds on Farm 1.*

was conducted on the nine farms nearby (involving 212 000 chickens) on 23 January 2003.

The remaining chickens on the farm were checked daily for mortalities, which were investigated immediately to determine the cause of death. Blood samples were collected from 14 chickens in all batches of market age chickens to measure H5 antibody responses. From each batch of market-aged chickens between February and April 2003, 60 cloacal swabs were randomly collected for H5 RRT-PCR testing and virus culture.

Laboratory test procedures. Dead birds were subjected to postmortem examination as described for previous H5N1 outbreak investigations (Ellis *et al*., 2004a). Pooled cloacal and tracheal swabs from each dead bird were suspended in antibiotic containing viral transport media and subsequently inoculated into the allantoic cavity of 9-day-old to 11-day-old specific pathogen free chicken embryos following standard procedures (Alexander, 2000). Cloacal swabs, throat swabs and swabs of faecal droppings from clinically healthy chickens that were collected for avian influenza virus surveillance in the chicken sheds were similarly tested. Allantoic fluid from eggs with dead embryos and all eggs at 4 days post-inoculation were tested for presence of haemagglutinins (HA) of chicken red blood cells, and HA-positive allantoic fluid was routinely subjected to HI tests using reference antisera (Veterinary Laboratory Agency, Weybridge, UK and USDA, Ames, IA, USA) to avian influenza subtypes H5 and H9 and Newcastle disease virus by standard procedures (Alexander, 2000).

Cloacal and faecal dropping swabs for surveillance testing of chicken sheds were also tested for presence of the H5 HA gene by the RRT-PCR test described by Spackman *et al*. (2002). HA-positive allantoic fluids from the virus cultures were also tested for the H5 gene by the RRT-PCR. Any isolated H5 virus was further characterized by sequencing of virus gene segments at the Department of Microbiology, University of Hong Kong using procedures described previously (Guan *et al*., 2002).

Antibody levels to H5 avian influenza virus in chicken sera were tested by the standard HI test procedures described by Alexander (2000) using avian influenza A/chicken/Hong Kong/97 (H5N1) virus antigen.

Results

Affected dead chickens from all three farms had gross and microscopic pathology changes consistent with previous H5N1 HPAI cases in chickens in Hong Kong. The chickens shown as H5N1 positive in Tables 1 and 2 gave positive results by H5 RRT-PCR tests on cloacal swabs and H5N1 virus was isolated by chick embryo allantoic cavity inoculation. Partial gene sequencing of eight gene

Table 1. *Record of the number of dead chickens in respective sheds on Farm 1 (Tai Kong Po)*

Date	Sheds 7, 8, 11	Shed 10	Sheds 3, 6	Sheds 4, 5	Shed 2
Number of chickens (age)	10 300 (65 to 98 days old)	8000 (26 days old)	3000 (62 days old)	10 300 (28 days old)	19 000 (62 to 74 days old)
3/1/03	7 to 8				
4/1/03	5 to 6				
5/1/03	11 to 12				
6/1/03	194				
7/1/03	403	Vaccinated	Vaccinated	Vaccinated	Vaccinated
8/1/03	318	36	7	0	0
9/1/03	493	0	4	0	0
10/1/03[a]	819	700	15	0	0
11/1/03	Depopulated	210	0	0	7 (negative)[b]
12/1/03		220	12	0	0
13/1/03		250	0	0	4 (negative)
14/1/03		600	18	0	0
15/1/03[c]		2500	26	6000	2 (negative)
16/1/03		Depopulated	Depopulated	Depopulated	10 (H5-positive)[d]
17/1/03					4 (H5-positive)
18/1/03					4 (H5-positive)
19/1/03					3 (H5-positive)
20/1/03					6 (negative)
21/1/03					8 (H5-positive)
22/1/03					10 (H5-positive)
23/1/03					11 (H5-positive)
241/03					12 (H5-positive)
25/1/03					6 (H5-positive)
26/1/03					6 (negative)
27/1/03					4 (negative)
28/1/03					5 (negative)
29/1/03					5 (negative)
30/1/03					4 (negative)
31/1/03					6 (negative)
1/2/03 to 7/2/03					All 0

[a]A total of 8000 chickens were culled from sheds 7, 8 and 11 on 10 January 2003.
[b](negative), negative for H5 virus isolation.
[c]A total of 12 000 chickens were culled from sheds 3, 4, 5, 6 and 10 on 15 January 2003.
[d](H5-positive), H5N1 avian influenza virus was isolated from dead chickens.

segments from representative viruses from these farms showed that all viruses were similar and they were also similar to H5N1 viruses that had been detected from the outbreak in waterfowl at one water bird park and in wild Grey Herons in December 2002.

Farm 1 investigation. H5N1 infection was detected in shed 2 on 16 January, 9 days post-vaccination, and dead chicken continued to be detected until 25 January (day 18 post-vaccination). However, subsequently no sick or dead birds were diagnosed as H5 avian influenza in shed 2 (Table 1). No subclinical infection with H5N1 virus was detected by virus culture of random samples of cloacal and throat swabs from 60 clinically normal chickens from this shed by AFCD on days 15, 22 and 28 post-vaccination or from cloacal and throat swabs collected from 300 clinically normal chickens on days 28, 33 and 37 post-vaccination in the cross-sectional sampling in this shed by University of Hong Kong.

The sequential antibody responses to H5 avian influenza in vaccinated chickens in shed 2 are indicated in Table 3. By day 22 post-vaccination, 81.7% chickens had H5 antibody titre ≥ 16 and the overall GMT for these birds was 33.9. By this time there had been no mortalities due to H5 avian influenza or H5 virus isolations for 4 days.

Farm 2 investigation. The dead bird monitoring in sheds 2, 4 and 5 showed that a small number of chickens in each of these sheds died of H5N1 HPAI (diagnosed by postmortem examination, RRT-PCR and virus culture) on 25 to 26 January 2003, indicating that these sheds had also been exposed to H5N1 virus. Two other more isolated sheds of younger chickens on the farm showed no H5N1 HPAI (Table 2).

By the time the affected chickens were detected in sheds 2, 4 and 5, these batches were already 13 to 17 days post-vaccination and there was only limited disease in these sheds over a 2-day period.

Prior to marketing of the first batch of chickens from this farm, virus culture testing of cloacal swabs collected from 60 randomly sampled chickens and swabs of 100 randomly collected fresh faecal droppings from the cage trays were shown to be negative for H5N1 viruses. Subsequently, no H5N1 virus was detected by H5 RRT-PCR or by virus culture from 60 randomly sampled cloacal

Table 2. *Record of the number of dead chickens in respective sheds on Farm 2 (Tai Kong Po)*

Date	Shed 1	Shed 3	Shed 2	Shed 4	Shed 5	Sheds A, B
Number of chickens (age)	2800 (46 days old)	2 500 (39 days old)	5000 (95 to 102 days old)	5800 (52 to 88 days old)	10 200 (59 to 81 days old)	8700 (16 to 32 days old)
Date vaccinated	14/1/03	8/1/03	9/1/03	12-13/1/03	10-11/1/03	15/1/03
20/1/03	150 (14 H5-positive pools)[a]			0		0
21/1/03	Depopulated			0		0
22/1/03				0		0
23/1/03				0		0
24/1/03				0		0
25/1/03				2 (1 H5 +ve pool)		0
26/1/03				8 (2 H5 +ve pools)		2 (−ve)[b]
27/1/03				0		5 (−ve) (1 NDV +ve)[c]
28/1/03 −1/2/03				0		0
2/2/03				0		20 (−ve) (1 H9 +ve)[d]
3/2/03				0		3 (−ve)
4/2/03				0		3 (−ve)
5/2/03 to 6/2/03				0		0
7/2/03				0		2 (−ve)

[a](H5-positive pool), H5N1 avian influenza virus was isolated from pooled cloacal and throat swabs from the dead chickens.
[b](negative), negative for H5 virus isolation.
[c]NDV, Newcastle disease virus.
[d]H9, avian influenza H9N2 virus.

swabs from the next seven batches of market-aged chickens from this farm.

H5 HI antibody titre $\geq$16 was detected in six of 14 (42.9%) of chickens in shed 4 at 10 days post-vaccination, four of 14 (28.6%) of chickens in shed 2 at 13 days post-vaccination and in all chickens from both shed 2 and 4 at 30 to 33 days post-vaccination (57 of 60 had H5 HI titre $\geq$32 and three chickens had titre = 16).

Farm 3 investigation. No H5N1 HPAI occurred in other sheds on the farm or in the nearby nine farms subsequent to this outbreak. H5N1 virus was not detected by H5 RRT-PCR or by virus culture from 60 randomly sampled cloacal swabs from all batches of market-aged chickens from this farm between February and April 2003.

The first batch of vaccinated chickens was marketed on day 37 post-vaccination, and blood tests on these chickens showed that all (14/14) had H5 HI antibody titres $\geq$32.

Discussion

One of the concerns in the use of vaccine to control HPAI in poultry farms is the possibility that while vaccine may protect from disease, asymptomatic virus circulation may continue, resulting in spread of infection to other farms. The monitoring and surveillance conducted on these three chicken farms showed that use of this killed H5N2 vaccine in the face of HPAI H5N1 virus challenge was able to protect chickens from disease and interrupt virus transmission. The protective effect of vaccine became apparent after day 18 post-vaccination. On farms 1 and 2, clear evidence of H5N1 infection was demonstrated in sheds of vaccinated chickens, and subsequently extensive surveillance by clinical inspection and virus detection tests, both H5 RRT-PCR and virus culture, showed that the virus transmission had been interrupted. For farm 3, the rapid depopulation of the affected shed and strict biosecurity measures applied combined to minimize the level of challenge to other sheds. No

Table 3. *Antibody responses to H5 virus[a] in vaccinated chickens on Farm 1*

Number of days post-vaccination (date)	Number of chickens	Titre 4	Titre 8	Titre 16	Titre 32	Titre 64	Titre 128	Titre 256	% birds positive	Geometric mean titre
15 (22/1/03)	60	28		11	15	4	2		53.3	11.7
22 (29/1/03)	60	11		6	16	15	7	5	81.7	33.9
28 (4/2/03)	60		6	5	49[b]				90	n/a
34 (10/2/03)	60		4		56 [b]				93.3	n/a
38 (14/2/03)	14				14 [b]				100	n/a
42 (18/2/03)	14				14 [b]				100	n/a

[a]Antibody responses were detected by HI tests using A/chicken/Hong Kong/97 (H5N1) antigen.
[b]Tested in a three-dilution test (8, 16, 32) only.
n/a indicates geometric mean titre was not calculated because end-point titres were not available.

evidence of clinical disease or H5N1 infection was demonstrated in the sheds of vaccinated chickens so it is possible that the other sheds on this farm may not have received significant exposure to the H5N1 virus from the initial infected shed.

Vaccines have been used in other countries to assist in the control of avian influenza. Countries that have used vaccines for avian influenza control include Italy (Capua *et al.*, 2002), the US (Halvorson, 2002), Mexico (Villarreal & Flores, 1998) and Pakistan (Naeem, 1998). Mostly vaccination has been directed against low pathogenic strains of avian influenza virus but Mexico and Pakistan have successfully used vaccine against highly pathogenic H5 or H7 avian influenza viruses. Experimental studies have shown that commercially available H5 avian influenza vaccines could protect poultry from 1997 Hong Kong strains of H5N1 HPAI virus (Swayne *et al*., 2001).

On Farm 2, avian influenza H9N2 virus was detected in the sheds containing 16-day-old to 32-day-old chickens. Recent experimental studies have suggested that infection with H9N2 virus may stimulate cell-mediated immune responses that could cross-protect chickens from intranasal H5N1 virus challenge that was lethal in uninoculated controls (Seo & Webster, 2001). This cross-protectivity was effective at 15 days after intranasal inoculation with H9N2 virus given in a low challenge dose (10 50% lethal chicken doses), but its effectiveness was diminished by 30 days post-inoculation. Infection of chickens with H9N2 avian influenza viruses is quite common in chickens in Hong Kong based on monthly serological surveillance conducted by our laboratories on local and imported chickens between 1999 and 2001. The H9N2 viruses isolated from chickens in Hong Kong belong to a lineage of viruses related to A/Duck/Hong Kong/Y280/97 (H9N2) (Guan *et al*., 2000), which generally causes mild or inapparent infections of the upper respiratory tract in chickens. On local farms where H9N2 infection has been monitored, it generally occurs in chickens under 30 days that are reared on litter. By the time they are moved to the A-frame cages infection is less common and chickens of multiple ages on affected farms are H9N2 antibody positive. On farm 2 with H9N2 infection circulating in the 16-day-old to 32-day-old birds it would be highly probable that the older birds (39 to 46 days old) in sheds 1 and 3 would have been exposed to this virus, but this did not prevent the H5N1 outbreak in these sheds. During the 2002 H5N1 outbreak on chicken farms in Hong Kong there appeared to be no correlation between exposure to H9N2 virus, measured by serology, and the severity of the outbreak. The H9N2 AI virus exposure and resulting immunity had no protective effect against the field challenge by H5N1 AI virus possibly because of either short-lived cross-protective cellular immunity or a high environmental challenge dose of H5N1 AI virus.

Avian influenza vaccination has generally been used in uninfected flocks in control areas around but not including infected flocks. From this investigation we are definitely not suggesting that the use of vaccination to assist in the control of an avian influenza outbreak could be delayed in the control area until evidence of spread from infected farm(s) occurs. Nor do we recommend the use of partial depopulation plus vaccination on an infected farm as a normal practice. In the first Tai Kong Po farm, five sheds with 22 000 chickens had to be killed before vaccination had a chance to work in the final shed, and in the meantime outbreaks occurred on two nearby farms that were ring vaccinated at the same time as the initial farm. Generally, when ring vaccination is used for avian influenza control, the infected farm and high-risk contact farms within an epidemiologically sustainable perimeter (usually several kilometres) are quarantined, monitored and possibly depopulated. Ring vaccination is used outside this zone where there is a good chance for immunity to develop to the virus before exposure occurs. The close proximity of farms and limited land availability makes this approach difficult in Hong Kong. For the three farms involved in this investigation the individual circumstances at the time, together with expanding use of preventative vaccination throughout Hong Kong, led to an unusual control strategy involving quarantine, partial depopulation and vaccination of unaffected sheds and surrounding farms. As part of this strategy very strict attention had to be paid to movement control of birds, people and materials onto and from the farm and strict biosecurity practices had to be maintained. This was combined with an intensive monitoring programme on the vaccinated sheds and the surrounding farms to rapidly detect any spread of the infection. This strategy was very resource intensive and would have been very difficult to sustain in a more widespread outbreak.

Another factor that should be considered with vaccinating in the face of an outbreak is the possibility of selection of variant viruses when the virus is replicating rapidly in the presence of partial or incomplete flock immunity. The chance of this occurring will clearly be lower if virus is introduced to a fully vaccinated flock that has had time to develop its immunity. However, concerns expressed about the risk of enhanced H5N1 virus evolution in the presence of a vaccinated antibody-positive chicken population needs to be kept in perspective. If you do not vaccinate, all exposed chickens have the potential to become infected with H5N1 viruses that will replicate to high titres and shed large quantities of virus in faeces and respiratory secretions that will infect further chickens. Each replication cycle increases the number of mutations and

the potential for antigenic variation. There are also many examples of emergence of HPAI avian influenza viruses from low or medium pathogenic avian influenza viruses without any influence from vaccination (Alexander *et al*., 2000). Inactivated oil emulsion avian influenza vaccines have given good protection despite variation of up to 10.9% in haemagglutinin-deduced amino acid sequence (Swayne *et al*., 1999, 2000). Avian influenza vaccination has been most widely practiced in Mexico, beginning in January 1995, and it continues to be used. Over 1.4 billion doses of inactivated vaccine and 500 million doses of fowlpox-AI-H5 recombinant vaccine have been used and the vaccines are still considered protective (Villarreal-Chavez & Rivera-Cruz, 2003).

The ultimate goal of any control programme for avian influenza should be to eradicate HPAI. This was also the goal in Hong Kong during this outbreak, and this goal was achieved. With the presence of these viruses in wild water birds in the region and the large daily cross-border movement of poultry the risk of H5N1 virus incursions infection in Hong Kong is very high. A comprehensive package of measures including enhanced biosecurity programmes for farms, wholesale and retail poultry markets, the use of rest days in markets to break cycles of infection and a comprehensive monitoring and surveillance programme for early detection of any H5 avian influenza virus incursions have been in place since 2001 and were enhanced after the February to April 2002 outbreak. As stressed by international animal health authorities (Alexander *et al*., 2000), avian influenza vaccination in Hong Kong is used to complement the strict biosecurity measures and a comprehensive monitoring and surveillance programme already in place. Comprehensive vaccination of all chicken farms supplying the local retail markets was introduced as an additional layer of protection after a one year long vaccination evaluation trial (Ellis *et al*., 2004b). This investigation showed that the use of killed H5N2 vaccine on three farms undergoing H5N1 HPAI outbreaks was able to protect chickens against disease and also to interrupt asymptomatic virus shedding. This is particularly relevant when dealing with viruses such as H5N1 where the virus also poses a significant risk to human health.

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Review

Success Factors for Avian Influenza Vaccine Use in Poultry and Potential Impact at the Wild Bird-Agricultural Interface

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Abstract: Thirty-two epizootics of high pathogenicity avian influenza (HPAI) have been reported in poultry and other birds since 1959. The ongoing H5N1 HPAI epizootic that began in 1996 has also spilled over to infect wild birds. Traditional stamping-out programs in poultry have resulted in eradication of most HPAI epizootics. However, vaccination of poultry was added as a control tool in 1995 and has been used during five epizootics. Over 113 billion doses of AI vaccine have been used in poultry from 2002 to 2010 as oil-emulsified, inactivated whole AIV vaccines (95.5%) and live vectored vaccines (4.5%). Over 99% of the vaccine has been used in the four H5N1 HPAI enzootic countries: China including Hong Kong (91%), Egypt (4.7%), Indonesia (2.3%), and Vietnam (1.4%) where vaccination programs have been nationwide and routine to all poultry. Ten other countries used vaccine in poultry in a focused, risk-based manner but this accounted for less than 1% of the vaccine used. Most vaccine ''failures'' have resulted from problems in the vaccination process; i.e., failure to adequately administer the vaccine to at-risk poultry resulting in lack of population immunity, while fewer failures have resulted from antigenic drift of field viruses away from the vaccine viruses. It is currently not feasible to vaccinate wild birds against H5N1 HPAI, but naturally occurring infections with H5 low pathogenicity avian influenza viruses may generate cross-protective immunity against H5N1 HPAI. The most feasible method to prevent and control H5N1 HPAI in wild birds is through control of the disease in poultry with use of vaccine to reduce environmental burden of H5N1 HPAIV, and eventual eradication of the virus in domestic poultry, especially in domestic ducks which are raised in enzootic countries on range or in other outdoor systems having contact with wild aquatic and periurban terrestrial birds.

Keywords: Avian influenza, disease, poultry, vaccination, vaccines, wild birds

Introduction

Since 1959, high pathogenicity avian influenza (HPAI) has caused 32 epizootics in avian species, mostly domestic poultry, including the H5N1 HPAI panzootic that began in Guangdong China in 1996 and has spread to affect 63 countries in Asia, Africa, and Europe in the past 17 years (OIE 2012a; Swayne et al. 2013). HPAI has affected wild birds in three epizootics: H5N3 of common terns (*Sterna hirundo*) in South Africa during 1961; H5N1 in a variety of wild bird species in Asia, Africa, and Europe since 2002; and H7N3 in a few passerine and columbiforme birds in Mexico during 2012 (Becker 1967; Ellis et al. 2004; OIE

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2012c). Historically, the reservoir of all avian influenza virus (AIV) genes, including all 16 hemagglutinin and 9 neuraminidase subtypes, are found in low pathogenicity avian influenza (LPAI) viruses (LPAIV) circulating in the wild waterfowl reservoir, mainly in birds of the orders Anseriformes and Charadriiformes, although rare infections with LPAIV have been documented in other aquatic birds. On sporadic occasions, these wild bird LPAIV have been transferred to poultry within agricultural systems and, through a process of exposure and successive adaptation especially involving village, backyard, and semi-commercial poultry, have resulted in the LPAIV adapting to domestic poultry with sustained transmission within agricultural systems (Swayne 2008b). In contrast, infections by HPAI virus (HPAIV) are less common in domestic poultry than LPAIV and arise following circulation of H5 or H7 LPAIV in poultry resulting in mutation from low to high virulence (Rohm et al. 1995). These HPAIV have not been maintained in wild birds as has LPAIV (i.e., wild birds are not the reservoir for HPAIV), although HPAIV have occasionally been transferred back to wild birds, especially with the H5N1 HPAIV of Guangdong lineage, causing sporadic to epizootic deaths in some wild bird species (Feare 2010). The finding of rare infections of H5N1 HPAIV in wild birds during extensive surveys in Asia, but common infections in live poultry markets in the same geographic region, suggests that the true reservoirs of H5N1 HPAIV in Asia are domestic poultry, especially asymptomatic domestic ducks. H5N1 HPAIV is lethal to chickens; however, in domestic ducks these viruses can produce a range of clinical disease from mild infections to severe disease with mortality. The way domestic ducks are raised in many Asian countries allows them to serve as bridging species in the transmission of H5N1 HPAIV between wild waterfowl and gallinaceous poultry. Occasionally, spill-over of H5N1 HPAI has occurred into wild birds. For example, intermediate-distance migrants may have transmitted the virus from Mainland Asia to Japan and Korea (Feare 2010; Guan et al. 2009; Hulse-Post et al. 2005; Pepin et al. 2012; Sturm-Ramirez et al. 2005). Asymptomatic infected migratory ducks are also suspected of contributing to the spread of H5N1 HPAIV from Asia to other parts of the world (Cattoli et al. 2009; Keawcharoen et al. 2008; Kim et al. 2009). In addition, periurban terrestrial birds such as sparrows, pigeons, and starlings that enter agricultural housing and access feed for domestic poultry, have been infected with H5N1 HPAIV and can be either mechanical vectors or biological vectors of H5N1 HPAIV between farms or farming systems (Brown et al. 2009; Kou et al. 2005). Therefore, control of H5N1 HPAIV infection in agricultural systems will have a profound effect on reducing and eliminating HPAIV exposure and infections of diverse aquatic and terrestrial wild birds.

Since 2003, the H5N1 HPAIV has become enzootic in poultry within several countries which has necessitated two main changes for HPAI control and eradication strategies; (1) development and implementation of rapid diagnostic tests to accelerate diagnosis before the virus spreads, which permits a quicker stamping-out action leading to eradication, and (2) addition of vaccines and vaccination as a control tool to manage clinical disease, prevent human infections, and maintain food security, especially in economically disadvantaged countries.

High Pathogenicity Avian Influenza Eradication Programs

Historical Strategies

The primary goal for HPAI epizootics in agricultural systems had been rapid eradication. For 26 HPAI epizootics, this has been achieved through comprehensive, integrated control programs that utilized education, diagnostics and surveillance, enhanced biosecurity, and elimination of infected poultry (Swayne et al. 2013). This successful strategy, often termed "stamping-out" relies upon: (1) educating farmers, service personnel, and governmental officials in disease control methods including changes in high-risk behaviors that can spread the virus; (2) using rapid diagnostics and surveillance methods to identify infected flocks; (3) implementing better biosecurity through quarantining infected flocks, imposing movement controls within the outbreak zone, and employing programs that clean and disinfect premises and equipment to limit virus spread, and (4) eliminating the source of infection by culling poultry on infected farms (Swayne et al. 2013). The success of stamping-out programs to eradicate HPAI has been associated with effective and efficient governmental veterinary services, sufficient economic resources for rapid mobilization and implementation, transparency of government in reporting outbreaks and good governance (Pavade et al. 2011).

Vaccines and Vaccination as a New Control Tool

The paradigm of HPAI eradication changed in 1994–95 when epizootics of H5N2 HPAI in central Mexico and

H7N3 HPAI in Pakistan overpowered the resources of the respective governments and commercial poultry industries in stamping-out programs, requiring the addition of a fifth control tool (i.e., vaccination) to permit interim management of the clinical disease and allow continued food security until eradication was achievable in the long-term (Swayne et al. 2011). Since this initial vaccine use, vaccination has been used in HPAI control programs for poultry and captive birds in thirteen Asian, European, and African countries for H5N1 HPAI (2002–present); North Korea for H7N7 HPAI (2005); and Mexico for H7N3 HPAI (2012–present) (OIE 2012b; Swayne et al. 2011). The use of vaccination in poultry has become a valuable tool for temporary management of HPAI, supporting national food security and promoting the livelihood of rural poor, especially in underdeveloped countries (Swayne 2012a; Swayne et al. 2011). Ninety-nine percent of the vaccine used in birds against HPAI has been in the four countries where H5N1 HPAI is enzootic; i.e., China (including Hong Kong), Egypt, Indonesia, and Vietnam (Swayne et al. 2011). H5N1 HPAI was already enzootic in these four countries before vaccination was implemented, indicating that vaccination did not create enzootic infections (Swayne et al. 2011). However, routine use of vaccines and improper vaccination has delayed eradication, by contributing to complacency, and has complicated surveillance (Swayne and Spackman 2013).

Vaccines and Vaccination for High Pathogenicity Avian Influenza

Role of Vaccines and Vaccination

The role vaccines and vaccination can play in control of avian influenza has been assessed in multiple experimental studies in poultry. In the field, vaccines and vaccination have been shown to increase resistance of poultry to virus infection thereby preventing infection in a large percentage of poultry within the housing operation, and among any infected birds, prevent illness and death, and reduce the amount of virus replicating in respiratory and gastrointestinal tracts [reviewed by (Swayne 2012a)]. These data translate to reduced quantity of virus contaminating the environment (Gilbert et al. 2008; OIE 2007), which will reduce virus exposure and infections to birds (Bouma et al. 2008; Goot et al. 2003) and humans (OIE 2007; Swayne et al. 2011), and, therefore, maintaining livelihoods and food security of rural poor (OIE 2007). However, vaccines and vaccination alone will not eradicate HPAI because eradication can only be achieved through a comprehensive strategy coordinating vaccines and vaccination with the other four control components of stamping-out programs.

Assessing the Protection from Vaccines and Vaccination

The assessment of protection induced by AI vaccines is best accomplished using an in vivo challenge model and measuring quantifiable criteria that mimics protective effects in the field (Swayne 2008a) which includes prevention of clinical signs and death following HPAIV challenge (Stone 1987), prevention of egg production drops following LPAIV and HPAIV challenge (Brugh et al. 1979; Stone 1987; Swayne et al. 2012), reduction in quantity of LPAIV or HPAIV challenge virus shed from respiratory and gastrointestinal tracts (Swayne et al. 1999; Swayne et al. 1997), and prevention of contact transmission (LP and HPAIV challenge) (Swayne et al. 1997).

Experimentally, efficacious AI vaccines have been shown to have the following ideal traits: (1) protect against high environmental virus exposure or challenge dose (Swayne et al. 1997); (2) provide protection for long periods of time, usually a minimum of 6–12 months (Swayne and Spackman 2013); (3) provide reproducible protection through a defined vaccination method such as subcutaneous injection, wing web administration, coarse or fine spray, eye drop, in ovo, etc. (Swayne and Spackman 2013); (4) protect with a minimum number of vaccinations, ideally two but some species (e.g., turkeys) and long-lived birds (e.g., layers), may require three or more vaccinations (Eggert and Swayne 2010); and (5) broadly usable in multiple bird species (Swayne and Spackman 2013).

Protection in Chickens and Ducks

Antigenic matching between the vaccine and field virus is another critical factor in achieving optimal vaccine efficacy. Within the H5 and H7 subtypes, there can be enough variation that vaccines will not provide adequate protection against all challenge viruses because of poor match between vaccine and field strains (Abbas et al. 2011; Eggert et al. 2010; Grund et al. 2011; Pfeiffer et al. 2010). Therefore, selecting an initial vaccine that is a good antigenic match to the challenge virus is crucial. Antigenic drift with loss of protection has been observed in numerous cases where AIV has persisted in a population for a long time and vaccine

has been used long-term (Chen 2009; Escorcia et al. 2008; Grund et al. 2011; Lee et al. 2004). In order to maintain the most effective vaccination program, the field virus should be monitored for antigenic changes and the vaccine should be tested against new variants or at a minimum vaccines should be re-evaluated every 2–3 years for protection against current circulating field viruses.

Experimental studies in chickens and ducks evaluating several of the factors cited above have been conducted; however, very few studies have been reported for turkeys for protection from HPAI (Bublot et al. 2010; Cagle et al. 2011; Eggert and Swayne 2010; Kilany et al. 2011; Middleton et al. 2007; Pantin-Jackwood et al. 2012; Pfeiffer et al. 2010; Qiu et al. 2007; Steensels et al. 2007; Tian et al. 2005; Toffan et al. 2007; Webster et al. 2006; Yamamoto et al. 2010; Zhang et al. 2005). Few vaccines have achieved all the factors cited above, but still have been used successfully in the field. Importantly, it should be noted that vaccine studies in the laboratory cannot completely simulate field conditions and protection in the field is reported to be less effective, necessitating booster vaccinations (Eggert and Swayne 2010).

Due to the practical difficulty in evaluating the duration of immunity experimentally, a few studies have looked at the course of antibody levels in chickens in the field after vaccination with inactivated vaccines; however, general trends are difficult to establish because of numerous variables, including differences in genetic lines of chickens, number of times the vaccine was administered, vaccine dose, and different adjuvants (Boltz et al. 2009; Hwang et al. 2011; Sasaki et al. 2009). In situations where vaccination is used as an adjunct to other control methods, duration of immunity may be less critical if virus spread is controlled promptly. Although it can provide important detailed information on the performance characteristics of a vaccine, direct assessment of vaccine efficacy by in vivo testing is time consuming and expensive. A practical alternative for determining a minimal protection level is by indirect assessment using virus neutralization or, more commonly in poultry, hemagglutination inhibition tests to evaluate the antibody titers in vaccinated populations. If an adequate proportion of the flock has a minimum titer of antibody to the current field virus, they are expected to be protected. This is also why it is important to maintain adequate surveillance of vaccinated populations for exposure to the virus. This ensures that new field variants are promptly detected and can be characterized for changes which affect their antigenic traits.

Given the widespread infection of domestic ducks with H5N1 HPAIV in certain parts of the world, reducing the risk of virus infection in ducks is considered crucial for controlling the spread of H5N1 HPAI. In much of the developing world, domestic ducks are usually farmed in open fields, flooded rice paddies, or on ponds or other bodies of water, allowing direct exposure to wild waterfowl, and domestic ducks are frequently moved between fields and to live poultry markets, aiding to maintenance and spread of the virus in agricultural production systems. Since in most cases biosecurity measures are impractical or impossible to implement and enforce, vaccination is one of the few control tools available to protect domestic ducks against H5N1 HPAI. In laboratory studies with moderate to high challenge doses, vaccination has proven effective in protecting domestic ducks against clinical signs of disease; however, different species of domestic ducks respond differently to vaccination, and shedding of the virus may still occur in clinically healthy vaccinated ducks, but the titer of virus shed is reduced (Cagle et al. 2011; Steensels et al. 2007, 2009). The difficulty of adequately vaccinating sufficient number of ducks to maintain "herd immunity" is a big obstacle in the control of H5N1 HPAIV. In situations in which ducks are reared in open fields, vaccination coverage is poor, i.e., low vaccination rate in the population, and, therefore, high numbers of domestic ducks remain susceptible and serve as reservoirs and disseminators of H5N1 HPAIV.

Current vaccines and vaccination practices for the control of H5N1 HPAIV infection in domestic waterfowl should take into account different variables including susceptibility of the ducks to different circulating viruses, effect of species, and husbandry practices. Not many studies have been conducted evaluating vaccination of domestic ducks in the field. In a study examining virus transmission within infected flocks before and after vaccination, it was found that apart from issues related to the quality of protection provided by the vaccine, the overall effectiveness of the vaccination campaigns was undermined by factors that deter farmers from vaccinating their flocks and operational issues for vaccine delivery (Magalhaes et al. 2010). The authors suggested that if vaccination continues to be included as part of a sustainable disease control program, efforts should be focused on training farmers in disease prevention in addition to disease recognition, as the latter is likely to be compromised in a vaccinated population. Results from field and laboratory evaluation of vaccines against H5N1 HPAI in domestic ducks indicates that

factors such as duck species and/or breed, vaccination protocols (number of doses, age), and proper use of vaccines may significantly influence the success or failure of the H5N1 vaccination program. Other factors, including the role of maternally derived antibodies, co-infection with other pathogens, and use of adjuvants not optimized for ducks, remains to be determined. Continuous new outbreaks of H5N1 HPAI emphasize the need for a comprehensive domestic waterfowl vaccination strategy and the development of domestic waterfowl-specific efficacious vaccines.

Immunity and Protection of Wild Bird Populations

There are two viable and interrelated questions concerning protection of wild bird populations from HPAI: (1) can wild bird populations be actively vaccinated to protect from HPAIV infection and disease, and (2) will natural exposure to H5 or H7 LPAIV induce immunity and protection from HPAIV infection and disease? There is no data on capture of wild birds, individual vaccination against AIV, and release back to natural habitats. However, zoo, hunting, companion, conservation, and endangered species of birds of diverse species, including aquatic birds of the orders Anseriformes, Charadriiformes, Ciconiiformes, Pelicaniformes, and Phoenicopteriformes, on over 292 premises in 20 countries have been vaccinated with inactivated poultry vaccines against H5 and/or H7 subtypes (Bertelsen et al. 2007; Furger et al. 2008; Philippa et al. 2005, 2007). These poultry vaccines produced variable levels of H5 and/or H7 hemagglutinating antibodies with 50 and 82% of the birds seroconverting (HI titer of $\geq$1:16) following a single and booster vaccination, respectively (EFSA 2007). The presence of such HI antibodies levels has been associated with protection in chickens and turkeys, but the specific HI antibody levels needed for protection in most non-poultry species, i.e., captive ''wild'' bird species, are unknown (Koch et al. 2009). Capture of wild species from their native habitat for administration of a killed vaccine would be logistically unrealistic and likely to cause unacceptable mortality in the birds from the capture and handling process. In addition, recapture of individual birds for a second immunization would be even more unrealistic. The only practicality of vaccination of non-poultry species would be those birds already held in captivity.

The second issue would be the immunity provided by natural exposure to LPAIV and resulting protection against HPAIV. In a recent study in Alaska, 44% of 11 species of Anseriformes birds and 80–95% of Emperor geese and three eider species tested had anti-AIV antibodies, based on anti-nucleoprotein ELISA test (Wilson et al. 2013). However, the anti-AIV antibody positive rates varied with species, age, year, and season (Ely et al. 2013). Protection is not based on the broadly reactive anti-nucleoprotein antibodies but on the more specific anti-hemagglutinin or anti-neuraminidase antibodies. The prevalence of H5 antibodies is rarely reported but in one study in wild ducks in the USA, a 27% prevalence of anti-H5 antibodies was found (Nettles et al. 1985) while a study in Europe showed 49–69% anti-H5 antibody prevalence in mute swans, 64% in sacred ibis, 28% in mallards, and 27% in common pochards (Niqueux et al. 2010). These studies were spatially and geographically associated with outbreaks of H5 HPAI and may not reflect the general seroprevalence of anti-H5 antibodies of all aquatic species in all geographic regions, and seroprevalence of anti-H7 antibodies is unknown.

In one experimental study, Costa et al. (2010) using wood ducks (*Aix sponsa*) determined that exposure to LPAIV could provide protection from H5N1 HPAIV challenge, but the protection required the exposure to a H5 LPAIV and that virus must be adequately adapted to the bird species to replicate to sufficient titer to stimulate a detectible immune response based on H5 HI antibodies. They suggested that in naturally occurring outbreaks of H5N1 HPAI, birds with pre-existing immunity to homologous hemagglutinin or neuraminidase subtypes of AI virus may either survive H5N1 HPAIV infection or live longer than naive birds and, consequently, could pose a greater risk for contributing to viral transmission and dissemination, if titers of H5 and N1 antibodies are low and provide protection only from death but do not completely prevent virus replication. In addition, the ability to capture and induce protection to all susceptible wild waterfowl through timed exposure of wild birds to live LPAIV would not be acceptable because of the need for multiple individual strains adapted to individual wild bird species, which would be prohibitive and could produce unintended and unknown adverse effects. Furthermore, the presence of antibodies to H5 and H7 due to natural LPAIV infections cannot be relied upon to protect wild birds from infection and disease following HPAIV exposure, if the field virus were variants, antigenically distant from the LPAIV. For wild birds, the only realistic means to protect from HPAIV would be to prevent exposure to agricultural reservoirs, and the adjunct of controlling and eradicating the HPAIV from the agricultural system.

Vaccines in the Field for Poultry

For 2002–2010, over 113 billion doses of AIV vaccine were used in poultry within 15 different countries/special administrative regions (Swayne et al. 2011). The majority of the vaccine was used in poultry within four H5N1 HPAI enzootic countries, utilizing nationwide vaccination programs with the goal of reaching all poultry within the country (Swayne et al. 2011). China used >103 billion doses (90.99%), Egypt 5 billion doses (4.65%), Indonesia 2.6 billion doses (2.32%), and Vietnam 1.6 billion doses (1.43%). With these four countries, the vaccine use was proportional to the country's poultry production with China being the number one poultry producer and consumer in the world with a production of 14.9–16.4 billion birds per year (2004–2010). The remaining 10 countries/regions (Mongolia, Kazakhstan, France, The Netherlands, Cote d'Ivoire, Sudan, North Korea, Israel, Russia, and Pakistan) used 698 million doses of vaccines (0.6%) in poultry for targeted preventative or emergency vaccination programs; focusing to either specific geographic areas, around outbreak zones or to specific types of poultry or farming systems. In mid-2012, Mexico began a AIV vaccine program in laying chickens within the defined control zone of the state of Jalisco in response to the H7N3 HPAI epizootic (OIE 2012b). By contrast, AIV vaccine has had minimal use in non-poultry birds, with 271,690 doses being used during 2002 and 2010 in zoo, hunting, companion, conservation, or endangered birds to protect from H5 and/or H7 HPAI, which represents 0.00024% of the total AIV vaccine used in birds (Swayne et al. 2011).

The vast majority of the 113 billion doses of vaccine used have been inactivated oil-emulsified whole AIV vaccines (95.5%) which require handling and injection of individual birds, while live recombinant virus vaccines (4.5%) have had a more restricted, focused use within some poultry populations (Swayne et al. 2011). The live recombinant vaccines have included Newcastle disease virus (rNDV)-vectored vaccine with H5 influenza gene insert (rNDV-H5-AIV) which can be administered by spray respiratory application, and two fowlpox virus (rFPV)-vectored vaccines with either an H5 AIV gene insert (rFPV-H5-AIV) or an H5 and N1 AIV gene inserts which are administered only to chickens at 1 day-of-age by injection. Two new recombinant vaccines have been developed and licensed whose potential will improve application and protection; herpesvirus turkey (rHVT)-vectored vaccine with H5 AI virus gene insert for use in chickens and turkeys, and a duck virus enteritis (rDVE)-vectored vaccine with an H5 gene insert for use in domestic ducks (Liu et al. 2011; Rauw et al. 2011). Between 1998 and 2005, over two billion doses of an rFPV-H5-AIV were used in chickens in Central America to protect against H5N2 LPAIV, (Bublot et al. 2006), and its use has continued through 2013.

Since 2002, large quantities of AI vaccines have been used against H5N1 HPAI, but this tool alone has not resulted in eradication within the four enzootic countries, but has positively contributed to the eradication or prevention of HPAI in the other 11 countries/regions. Within the four enzootic countries, reports of AIV vaccine "failures" have been made, specifically reporting of clinical disease consistent with HPAI or isolation of H5N1 HPAIV in vaccinated flocks or in regions that vaccinate (Swayne 2012a). These vaccine "failures" have resulted from two categories of problems: (1) failure of the vaccine and (2) failure of vaccination. Vaccine "failures" have resulted from poor-quality vaccines with inadequate quantity of H5 antigen, or vaccine containing a seed strain that does not protect against a field virus because of antigenic drift. Vaccination "failures" have resulted from the lack of proper administration of vaccine or inability to vaccinate and produce a protective immune response in the at-risk poultry population; i.e., a failure to achieve population immunity because of inability to vaccinate poultry properly (Bouma et al. 2007; Swayne 2012b). Delivery of vaccine to billions of poultry owned by millions of people is a huge logistic problem.

Vaccine Technologies

Five different categories of vaccine technologies have been used to develop AIV vaccines in the laboratory and study their ability to protect birds: (1) inactivated whole AIV, (2) live AIV, (3) live vectors, (4) in vitro produced hemagglutinin, and (5) DNA vaccines (Table 1) (Swayne and Spackman 2013). However, application in the field through licensing and use has only been accomplished with a few technologies and products: i.e., inactivated whole AIV vaccines and live vectored vaccines (rNDV, rFPV, rHVT, and rDVE). The inactivated vaccine requires catching, handling, and injecting each individual bird as also when using rFPV, but the rNDV can be mass applied by spray administration and rHVT can be applied at 1 day-of-age to chickens in the hatchery or in ovo, saving time and labor cost. Adoption of new technologies for commercial vaccines requires satisfying multiple ideal traits for AIV

Table 1. Experimental and Licensed Vaccines for HPAI in Poultry and Other Avian Species [Modified from (Swayne and Kapczynski 2008; Swayne and Spackman 2013)].

Vaccine category	Vaccine	Species	Route	AI subtypes	HPAIV challenge tested	Licensed	Comments	Additional references
Inactivated AIV	Adjuvanted whole AIV	Chicken (layer and broiler), turkey, duck, goose, other poultry, zoo birds	SQ, IM, in ovo	H5, H7	Yes	Yes	Mostly oil-emulsified; some with aluminum hydroxide. Includes H5 and H7 LPAIV, H5 and H7 HPAIV, and H7 reverse genetic LPAIV seed strains. Requires parenteral administration	
Live AIV	Live wild-type LPAIV	Chicken	IM, IT spray	H5, H7	Yes	No	Rumors of intentional exposure with LPAIV to protect from HPAIV have been reported in H5N1 and H5N2 HPAI outbreaks in 1990s and 2000s	(Swayne, unpublished information)
	Attenuated LPAIV	Chicken	Spray	H5, H7	Yes	No	Temperature sensitive mutant or replace HA with ectodomain of NDV HN gene; risk assessment needed for reassortment potential	Park et al. (2006), Zhang et al. (2012)
Live vector	rd-Adenovirus	Chicken	SQ, IN, in ovo	H5	Yes	No	rd = Replication defective, only 1 round of replication occurs after injection. SQ and in ovo protected	Gao et al. (2006)
	Avian leukosis virus	Chicken	IM	H7	Yes	No		Hunt et al. (1988)
	Avian paramyxovirus type 1 (rNDV)	Chicken	Eye, IN	H5, H7	Yes	Yes	Licensed in Mexico and China	Ge et al. (2007), Swayne et al. (2003)
	Duck enteritis virus (rDVE)	Duck	IM	H5	Yes	No	Submitted for license in mid-2012 for ducks (China)	Liu et al. (2011)
	Fowlpox virus (rFPV)	Chicken (layer and broiler), goose, Muscovy ducks	SQ, WW	H5, N1	Yes	Yes	Licensed 1997 for chickens (USA, Mexico); used primarily in Central America against H5N2 LPAI; limited use in China	

Table 1. continued

Vaccine category	Vaccine	Species	Route	AI subtypes	HPAIV challenge tested	Licensed	Comments	Additional references
	Herpesvirus Turkey (rHVT)	Chickens	SQ	H5, N1	Yes	Yes	Licensed 2012 for chickens (USA, Egypt). Used Egypt	CEVA (2013), Rauw et al. (2011)
	Infectious laryngotracheitis virus vector	Chicken	Eye	H7, H5, N1	Yes	No	N1 did not protect	Pavlova et al. (2009), Veits et al. (2003)
	att-*Salmonella typhimurium*	Chicken	OR	H5, M2e	Yes	No	Attenuated vaccine strain. Failed to protect from HPAIV challenge with single oral immunization	Layton et al. (2009), Pan et al. (2009)
	Vaccinia	Chicken	IM, IP	H5	Yes	No	Low to no antibody response	Chambers et al. (1988)
	rd-Venezuelan Equine Encephalitis virus		SQ, in ovo	H5	Yes	No	rd = Replication defective, only 1 round of replication occurs after injection	Schultz-Cherry et al. (2000)
In vitro produced hemagglutinin	Baculovirus in insect cell culture	Chicken, Muscovy duck	SQ	H5, H7	Yes	No		Crawford et al. (1999)
	Eukaryotic systems (plants or cells cultures)	Chicken	IM	H5	Yes	No	*Nicotiana* sp.	Kalthoff et al. (2010)
DNA	Naked DNA	Chicken	IM	H5	Yes	No	Not financially viable. Improvements needed in promoters and adjuvants to decrease quantity of nucleic acid needed and reduce number doses for protection	Rao et al. (2008), Suarez and Schultz-Cherry (2000)

Eye conjunctival sac, *IM* intramuscular, *IN* intranasal, *in ovo* into embryonating egg, *IP* intraperitoneal, *IT* intratracheal, *OR* oral, *spray* fine or coarse droplet into air space, *SQ* subcutaneous, *WW* wing web.

Table 2. Properties of Ideal AIV Vaccines and Vaccination Methods for Poultry [Modified from (Swayne and Spackman 2013)].

Desired property	Current situation
Inexpensive	Current cost for inactivated AIV vaccine: $0.05–0.10/dose plus cost of administration ($0.05–0.07 per dose for individual handling and injection) (Swayne and Kapczynski 2008)
Use in multiple avian species	Most used in meat, layer, and breeder chickens, but large quantity also used in ducks; minor amounts in turkeys, geese, quail, etc. (Swayne et al. 2011)
Single dose protection	Most situations require minimum of two doses; prime-boost scenario is optimal with additional boost in long-lived birds at 6–12 month intervals (Steensels et al. 2009; Swayne 2006)
Easy, mass application	95.5% is inactivated vaccine administered by handling and injecting individual birds, with 4.5% as vectored vaccine given by mass spray vaccination (rNDV vector) (Swayne et al. 2011)
Identify infected birds in vaccinated population	Serological differentiation tests are available, but only minor use. Most vaccine applied without using a DIVA strategy (Swayne 2006)
Overcome maternal anti body block	Maternal antibody to AIV hemagglutinin or virus vector inhibits primary immune response. Initial vaccination must be timed for declining maternal antibody titers to allow optimal primary immune response (Maas et al. 2011), as decline in active immunity before giving booster vaccinations is also needed (Swayne et al. 2000)
Given at 1 day-of-age in hatchery or in ovo	Inactivated vaccines provide poor protection if given at 1 day-of-age. Vectored vaccines can be given at 1 day-of-age, but generally require a field boost with inactivated vaccine 10 days or more later
Antigenically close to field virus	The majority of inactivated whole AIV vaccine uses reverse genetic generated vaccine seed strains to antigenically match field viruses (Swayne 2012b; Swayne et al. 2011)

vaccines including practical field application to solve poultry health problems (Table 2). In addition, the reader must understand that an ideal vaccine for humans may not be ideal for poultry.

Any new vaccine technologies will only be adopted for licensing and field use if the new vaccine will provide protection in experimental trials that is equivalent to or better than the "gold standard," i.e., oil-emulsified whole AIV vaccine (Swayne and Spackman 2013). Vaccine development and field implementation in commercial poultry is driven by economics with adoption of new technologies occurring only if a financial advantage is provided such as the cost of the new vaccine is less than the loss from disease with no vaccination, or the cost differential of new vaccine over the existing vaccine is less than the savings from additional protection from disease. Historically, most AIV vaccines have been based on inactivated whole AIV with the seed virus being produced in embryonating chicken eggs. This mature, standard technology has been used successfully for over 40 years to produce trillions of doses of killed or live-attenuated vaccines to other poultry viral diseases such as Marek's disease, reoviral arthritis, Newcastle disease, infectious bronchitis, and infectious bursal disease. This low-cost technology has produced efficacious, potent vaccines without the additional cost of royalties for patents or the purchase of new manufacturing equipment which will be needed for implementation of newer vaccine technologies. However, newer technologies will be and have been utilized at the higher cost when they have addressed one or more critical traits which have made the new technologies produce a product closer to the ideal vaccine (Table 2). As an example, 66.6 of 73 billion doses (91%) of inactivated H5 AIV vaccines used from 2007 to 2009 were based on vaccine seed strains produced through reverse genetic technology (Swayne 2012b). These vaccines are closer antigenically to H5N1 viruses encountered in the field, and provide better protection than historic inactivated vaccine seed strains based on LPAIV.

In developed countries, inactivated whole AIV vaccines have been limited to use in valuable, long-lived, or specialty poultry because of the high cost of individual bird administration and long withdrawal period for oil adjuvant in any meat poultry. By contrast, in less developed and developing/transitional countries with low labor cost for vaccination and shorter withdrawal periods for oil adjuvants, inactivated vaccines have been administered to the much larger populations of meat chickens and ducks. Experimental studies have demonstrated the possibility for low-cost mechanized in ovo injection for oil-emulsified, inactivated whole AIV vaccines (Stone et al. 1997), adenovirus-vectored vaccines (Breedlove et al. 2011),

VEE-vectored vaccine (Schultz-Cherry et al. 2000), attenuated AIV vaccine (Song et al. 2007), and rNDV-vectored vaccines (Steel et al. 2008) that could be commercially viable and allow for more use of AIV vaccines in developed countries. In addition, a superior approach would be new delivery technologies for easier, mass application such as administration by spray (respiratory delivery of fine droplets) or per os (oropharyngeal and upper digestive tract delivery in feed or water).

Even with new breakthroughs in technologies, important fundamental questions must be first answered; i.e., that is whether vaccination is needed as a control tool, or if other control tools such as prevention through management biosecurity or, if immediate stamping-out is the better approach. In a recent survey (Swayne et al. 2011), most countries preferred rapid eradication of HPAI by using a stamping-out program without vaccination and indicated that they would only use vaccination if the HPAI epizootic was large and stamping-out was not effective in producing immediate eradication. Alternatively, if the threat of an epizootic was high, vaccination might be used as a preventative measure for valuable poultry, and endangered or valuable captive bird species within zoos or other collections (Swayne et al. 2011). Historically, the decision point for implementing vaccination for HPAI was reached earlier with the least developed and developing/transition countries (13 of 15 countries that vaccinated), than in developed countries where only two countries (France and The Netherlands) vaccinated and they used a small, time-limited targeted vaccination program (Pavade et al. 2011; Swayne et al. 2011).

Vaccination of Poultry: Coverage and Population Immunity

Protection in the field can only be achieved if the at-risk poultry are able to mount an effective immune response and if individual birds receive the vaccine in the proper dose, correct number of vaccinations, and administration by the correct route. Population immunity of at-risk poultry is the goal, which is only achieved if greater than 60–80% of the poultry have a protective immune response (Bouma et al. 2007; Swayne et al. 2011). If we look at an entire country conducting routine vaccination of all poultry, the goal of national population immunity is difficult to achieve because of limitations in financial and human resources. This conclusion is based on the 113 billion doses of AIV vaccine used in poultry during 2002–2010 which resulted in only a 41.9% coverage rate among the at-risk national poultry population of the 15 vaccinating countries/regions (Swayne et al. 2011). Five of the 15 countries/regions conducted routine vaccination campaigns of all poultry with national coverage rates of 47.1% for China, 86.2% for Hong Kong, 69.9% for Egypt, 14% for Indonesia, and 52.3% for Vietnam (Swayne et al. 2011). This initial data suggest that only two countries/regions achieved population immunity (Hong Kong and Egypt), but more detailed analysis using more accurate estimates of higher village poultry populations in Egypt suggests that Egypt did not achieve a national population immunity with revised vaccination coverage rates between 27.8 and 48.6% (Swayne et al. 2011). Furthermore, the use of 1 day-of-age vaccination in broilers in Egypt using inactivated oil-emulsified vaccines may have also contributed to inadequate immune responses, even further decreasing the effective immunity in the population. Therefore, cases of H5N1 HPAI in poultry continue to occur in China, Egypt, Indonesia, and Vietnam because of the lack of population immunity, but Hong Kong did achieve national population immunity, with only one farm having H5N1 HPAI in poultry during 2003–2012 (Swayne 2012a). These findings suggest that national population immunity in poultry, with its intensive financial and human resource requirements, is not realistic in most countries. Alternatively, vaccination should be targeted to poultry at the greatest risk for exposure to HPAI and/or to specific geographic regions. Decisions on which poultry and/or geographic regions to vaccinate require both ongoing field surveillance and epidemiological data and modeling in order to design and implement effective vaccination programs. The historical yearly vaccination campaigns, used more commonly in cattle and pigs for transboundary diseases, are not effective with commercial poultry because of the shorter replacement period (i.e., chickens and ducks have a 5-month generation time) which result in production of a large naïve poultry populations between the vaccination campaigns, thus providing susceptible host to maintain the virus in the population. In addition, countries with large populations of poultry produced in the semi-commercial and village sectors must develop unique programs that will reach the large number of households with low numbers of birds. Initially, expectations were high that a spray vaccination of rNDV-H5-AIV would provide single dose, uniform protection in all poultry. Although rNDV-H5-AIV by respiratory mass application in experimental studies with specific pathogen-free chickens did provide protection from HPAIV

challenge, when transferred to the field, the presence of high levels of maternal antibody to NDV and H5 AIV inhibited rNDV-H5-AIV replication and failed to provide protection with the single vaccine dose (Swayne and Spackman 2013). The rNDV-H5-AIV has best been used as a priming vaccine followed by inactivated whole AIV booster vaccination. Additional research is needed on optimizing vaccination protocols for different poultry species and ages to achieve low-cost immunity.

Conclusions

Based on the information presented and discussed, the following conclusions may be drawn:

1. Historically, infection of wild birds by HPAIV has been rare, but wild bird infections have become more common with the emergence of H5N1 HPAIV (Guangdong lineage) in China which has spread across three continents, causing notable infections and deaths in a variety of wild bird species. However, the major source and reservoir of H5N1 HPAIV is domestic poultry, especially domestic ducks.
2. Vaccines have been used as a tool in HPAIV control and eradication for poultry in five of 32 epizootics. Most of the vaccine has been used in poultry to protect against H5N1 HPAIV (Guangdong lineage) and have been used in enzootic countries/regions (China, Egypt, Indonesia, and Vietnam) as part of nationwide vaccination campaigns. Targeted vaccination, based on geography, bird type, and/or time limitations, has been practiced in another 10 countries and regions, but accounted for less than 1% of all AI vaccine used.
3. Most poultry AI vaccines have been the traditional, inactivated oil-emulsified whole AIV vaccines, with $<5\%$ of AI vaccines being live vectored vaccines. The inactivated vaccine requires labor-intensive catching and individual bird vaccination.
4. Vaccines have been used to protect some non-poultry species, but only in captive birds on 292 premises in 20 countries; i.e., mostly for zoo, hunting, companion, conservation, or endangered birds held in captivity. Vaccination of wild birds in natural habitats has not been attempted and is neither practical nor feasible.
5. LPAIV infection in wild birds can confer protection against HPAIV if the hemagglutinin and/or neuraminidase subtype of the LPAIV matches the HPAIV and if the LPAIV infects and produces a robust immune response. However, in practice, any protection against H5 and/or H7 HPAIV in wild bird populations is dependent upon the geographic area, bird species, year, and season. Predictability of any such natural protection is unknown.
6. Control and eradication of HPAIV from the domestic poultry reservoirs is the most effective means to protect wild bird populations from HPAIV.

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Poultry vaccination directed evolution of H9N2 low pathogenicity avian influenza viruses in Korea

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ABSTRACT

Significant economic losses in the poultry industries have resulted from H9N2 low pathogenic avian influenza virus infections across North Africa, the Middle East and Asia. The present study investigated the evolutionary dynamics of H9N2 viruses circulating in Korea from 1996 to 2012. Our analysis of viral population dynamics revealed an increase in genetic diversity between the years 2003 and 2007, corresponding to the spread and diversification of H9N2 viruses into multiple genetic groups (named A and B), followed by a sudden decrease in 2007, which was associated with implementation of vaccination using a Clade A virus. Implementation of the H9N2 vaccination program in Korea has dramatically reduced the diversity of H9N2 virus, and only one sub-lineage of clade B has survived, expanded, and currently circulates in Korea. In addition, the antigenic drift of this new genetic group away from the current vaccine strain suggests the need to update the vaccine seed strain.

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Introduction

Sixteen hemagglutinin (HA) subtypes (H1–H16) and nine NA subtypes (N1–N9) have been identified among avian influenza viruses (Swayne et al., 2013). The H9N2 low pathogenic avian influenza virus (LPAIV) was first identified in poultry in the 1960s and became widespread in Asian poultry in the 1990s (Guan et al., 1999). The first outbreak of the H9N2 LPAI in China occurred in Guangdong province of Southern China during November 1992 to May 1994 and rapidly spread to become the most prevalent LPAIV in domestic poultry (Lee and Song, 2013; Sun and Liu, 2015; Zhang et al., 2009). This H9N2 LPAIV lineage has resulted in great economic losses due to decreased egg production and increased mortality. In addition, the H9N2 LPAIV has caused sporadic human infections in Asia since 1998, raising concerns about a pandemic potential with this lineage of virus (Butt et al., 2005; Lin et al., 2000; Matrosovich et al., 2001). Phylogenetic and antigenic analysis have identified several groups of H9N2 LPAI virus in Eurasia: the G1 lineage, represented by A/quail/Hong Kong/G1/97 (G1-like); the Y280 lineage, represented by three prototype viruses A/duck/Hong Kong/Y280/97 (Y280-like), A/chicken/Beijing/1/94 (BJ94-like), and A/chicken/Hong Kong/G9/97 (G9-like); and the Y439 or Korean lineage, represented by A/duck/Hong Kong/Y439/97 (Y439-like) and A/chicken/Korea/38349-p96323/96 (Korean-like) (Butt et al., 2010; Guan et al., 1999; Matrosovich et al., 2001).

The first field outbreak of the H9N2 LPAIV in Korea occurred in 1996 with A/chicken/Korea/96006/96 (H9N2) being the reference strain, a virus genetically closely related to the Y439-like lineage virus later isolated from aquatic birds. Since then, H9N2 LPAIV has become endemic in domestic poultry in Korea and has formed the distinct Korean-like lineage (Kwon et al., 2006; Lee et al., 2000, 2007; Lee and Song, 2013). The Korean-like lineage has continued to evolve, exhibiting antigenic drift of the hemagglutinin and reassortment of internal gene segments with other LPAIV circulating in the Korean live bird markets (Choi et al., 2005; Kim et al., 2010, 2006; Lee et al., 2010, 2007; Park et al., 2011). To control H9N2 LPAI outbreaks, the Korean veterinary authorities utilized government stamping-out and compensation policies between 1996 and 1999, but complete eradication was not achieved. Since 2007, the veterinary authority has permitted the use of the inactivated oil adjuvant H9N2 LPAI vaccine derived from a Korean H9N2 isolate (A/chicken/Korea/01310/2001) in commercial layer and broiler breeder chickens (Choi et al., 2008).

A major determinant of variation in substitution rates among hemagglutinin genes of influenza A viruses seems to be the strength of immune selection pressure; at approximately one mutation per each genome replication, this translates into substitution rates of 10^{-4}–10^{-3} nucleotide substitutions per site per year (Nelson and Holmes, 2007). Thus, strong humoral immunity induced by vaccination can be an important factor promoting

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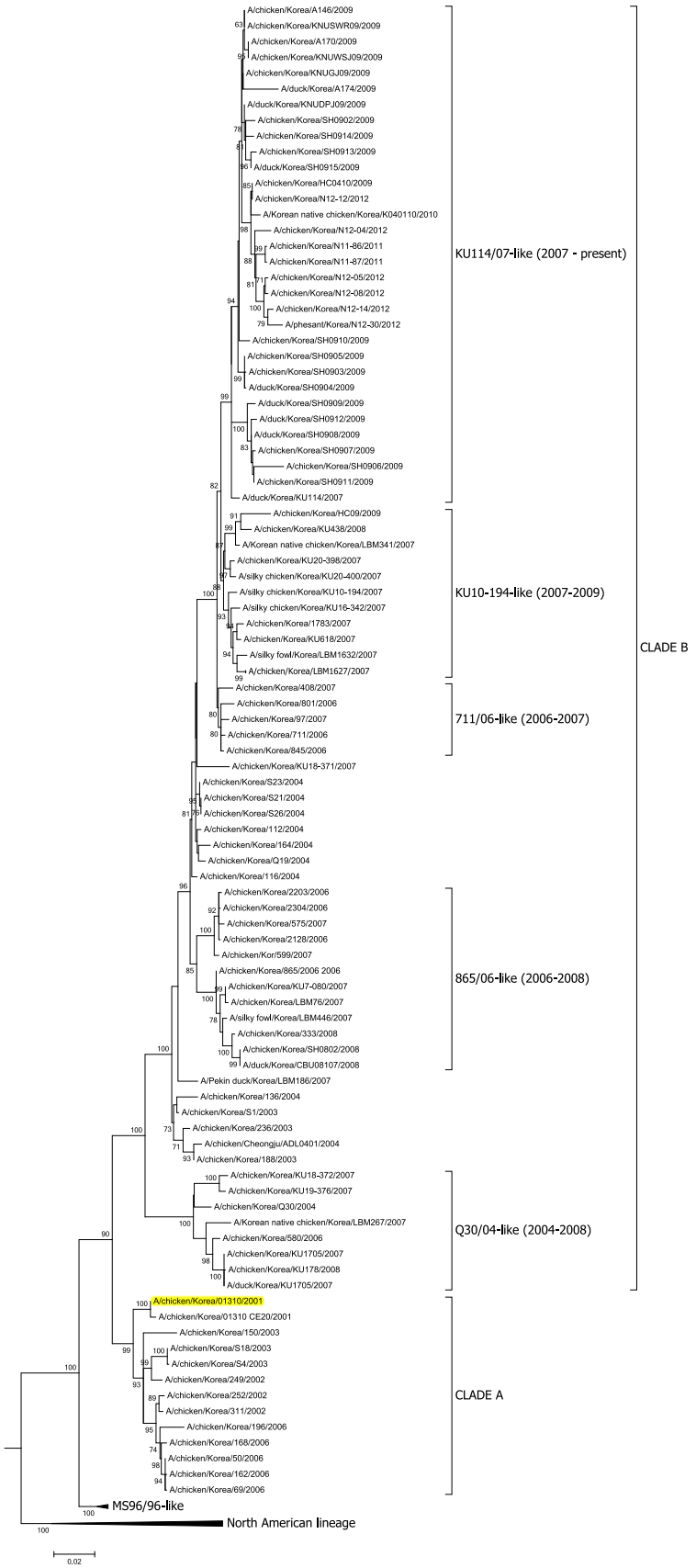

Fig. 1. Phylogenetic tree created by the Maximum-likelihood algorithm for the HA gene of H9N2 avian influenza viruses from Korea. The current commercial vaccine strain, A/chicken/Korea/01310/2001(H9N2), is highlighted.

selection of escape mutants in vaccinated animals. In a previous study, Cattoli et al. proposed a difference in evolutionary dynamics of H5N1 high pathogenicity avian influenza viruses (HPAIV) among countries where vaccination was or was not adopted. Particularly, evolutionary rates and the number of positively selected sites were higher in virus populations from countries applying vaccine for H5N1 HPAIV, compared to viruses populations in countries which had never used vaccination (Cattoli et al., 2011a). In the present study we investigated the evolutionary change and phylodynamics of Korean H9 HA genes isolated from 1996 through 2012, and analyzed selection pressure and point mutations related to antigenic features before and after the introduction of vaccination in Korea.

Results

The topology of the HA tree of representative H9N2 lineages (351 sequences) indicated that the Korean viruses formed a well-supported monophyletic group within the Y439-like or Korean-like lineage (Supplemental Fig. S1). To explore the evolutionary dynamics of the HA gene in Korea, a ML phylogenetic tree was inferred for a total of 100 H9N2 LPAIV identified from 1996 to 2012, before and after implementation of field vaccination (Fig. 1). The Korean H9N2 viruses can be mostly divided into four distinct clades, defined by high bootstrap values (> 80%) and long branch lengths in the HA phylogeny: MS96/96-like, 01310/01-like (Clade A), 116/04-like (Clade B), and North American (Fig. 1). The MS96/

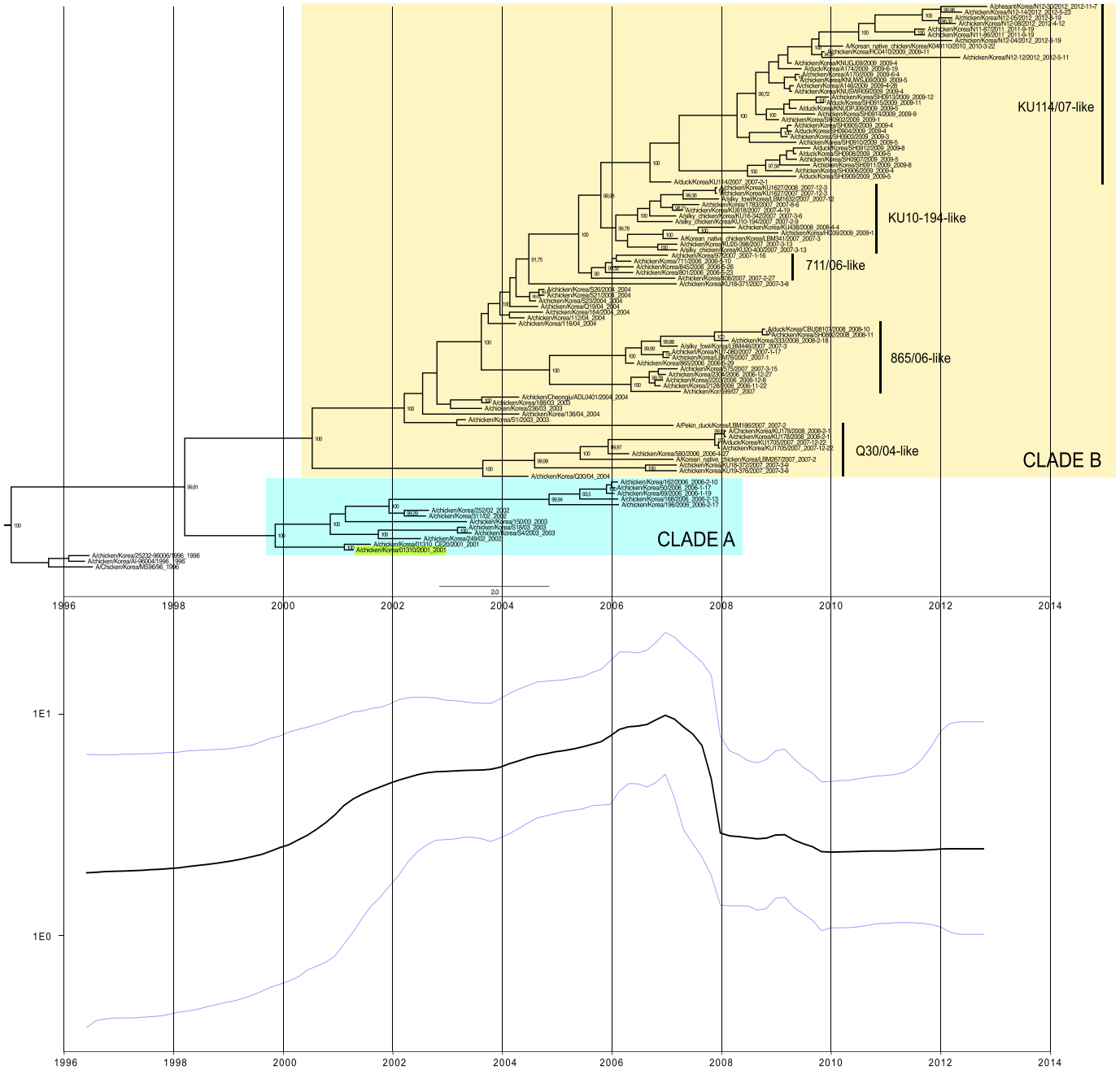

Fig. 2. Temporally structured maximum clade credibility phylogenetic tree and Bayesian Skyline plot of the HA gene showing changes in genetic diversity in Korean H9N2 viruses (1996–2012). A measure of genetic diversity (Net) in Bayesian Skyline plot is given on the *y*-axis with 95% HPD values shown in thin line. The current commercial vaccine strain, A/chicken/Korea/01310/2001(H9N2), is highlighted.

Table 1
Evolutionary profiles of Korean H9N2 LPAI viruses.

Year	No. of sequences	Evolutionary rate sub/site/year $\times 10^{-3}$ (95% HPD)	Mean d*N*/d*S*	Positively selected sites (p-value < 0.1)	
				N	Amino acid position (H9 numbering)
1996–2012	100	5.60(4.67–6.49)	0.236	7	12, 131, 133, 153[a], 166[a,b], 287, 498
2001–2006	34	5.79(3.60–8.21)	0.214	1	230[a]
2007–2012	63	5.80(3.55–8.42)	0.219	4	131, 133, 437, 498

[a] Sites for previously reported antigenic escape mutant.
[b] Potential glycosylation sites.

96-like group contained H9N2 LPAI viruses that caused the first H9N2 outbreak on Korean chicken farms in 1996, represented by A/chicken/Korea/96006/96 (H9N2) which was closely related to the A/duck/Hong Kong/Y439/97(H9N2) (Lee et al., 2000). H9N2 viruses isolated in Korea from 2001 to 2012 fell within two separate clades, designated as clade A (01310/01-like lineage), which contains viruses collected from 2001 to 2006, and clade B (116/04-like lineage), which comprises the majority of the recent isolates (2003–2012). Clade B can be further divided into five clusters, named Q30/04-like, 865/06-like, 711/06-like, KU10-194/-like, and KU114/07-like. Interestingly, these clades include only H9N2 viruses from Korea, suggesting that they were originally derived from a single viral introduction into poultry. By contrast, multiple introductions of H9N2 viruses with some genes of the North American lineage were found in Korea in wild birds [A/wild bird/Korea/8g-39/2005(H9N2), A/white-fronted goose/Korea/20-36/2007(H9N2), and A/bean goose/Korea/220/2011(H9N2)], but with only a limited spread across the country and the lack of transmission to poultry (Fig. 1) (Lee et al., 2014, 2013a, b).

As shown in Fig. 2, Korean H9N2 LPAI viruses were distinctly separated into clades A and B in MCC tree, consistent with the phylogenetic tree obtained by the ML algorithm. The time-scaled phylogeny indicated that clade A disappeared during 2006, and was replaced with clade B that evolved into several sub-lineages. However, except for KU114/07-like viruses, none of the clade B sub-lineages were detected after early 2009. The analysis of virus population dynamic revealed a gradual increase in genetic diversity from the beginning of the epidemic to the end of 2006 followed by a sudden decrease during 2007. The increasing population size corresponded to the appearance of clade A and B viruses and their diversifications into multiple sub-lineages, while the sudden decrease corresponded to the start of mass vaccination and the extinction of clade A viruses (Fig. 2), the source of the seed strain used in the vaccination campaign.

The evolutionary rate estimated for the HA gene of the Korean H9N2 viruses was 5.6×10^{-3} substitutions/site/year (95%highest posterior density, HPD, from 4.67×10^{-3} to 6.49×10^{-3}). The mean time of the most recent common ancestor (tMRCA) of H9N2 viruses was September 1994 (95% HPD, January 1992–March 1996), when H9N2 virus was first isolated in Southern China. To characterize the viral population dynamics of each clade, we calculated the evolutionary rates of the clades A and B separately. Interestingly, the rates of the two clades were different: a mean rate of 1.97×10^{-3} substitutions/site/year (95% HPD from 1.23×10^{-3} to 3.45×10^{-3}) for clade A, and a rate of 5.28×10^{-3} substitutions/site/year (95% HPD from 4.22×10^{-3} to 6.36×10^{-3}) for clade B, making the latter clade the faster evolving group. The estimated tMRCA was October 1999 (95% HPD, May 1998–November 2000) for clade A and June 2000 (95% HPD, September 1998–December 2001) for clade B, suggesting that the detection of these two clades occurred several months after their appearance in the country.

We analyzed the selection profiles of the HA protein of Korean H9N2 viruses. Overall, the ratio of nonsynonymous to synonymous substitutions per site (d*N*/d*S*) was 0.236, indicating that Korean H9N2 viruses had evolved under purifying selection. However, we found seven individual codons (p-value < 0.1) that may be under positive selection in the HA protein of the Korean H9N2 viruses, one putatively positive selected residues in viruses collected before the implementation of vaccination (2003–2006) and four in viruses collected from vaccinated poultry populations (2007–2012) (Table 1, Fig 3). In reference to the antigenic sites described in an earlier report, three positively selected sites (positions 153, 166 and 230) were antigenic escape mutant sites; the position 153 and 166 were located in antigenic site II and I in H9, respectively, and the position 230 was located in the vicinity of the trimeric interface of the globular domains of HA1 (Kaverin et al., 2004; Okamatsu et al., 2008; Wan et al., 2014). In addition, the position 166 fell within a potential glycosylation site (166-168) (Supplemental Table S2). The KU114/07-like lineage had different amino acid sequences at antigenic escape mutant sites (positions N153G, N201S, and L230I) compared to most of the viruses isolated between 1996 and 2007 and compared to vaccine strain 01310_CE20/2001 (N145T, N201S, N145T, N166S, L230I) (Supplemental Table S1).

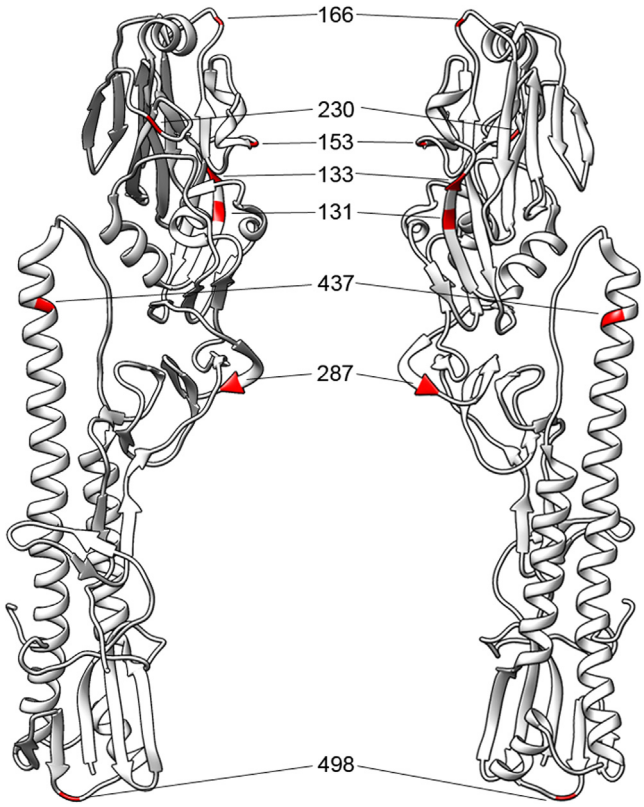

Fig. 3. Ribbon diagram of the monomer of H9 hemagglutinin. Front (a) and back (b) views are shown. Locations of positively selected amino acid changes of Korean H9 isolates are labeled with red color.

Discussion

Our phylogenetic analysis of the HA gene segment of the Korean H9N2 viruses collected between 1996 and 2012 revealed the occurrence of different introductions within the country by poultry or wild birds. However, only a single lineage of virus evolved and circulated extensively in Korean poultry, eventually giving rise to clades A and B. The origin dates of the most recent common ancestors for clades A and B were May 1998–November 2000 and September 1998–December 2001, respectively, suggest that they emerged almost simultaneously probably from the MS96/96-like group. No evidence of new introductions of H9N2 strains from Asia to Korean poultry during 2001–2015 was detected, as shown by our neighbor-joining phylogenetic tree of the HA sequences representative of the H9 lineages (Supplemental Fig. S1).

The analysis of population dynamic revealed a gradual increase of genetic diversity between the years 1996 and 2007 and showed a distinct decrease during 2007. The increase of genetic variability corresponds to the appearance of clade A and the multiple sub-lineages of clade B. The vaccine trials by Korean vaccine companies were conducted on chicken farms between the latter half of 2006 and early 2007 and widespread commercial vaccination commenced in February 2007. The timing of the remarkable decrease in genetic variability corresponded to the introduction and the widespread use of H9N2 clade A vaccine in Korea.

Implementation of H9N2 vaccine likely resulted in the selection and persistence of KU114/07-like lineage of clade B, and the loss of clade A and other sub-lineages of clade B. Clade A and most sub-lineages of clade B disappeared after vaccination, suggesting that the H9N2 vaccination program in Korea dramatically reduced the diversity of the H9N2 lineage. In particular, no clade A viruses were detected after the beginning of the vaccination program. The fact that the vaccine strain belongs to this clade may explain the sudden disappearance of this genetic group. However, KU114/07-like lineage of clade B survived after implementation of vaccination, and expanded in importance, including accumulation of three mutations at antigenic escape mutant sites. Similar changes to HA gene evolution have been reported after H5N2 LPAI vaccine implementation in Mexico (Lee et al., 2004). In 1995, widespread vaccination program was applied to commercial poultry for control of H5N2 HPAIV in Mexico, and has continued against H5N2 LPAIV. The antigenic variants that existed prior to implementation of vaccine were well controlled by vaccine-induced immunity, but new lineages arose after vaccination that replaced the original viruses. These newly emerged viruses in Mexico were antigenically distinct and commercial vaccination was not able to prevent virus shedding when chickens were challenged with these isolates. In addition, the long-term utilization of vaccines against H5 HPAI has been associated with emergence of vaccine resistant field viruses in China (Chen, 2009; Swayne, 2012), Egypt (Cattoli et al., 2011b; Grund et al., 2011), Hong Kong (Connie Leung et al., 2013), Indonesia (Swayne et al., 2015), and Vietnam (Cha et al., 2013).

A previous study reported that H9N2 LPAI virus which belonged to KU114/07-like lineage [A/chicken/Korea/K040110/10 (H9N2)], was isolated from a severe outbreak at a Korean chicken farm in 2010. Interestingly, this isolate replicated well and caused clinical signs (facial edema and diarrhea) in H9N2-vaccinated birds (Lee et al., 2011). In addition, Park et al. (2011) also showed that Korean H9N2 viruses belonging to KU114/07-like lineage showed lower hemagglutination inhibition titer (geometric mean titer (GMT)=20–160) against vaccine strain than that of clade A (GMT=640) and 865/06-like sub-lineage of clade B (GMT=320). Moreover, these isolates were able to replicate in H9N2-vaccinated birds under experimental condition. These reports suggested the KU114/07-like lineage arose following implementation of vaccination, antigenically drifting rapidly away from the commercial vaccine strain, A/chicken/Korea/01310/2001(H9N2), resulting in the recent KU114/07-like viruses being poorly protected by the vaccine.

Vaccine immunity can exert selective pressure for point mutations in the HA gene. Consequently, rapid antigenic evolution in the HA gene with a slightly changed antigenic structure may prevent effective immunity with existing vaccines (Hensley et al., 2009). Based on our results, three positively selected sites (positions 153, 166 and 230) identified in the HA gene segment were antigenic escape mutant sites identified in previous studies and KU114/07-like lineage had different amino acid sequences at these sites compared to viruses isolated before vaccination. In particular, the 2001 vaccine strain and a few field isolates possessed an additional potential glycosylation site at position 166-168 compared to most of the field isolates (Supplemental Tables S1 and S2). The H9N2 viruses collected after vaccination showed slightly higher mean d*N*/d*S* ratio and number of positively selected sites than before vaccination. Overall, these results suggest that Korean H9N2 viruses isolated after 2007 are undergoing increased antigenic drift that is most likely due to vaccination pressure. Although phenotypic effect of these positively selection and mutations in antigenic sites remains unclear, these sites should be closely monitored to identify the relatedness of antigenicity and genetic features.

Implementation of the H9N2 vaccination program in Korea has dramatically reduced the incidence and severity of H9N2 disease in poultry and reduced the genetic and antigenic diversity of H9N2 virus. However, KU114/07-like lineage of clade B has survived, evolving to a subclade that has shown poor antigenic match with the current 2001 vaccine strain. Enhanced surveillance of H9N2 viruses is needed to identify further increments in viral evolution and such data will help in more timely update of vaccine strain to antigenically more closely match the circulating field viruses. Implementation of timely change in vaccine seed strains to more closely match field viruses could reduce viral divergence and better control H9N2 associated poultry disease.

Materials and methods

Nucleotide sequencing

Viral RNA was extracted from 22 H9N2 virus stocks using the RNeasy Mini kit (Qiagen) and reverse transcribed with the Omniscript Reverse Transcriptase kit (Qiagen). PCR amplifications of the HA gene segment were performed as described previously (Hoffmann et al., 2001). The amplified DNA products were electrophoresed in a 1.0% agarose gel. Pieces of the gel containing DNA bands of the expected sizes were extracted using MEGA quick-spin (INtRON Biotechnology, Korea). Nucleotide sequencing was performed with a BigDye Terminator v3.1 Cycle Sequencing Kit and products were analyzed on the ABI PRISM 3730xl genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

A total of 103 HA gene segments were used in this study. Specifically, the nucleotide sequences of 22 H9N2 viruses from 2007 to 2012 generated for this study (accession numbers KT157792-KT157809 and KT165007-KT165010) were analyzed together with the HA segment (sequence length > 1590) of all H9N2 viruses isolated in Korea that were available in the GenBank (n=81) from 1996 to 2011. The nucleotide sequences of Korean H9 LPAIV were aligned using MUSCLE (Edgar, 2004) and manual editing of alignments were performed in MEGA 6 software

(Tamura et al., 2013). The maximum likelihood (ML) tree was estimated by the MEGA 6 software (Tamura et al., 2013) using the general time-reversible (GTR) model of nucleotide substitution with gamma-distributed rate variation among sites (with four rate categories, Γ4), and proportion of invariant sites (I) were estimated. Statistical analysis of phylogenetic tree was determined by bootstrap analysis with 1000 replicates. Additionally, to investigate whether sub-lineages diversified within Korea or some of variants represent novel introductions into Korea from other countries, we constructed a neighbor-joining phylogenetic tree using 351 representative H9 sequences identified available in the GenBank.

Molecular evolution and skyline plot

Molecular evolution rates and genetic diversity were analyzed as previously demonstrated (Davidson et al., 2014; Fusaro et al., 2011) for the complete dataset (100 sequences), as well as for clade A and B viruses. We excluded three viruses that had North American lineage hemagglutinin gene that were isolated in Korean wild birds because they were unrelated to the Korea-like H9N2 lineage. Rates of nucleotide substitution per site per year and time of most recent common ancestor (TMRCA) were estimated using the BEAST program version 1.8.1 (Drummond and Rambaut, 2007), which employs a Bayesian Markov chain Monte Carlo (MCMC) approach. For each analysis, we employed a codon-based SRD06 nucleotide substitution model and an uncorrelated lognormal relaxed clock. In addition, we utilized a Skyline coalescent tree prior (10 piece-wise constant groups), as this is the best descriptor of the complexity of the population dynamics of the H9N2 viruses (Drummond et al., 2005). Maximum clade credibility (MCC) phylogenetic tree was estimated from the posterior distribution of trees generated by BEAST using the program TreeAnnotator v1.8.1 (Drummond and Rambaut, 2007). The MCC tree was visualized using the program FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). A Bayesian Skyline plot was used to infer the population dynamics of Korean H9N2 viruses in terms of changing level of relative genetic diversity [$N_e t$] through time, in which N_e represents the effective population size and t the generation time.

Analysis of selection pressures and glycosylation

Gene- and site-specific selection pressures for the AIV HA protein of the Korean H9N2 viruses were measured as the ratio of non-synonymous (dN) to synonymous (dS) nucleotide substitutions per site for the complete dataset (100 sequences), as well as for viruses collected before (1996–2006) and after (2007–2012) the vaccination campaign. The dN/dS ratios and the selection pressures at individual codons were estimated using the single-likelihood ancestor counting (SLAC), fixed-effects likelihood (FEL), and mixed effects model of episodic diversifying selection (MEME) available at the DataMonkey online version of the HY-Phy package http://www.datamonkey.org (Kosakovsky Pond and Frost, 2005; Pond and Frost, 2005). All analyses utilized the GTR model of nucleotide substitution and employed input NJ phylogenetic trees. Positively selected sites that confirmed by at least two different methods were included in this study.

The positions of positively selected amino acids on the HA molecule were examined on the 3-dimentional structure obtained from the Protein Databank (PDB accession number, 1JSD) with the Chimera 1.10 program. Potential N-glycosylation sites were predicted using NetNGlyc server 1.0.

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Appendix A. Supplementary material

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VETERINARY RESEARCH

REVIEW Open Access

Current situation of H9N2 subtype avian influenza in China

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Abstract

In China, H9N2 subtype avian influenza outbreak is firstly reported in Guangdong province in 1992. Subsequently, the disease spreads into vast majority regions nationwide and has currently become endemic there. Over vicennial genetic evolution, the viral pathogenicity and transmissibility have showed an increasing trend as year goes by, posing serious threat to poultry industry. In addition, H9N2 has demonstrated significance to public health as it could not only directly infect mankind, but also donate partial or even whole cassette of internal genes to generate novel human-lethal reassortants like H5N1, H7N9, H10N8 and H5N6 viruses. In this review, we mainly focused on the epidemiological dynamics, biological characteristics, molecular phylogeny and vaccine strategy of H9N2 subtype avian influenza virus in China to present an overview of the situation of H9N2 in China.

Table of Contents

1 Introduction

Avian influenza (AI) is initially reported in 1878 in Italy to describe the disease resulted in massive poultry death, which was then termed as "Fowl plague" to distinguish from fowl cholera in 1880 [1]. Although had being identified as filterable virus in 1901, the causative agent is formally designated as influenza A virus until 1955 [1, 2]. Apart from the highly pathogenic forms, less virulent AI viruses have been successively detected in various countries since the mid-1900s that started with the first isolate from chickens in Germany in 1949 [A/chicken/Germany/1949(H10N7)] without being recognized and defined the specific subtype till 1960. As for the H9N2 subtype, with distinguished characteristics to challenge animal industry and even human health among the low pathogenic AI forces, the protovirus is generally considered as the early isolate from turkey flocks in Wisconsin in America in 1966 [A/turkey/Wisconsin/1/1966(H9N2)] [3]. The virus spread becomes more and more extensively at about 1990s, resulting continuous viral circulation in several countries in Asia, Middle East and North Africa [4]. On one hand, H9N2 AI virus could cause damage to birds with direct pathology, coinfection and immunosuppression [5, 6]. On the other hand, H9N2 viruses not only infect mankind directly, but also provide partial or even whole set of internal genes to emerging human-lethal

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H5N1, H7N9, H10N8 and H5N6 reassortants [7–11], posing a substantial threat to public health. Therefore, the study of H9N2 AI virus deserves great attention.

2 The etiology of AI

Avian influenza virus affiliates to the genus of type A influenza virus in the Orthomyxoviridae family, packaged with eight negative-sense and single-strand RNA segments in sequence of PB2, PB1, PA, HA, NP, NA, M and NS according to gene length [12]. Each viral gene encodes at least one protein, in which the three polymerase proteins (PB2, PB1 and PA) plus the nucleoprotein (NP) consist the minimal protein unit in forming the functional RNP structure essential for viral transcription and replication. Hemagglutinin (HA) and neuraminidase (NA) are the two major envelope glycoproteins indispensable in mediating influenza A virus to invade host cells and promoting matured newborn virions to disaggregate from cell surface, respectively [13]. Both M and NS genes utilize RNA splicing to synthesize two protein forms of matrix protein (M1) and ion channel protein (M2), nonstructural protein (NS1) and nuclear export protein (NS2), respectively. Through ribosomal frameshift, PB1 and PA genes can also be edited to generate additional PB1-F2 and PA-X proteins, effecting on virus pathogenicity [14, 15]. Based on the antigenic diversity, AI virus can be classified into 16 HA subtypes (H1–H16) and 9 NA subtypes (N1–N9), resulting in various subtype combinations. The criteria to discriminate highly pathogenic avian influenza virus (HPAIV) and low pathogenicity avian influenza virus (LPAIV) were defined at the First International Symposium on Avian Influenza in Beltsville in 1981 [1]. HPAIV only restricts to partial proportions of H5 or H7 subtype, whereas LPAIV covers all the remaining viruses. In particular, H9N2 is currently the most widely circulating and damaging LPAIV subtype in the world.

3 Outbreaks and prevalence of H9N2 in China

Isolation of AI virus in China has been documented since 1970s [16]. During November 1975 to October 1979, several different subtypes of AI viruses had been isolated from imported live poultry (duck, goose, chicken) in Guangdong and Guangxi provinces, of which the most prevalent subtype is H4N6 [17, 18]. In addition, domestic scholars also described type A influenza virus from duck flocks in some meat processing enterprise in Nanjing in 1980 [19]. However, those above mentioned AI viruses were all identified from apparently healthy birds, therefore insufficient to certify the actual existence of disease outbreaks.

Till 1992, Chen et al. isolated the first H9N2 subtype LPAIV strain $AID_{93\text{-}1}$ (once erroneously identified as H9N3 subtype then), also the earliest published report of AI outbreaks in mainland China [20]. During November 1992 to May 1994, a total of 17 chicken farms and two minor poultry farms had suffered from AI outbreaks in regions of Guangdong province [20, 21]. A few years afterwards, several other parts in China intermittently reported sporadic disease outbreaks caused by H9N2 [22–24]. However, a massive H9N2 epizootics occurred from fall to winter in 1998, initially starting from Hebei province and rapidly spreading to majority of poultry raising areas nationwide in only 2 months [25, 26]. According to the statistics, the ratio of chicken flocks subjected to H9N2 subtype AI infection accounted for 93.89% in the period of 1996–2000, thereby demonstrating that H9N2 was the predominant subtype affecting poultry farming from the end of twentieth century to the beginning of twenty first century [27]. Even to this day, H9N2 is still one of the three primary AI subtypes devastating poultry industry other than the notorious H5N1 and emerging rookie of H7N9.

Theoretically, emerging diseases could possibly be effectively controlled by a stamping-out policy before disseminating into vast areas [28]. However, the optimal eradication opportunity for H9N2 through timely culling of infected poultry was missed during 1992–1998 in China, as the disease has remarkably spread into large regions especially since 1998 and the vaccination strategy has been extensively executed since then [29]. Presently, H9N2 has become stably established in chicken flocks to acquire the endemicity in vast majority of China, accompanied with the substantial implementation of vaccination programs [5]. Moreover, the virus is yet prevalent in wild birds, live poultry markets, backyard flocks and environment [30, 31]. Generally, the inherited complex breeding and trading patterns of poultry industry contributed critically to the current epidemiological situation of H9N2 in China. On one hand, traditional small-scale and backyard-level raisings such as free ranging and mixed ranging still occupy certain ratio in poultry production nationwide, while their biosecurity condition and vaccination coverage are relatively unsatisfactory as compared with typical intensive operations. On the other hand, live poultry markets (LPMs) as a distinctive manifestation of the consumption style that freshly-killed poultry meat is much more preferred rather than chilled or frozen meat, has provided a tremendous gene pool of avian influenza viruses which is evidenced by the continued high virus detection rate including multiple HA/NA subtypes [32]. It is worth noting that interventions involving implementation of one or two rest days per month in the wholesale and retail LPMs could significantly reduce the H9N2 isolation rates [33]. As China is still located on the important flyways for migration, the

huge amount of domestic waterfowls which frequently contact the ecointerface with wild waterfowls when sharing common water or makeshift inhabitance also facilitated the persistence and evolution of H9N2 viruses in environment by means like inter-transmission and gene reassortment between birds [30]. Ecologically, at least those above mentioned intricate factors jointly shaped the enzootic status of H9N2 in China.

4 Genetic evolution of H9N2

H9N2 subtype AI virus is extensively distributed worldwide, generally divided into two major lineages of North-American lineage and Eurasian lineage. Specifically, the Eurasian lineage further blooms into various virus clusters, as represented by A/chicken/Beijing/1/1994(BJ/94-like) or A/duck/Hong Kong/Y280/1997(Y280-like), A/quail/Hong Kong/G1/1997(G1-like), A/duck/Hong Kong/Y439/1997(Y439-like), A/chicken/Shanghai/F/1998(F/98-like) and so on [34–36]. Comparing with the H9N2 viruses in Central Asia and the Middle East, Chinese isolates clustered independently as referred from the phylogenetic trees of HA and NA genes [36]. In China, G1-like circulated mainly in quails is of geography superiority in southern regions, whereas BJ/94-like and F/98-like prevailed in chicken flocks are regnant in northern and eastern areas, respectively [26, 35].

4.1 HA phylogenetic clades

To further systematically understand the evolutionary dynamics of H9N2 subtype AI virus globally, four stem evolutionary clades of h9.1–h9.4 have been designated by Jiang et al. to map the HA gene phylogeny through comparing more than 1000 HA sequences retrieved from GenBank, as referred to the nomenclature of the Asian H5N1 HPAIV defined by the WHO/OIE/FAO H5N1 working group [37, 38]. Particularly, h9.1 and h9.2 just corresponded to early North-American isolates in 1966 and the nineties, respectively. H9.3 covered the widest temporal span including Asia, Europe, Africa, Pacific and North America, so did expand the longest spatial range from 1976 until now. The most vast clade h9.4 included two subclades of h9.4.1 and h9.4.2, which coordinated to the G1-like (h9.4.1.1) and Y280-like (h9.4.2.4) H9N2 viruses prevailing in most Asian countries ever since 1994, respectively. In more detail, h9.4.1 contained isolates from Pakistan, India, Iran and Israel, whereas h9.4.2 accommodated exclusively Chinese strains. Chronologically, domestic H9N2 viruses before 2007 generally belonged to clades h9.4.2.1–h9.4.2.4, in which h9.4.2.1 equaled to the above mentioned F/98-like viruses. Thereafter, h9.4.2.5 represented by A/chicken/Guangxi/55/2005(H9N2) has become predominant step by step, whilst h9.4.2.6 distinguished by A/chicken/Guangdong/FZH/2011(H9N2) mainly in southern China has also acquired establishment and tended to spread readily across the country from about 2010. Hence, currently, h9.4.2.5 and h9.4.2.6 have co-circulated in China, while of which the former H9N2 viruses are yet superior over the latter ones.

4.2 Genotypic diversity

Owing to the segmented nature of AI virus genome, when two or more virus strains concurrently infect a single cell, exchange of gene segments would occur among different virus particles via gene reassortment to generate a series of newborn viral descendants inheriting parental components. Certainly, H9N2 subtype AI virus is also without exception that distinct virus clusters could reassort with each other or with other AI subtypes to produce various genotypes, which is defined on the basis of the combination of each individual gene phylogenies. For instance, virus harbored all the gene constellation from BJ/94-like is designated as genotype A, variant of three polymerase genes and NP gene from F/98-like while the remaining four genes from BJ/94-like is assigned for genotype H. Thus far, H9N2 subtype AI virus in China has evolved into diversified clusters and genotypes (A–W), showing clear spatio-temporal divergence (Table 1; Figure 1) [7, 26, 29, 39, 40]. Among the rest, three major genotypes of H9N2 subtype AI virus containing A, H and S, have predominated in chicken flocks during different periods since the nineties [6, 7, 29, 41]. In particular, early genotype A prevailing in the nineties had gradually been replaced by genotype H, evident of better adaptation in poultry and easier reassortment with other AI viruses, after 2000 [6]. However, genotype S with exogenous G1-like PB2 and M genes on the genetic backbone of F/98-like viruses emerged around 2007 and had become increasingly established in chickens afterwards, especially in the Yangtze River Delta region in eastern China [6, 7]. Updated epidemiological studies in more recent years also suggest the supreme of genotype S there [40]. Consistently, the additionally categorized genotype G57 (generally equivalent to genotype S) demonstrated greater infectivity than the other genotypes, and had been dominating ever since 2010 across China to cause severe damages to poultry farming [42].

5 Biological property variation of H9N2

The premier isolates of H9N2 just infected turkeys, rarely encroached on chickens, but have gradually adapted to chickens and acquired pathogenicity after years of evolution [1, 3]. Since the initial isolation of H9N2 virus in China, its host range and virulence have become increasingly wider and stronger, respectively [5, 26, 35, 42, 43]. As revealed by a continuous surveillance on H9N2

Table 1 Genotypes of H9N2 subtype avian influenza viruses in China.

Genotype	Emerged year	Gene constellation								References
		PB2	PB1	PA	HA	NP	NA	M	NS	
A	**1994**	**BJ/94**	**BJ/94**	**BJ/94**	**BJ/94**	**BJ/94**	**BJ/94**	**BJ/94**	**BJ/94**	[29]
B	1997	G1/97	G1/97	BJ/94	BJ/94	BJ/94	G9/97	BJ/94	BJ/94	
C	1999	G1/97	G1/97	G1/97	BJ/94	BJ/94	G9/97	BJ/94	G1/97	
D	1999	G1/97	G1/97	G1/97	BJ/94	BJ/94	BJ/94	BJ/94	G1/97	
E	2000	G1/97	G1/97	G1/97	TY/WI/66	TY/WI/66	G9/97	BJ/94	BJ/94	
F	2000	BJ/94	BJ/94	BJ/94	BJ/94	BJ/94	G9/97	BJ/94	BJ/94	
G	2000	G1/97	BJ/94	BJ/94	BJ/94	BJ/94	BJ/94	BJ/94	BJ/94	
H	**1998**	**F/98**	**F/98**	**F/98**	**BJ/94**	**F/98**	**BJ/94**	**BJ/94**	**BJ/94**	
I	2001	F/98	F/98	F/98	BJ/94	F/98	G9/97	BJ/94	BJ/94	
J	1999	F/98	F/98	F/98	BJ/94	F/98	BJ/94	BJ/94	d73/76	[26]
K	2003	BJ/94	BJ/94	Kor/323/96	BJ/94	BJ/94	BJ/94	BJ/94	BJ/94	
L	2005	F/98	F/98	F/98	BJ/94	F/98	BJ/94	BJ/94	Kor/323/96	
M	1998	BJ/94	BJ/94	F/98	BJ/94	BJ/94	BJ/94	BJ/94	BJ/94	[39]
N	2007	BJ/94	F/98	F/98	BJ/94	F/98	BJ/94	BJ/94	BJ/94	
O	2007	F/98	F/98	F/98	BJ/94	F/98	BJ/94	G1/97	BJ/94	
P	2008	F/98	F/98	F/98	BJ/94	F/98	G9/97	G1/97	BJ/94	
Q	2008	F/98	BJ/94	Y439/97	BJ/94	F/98	G9/97	G1/97	BJ/94	
R	2007	F/98	F/98	Y439/97	BJ/94	F/98	BJ/94	G1/97	BJ/94	[7]
S	**2007**	**G1/97**	**F/98**	**F/98**	**BJ/94**	**F/98**	**BJ/94**	**G1/97**	**BJ/94**	
T	2008	F/98	BJ/94	F/98	BJ/94	F/98	G9/97	G1/97	BJ/94	
U	2009	G1/97	BJ/94	Y439/97	BJ/94	F/98	G9/97	G1/97	BJ/94	
V	2014	G1/97	F/98	F/98	BJ/94	F/98	G9/97	G1/97	BJ/94	[40]
W	2014	Wild Waterfowls	F/98	F/98	BJ/94	F/98	F/98	G1/97	BJ/94	

Genotypes were defined according to the array mode of the eight gene phylogenies, those which have or had persisted for a long time in China are labeled in bold

BJ/94: A/chicken/Beijing/1/1994(H9N2)-like; G1/97: A/quail/Hong Kong/G1/1997(H9N2)-like; G9/97: A/chicken/Hong Kong/G9/1997(H9N2)-like; Y439/97: A/duck/Hong Kong/Y439/1997(H9N2)-like; TY/WI/66: A/turkey/Wisconsin/1/1966(H9N2)-like; F/98: A/chicken/Shanghai/F/1998(H9N2)-like; Kor/323/96: A/chicken/Korea/38349-p96323/1996(H9N2)-like; d73/76: A/duck/Hong Kong/d73/1976(H6N1)-like

subtype AI virus in eastern China from 1999 to 2008, most viruses before 2000 were competent to propagate in inoculated chickens but inadequate to be transmissible through respiratory droplets [26]. In contrast, variants after 2001 not only replicated well in vivo but also transmitted efficiently by respiratory droplets in chickens [44]. Comparing with the ones prior to 2010, H9N2 isolates circulating during 2010–2013 showed an obviously higher isolation rate and titers, as well as a longer period of virus shedding especially from cloaca in challenged chickens [42]. It was recently demonstrated that such improved viral fitness was resulted from the substitution of BJ/94-like M gene with the G1-like [45]. Specifically, H9N2 viruses containing G1-like M gene not only exhibited significantly efficient early augment of viral mRNA and vRNA to increase the amount of produced protein and benefit the release of progeny virions, but also conferred extrapulmonary virus spread in chickens [45]. Moreover, characterization of H9N2 viruses ranging from 2009 to 2013 in southern China indicated that natural H9N2 isolates of chicken origin had gradually acquired the preference for human-type α-2,6 sialic acid receptors, and several variants even developed the airborne transmissibility in ferrets [46].

6 Internal gene cassette reassortment of H9N2

It is acknowledged that the variation mechanism of AI virus mainly includes antigenic drift and genetic shift, with the former featured by point mutation of key amino acids in major immunoprotective proteins whereas the latter resulted from genomic reassortment [47]. As compared with genetic drift, gene reassortment poses a more radical effect on influenza virus by generating totally brand-new viruses with competitive advantage to spread widely such as those causing influenza pandemics in history [48–51]. According to literatures, H9N2 not merely donate partial gene segments but also the whole set of internal genes to reassort with other influenza A viruses [52, 53]. Especially in the past few years, the phenomenon that the six internal genes of H9N2 constituting a relatively stable community to transfer into other emerging reassortants as a whole cassette seems more

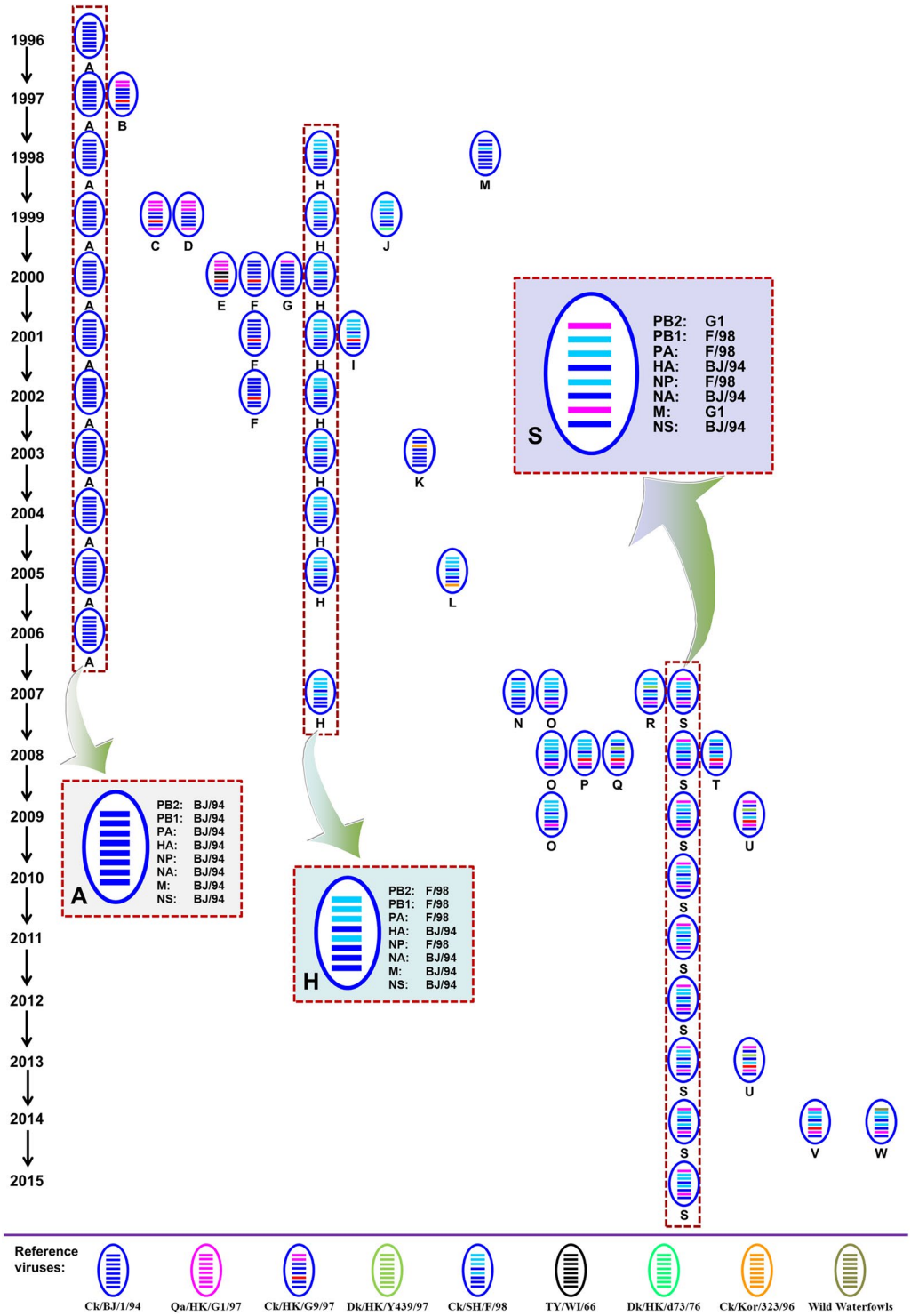

Figure 1 Genotypic diversity of H9N2 subtype avian influenza viruses in China during 1996–2015. The eight horizontal bars in oval (from top to bottom) represent PB2, PB1, PA, HA, NP, NA, M and NS genes, respectively. Each color represents a virus lineage. The resulting genotype designation is depicted below.

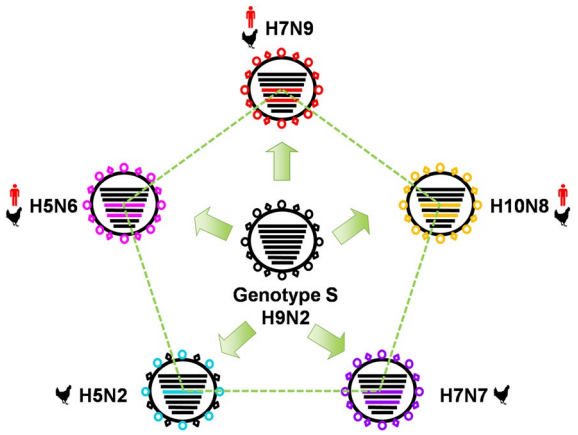

Figure 2 Illustrative scheme of the events induced by reassortment of whole set of internal genes from H9N2 to generate novel influenza A reassortants. The eight horizontal bars in circle (from top to bottom) represent PB2, PB1, PA, HA, NP, NA, M and NS genes, respectively. The ring and prismatic shape on outer surface of circle represent the HA and NA proteins, respectively. Each color represents a specific virus subtype. The black poultry label indicates that the generated influenza A virus challenges birds and the red figure label indicates that the reassortant can also infect human beings.

distinguished (Figure 2). For example, the newly detected chicken H7N7 viruses in Wenzhou city of Zhejiang province, the human-infecting H7N9 and H10N8 viruses initially reported in 2013, and the more recent clade 2.3.4.4 human-lethal H5N6 viruses, were all generated on the basis of complete internal genes from H9N2 subtype AI viruses [9, 10, 54–56]. In addition, H9N2 even dedicated all the other seven gene segments except HA to the clade 7.2 HPAI H5N2 natural reassortants in recent years [57, 58]. Despite diversity, those H9N2 donor viruses all pertain to the unique S genotype prevailing in chicken flocks in China since 2007 [7]. As influenza A virus proved to choose gene segments specifically for package when more than one kind of viruses co-infect the same host cell, whether the intrinsic vRNA–vRNA interaction contributed crucially to the molecular mechanism of this particular internal-gene-cassette re-assortment deserves further exploration [59–61].

7 Vaccine strategy for control of H9N2

Presently, H9N2 subtype AI virus has been widely spread in China, and has established stable lineages in commercial chicken flocks with endemicity [40]. Despite that the mortality caused by H9N2 generally not exceed 20%, it usually leads to respiratory and egg-drop symptom, as well as sever secondary infection of other respiratory diseases, affecting poultry productivity [5, 29]. Therefore, at current stage, vaccination is still one of the principle strategies to control H9N2 AI in China apart from biosecurity.

7.1 Conventional whole-virus inactivated vaccines

The majority of commonly used AI vaccines are killed whole-virus vaccines, prepared from formaldehyde inactivation of virus-containing allantoic fluids proliferated via chicken embryos and accompanied with adjuvants, manifesting favorable immune efficacy [62]. Domestically, various H9N2 strains have been used for inactivated vaccine development, wherein the F strain and SS strain respectively belonging to genotype H and genotype A are the two typical representatives [63, 64]. The F strain is a natural reassortant of chicken origin isolating from Shanghai in 1998, with its polymerase genes being replaced with counterpart gene segments from distinct H9N2 clusters in ducks. This F/98-like virus entirety had existed over a long time in chicken flocks in China, and even served as the donor to provide internal genes for further reassortants until recently [65, 66]. As for SS vaccine, it is developed from the seed isolated from Guangdong province in 1994, which is also the first commercial vaccine for control of H9N2 subtype AI in China. However, as the ongoing evolution of H9N2 viruses, vaccination failure due to infection with prevailing antigenic variants evidently challenges the efficacy of the vaccines in China, like that in many other countries such as Iran and Korea [40, 67–72]. Therefore, updated vaccine seed strains based on continuous surveillance data have gradually been preparing and permitting for clinical practice. To simplify the immune procedure to reach an ideal goal of "one injection preventing multiple diseases", a massive of double or multiple combined vaccines have been designed such as the triplex inactivated vaccines simultaneously against AI (H9 subtype), Newcastle disease and infectious bronchitis [73].

7.2 Recombinant and vector virus vaccines

Inactivated whole virus vaccine mainly elicits humoral immune response, deficient in inducing effective mucosal and cellular immunity. Furthermore, it also interferes with immunological surveillance and epidemiological investigation of AI virus under the condition of current technology. Therefore, novel DIVA (differentiating infected and vaccinated animals) vaccines against H9N2 come into being, including recombinant live virus vectored vaccine, subunit vaccine, DNA vaccine, VLPs (virus like particles) vaccine and so on. They could supplement certain shortages of traditional vaccines and are popular for AI vaccine development nowadays. Frequently used live virus vectors contain recombinant fowlpox virus, Newcastle disease virus, Marker's disease virus, etc [74–76]. Subunit vaccine is generally developed based on the extraction of immunogenic proteins (usually HA) of AI virus, without introducing viral particles. Large amounts of HA protein could be acquired by ligation of HA gene

with expressing plasmid vector for amplification, such as in the baculovirus expression system [77]. As for DNA vaccine, the exogenous gene encoding for protective antigen is initially cloned to eukaryotic expressing vector, followed by administrating the constructed DNA plasmids into animals to get expressed in vivo and to stimulate specific humoral and cellular immunity [78]. VLPs are self-assembled hollow protein particles by one or more viral structural proteins, containing no viral genetic materials but resembling integral viruses in appearance. Despite without infectivity, VLPs could still retain immunogenicity to provoke effective immune response and to serve as safe vaccines [79]. So far, a great number of novel genetically engineered AI vaccines have been designed in China, however, many of which are still in the stage of technical research and reserve, immature for clinical usage yet.

8 Interspecies transmission of H9N2

8.1 H9N2 in pigs

Apart from various kinds of poultry, H9N2 subtype AI viruses could also infect pigs, the long considered mixing vessel for mammalian and avian influenza variants. It is revealed by epidemiological survey that H9N2 viruses were isolated from pigs naturally when transported from southern China to Hong Kong for sale, as early as in 1998 [80]. Subsequently during 2001–2008, H9N2 had been detected incessantly in swine herds in several provinces covering Shandong, Fujian, Henan, Jiangxi, Guangdong, Guangxi, Hebei and so on [81–84]. In addition, the identified swine H9N2 isolates exhibited evident genetic and antigenic complexity with diversified genotypes [85]. Serological investigation also manifested the infection of H9N2 viruses in Chinese pig population [86–88].

8.2 H9N2 in humans

What's more noteworthy, H9N2 subtype AI viruses have already acquired the ability to break through species barrier and directly invade human beings without intermediate hosts. The first documentation of human-infecting H9N2 viruses in China traced back to 1998, as described that five H9N2 strains were cultured from laryngopharyngeal mucus of flu-like outpatients and inpatients in southern regions [89]. Further gene sequence analysis indicated that those H9N2 human isolates probably derived from local chicken flocks [90]. In March 1999 in Hong Kong, another two children were confirmed infection with H9N2 viruses, with their genomic sequences highly homologous with the quail strain A/quail/Hong Kong/G1/1997 [91, 92]. Therefore, quails had also been suggest to play important roles in cross-species transmission of H9N2 viruses [93]. Still in 1999, A/chicken/Hong Kong/G9/1997-like H9N2 virus repeatedly isolated from human population in November in southern China [94]. Again in December 2003, Hong Kong reported a second human infection event of H9N2 virus, of which all the eight gene segments were of avian origin and clustered most intimately with those extensively distributed in live poultry market there [95]. Yet recently, laboratory-confirmed human infection of H9N2 virus have continuously been reporting sporadically from WHO, with an apparently higher rate in the last few years and even one fatal case additionally suffering from chronic underlying conditions in 2016 [45]. Besides, quite a number of people prove to have been exposed to H9N2 viruses by serological data, especially those poultry workers [89, 96–98]. Distinct from HPAI H5N1 infection, the overall human symptoms induced by H9N2 are analogous to seasonal flu with rapid recovery and no lethality. However, just such mild infection has made H9N2 easily be negligible in clinical, facilitating to adapt further in the body by reassortment with other human influenza viruses to yield potential variants with high reproductivity and even efficient interpersonal transmissibility.

9 Conclusion

Although being classified as LPAIV, H9N2 subtype AI virus is extensively distributed in chicken flocks to pose a persistent challenge. In China, traditional raising system of livestock including free-ranging and polyculture, continuously occupies a crucial status yet. It is inevitable for chicken to contact with domestic or wild waterfowl, which harbored large amount of H9N2 viruses. These apparently healthy latent birds could serve as the "Trojan horses" in chicken flocks to cause the circulation of H9N2. Furthermore, the LPMs extending throughout China still played an indispensable role in hosting and disseminating of H9N2 AI virus, as evidenced by significant higher rates of virus isolation than other locations. However, focusing on LPMs management, innovative control measures targeting principally against the emerging avian influenza A(H7N9) virus such as closure of LPMs or other more sustainable but yet effective interventions including washing and cleaning once a day, disinfecting once a week, having rest days once a month and banning live poultry overnight, as well as separating of aquatic and non-aquatic live poultry, would certainly simultaneously reduce the risk of H9N2 contamination at source and deserve high priority in implementation. On account of incessant viral mutation and reassortment, natural variants with increased pathogenicity have been emerging periodically. Even though vaccination remains one of the primary strategies to control H9N2 subtype AI in China, the majority of vaccine recipients are actually still under siege of wild-type variants. Therefore, disease outbreaks would still occur in vaccinated flocks in

case of descended protection level or different kinds of immune failure. It is more prior important to establish favorable biosecurity management and take all practicable measures to control infection source, preventing virulent variants from intruding the poultry flocks. On the other hand, the human-infecting events of H9N2 AI virus deserve to be treated scientifically and rationally. Once animal influenza is controlled, should the risk of emerging human pandemic influenza be decreased to minimum level.

Abbreviations

AI: avian influenza; PB2: polymerase basic protein 2; PB1: polymerase basic protein 1; PA: polymerase acidic protein; HA: hemagglutinin; NP: nucleoprotein; NA: neuraminidase; M: matrix protein; NS: nonstructural protein; HPAIV: highly pathogenic avian influenza virus; LPAIV: low pathogenicity avian influenza virus; LPMs: live poultry markets; WHO: World Health Organization; OIE: World Organisation for Animal Health; FAO: Food and Agriculture Organization of United Nations; BJ/94: A/chicken/Beijing/1/1994(H9N2); F/98: A/chicken/Shanghai/F/1998(H9N2); G1: A/quail/Hong Kong/G1/1997(H9N2); SA: sialic acid; DIVA: differentiating infected and vaccinated animals; VLPs: virus like particles.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MG and LX drafted and revised the manuscript; XW helped in revision; XL designed the structure of the review. All authors read and approved the final manuscript.

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Review

A Decade of Avian Influenza in Bangladesh: Where Are We Now?

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Abstract: Highly pathogenic avian influenza (HPAI) has been a public health threat in Bangladesh since the first reported outbreak in poultry in 2007. The country has undertaken numerous efforts to detect, track, and combat avian influenza viruses (AIVs). The predominant genotype of the H5N1 viruses is clade 2.3.2.1a. The persistent changing of clades of the circulating H5N1 strains suggests probable mutations that might have been occurring over time. Surveillance has provided evidence that the virus has persistently prevailed in all sectors and caused discontinuous infections. The presence of AIV in live bird markets has been detected persistently. Weak biosecurity in the poultry sector is linked with resource limitation, low risk perception, and short-term sporadic interventions. Controlling avian influenza necessitates a concerted multi-sector 'One Health' approach that includes the government and key stakeholders.

Keywords: avian influenza; Bangladesh; biosecurity; H5N1; poultry; surveillance; vaccination

1. Background

Bangladesh reported its first outbreak of highly pathogenic avian influenza (HPAI) in poultry in 2007 [1]. Since then, a total 556 outbreaks of HPAI H5N1 in poultry have been reported in 52 of the 64 districts until 2013, and the virus has now became enzootic in poultry [1,2]. The other subtypes isolated were H1N2, H1N3, H3N6, H4N2, H5N6, H10N7, and the predominant low pathogenic avian influenza (LPAI) virus H9N2 [3,4]. Unusual mortalities caused by H5N1 have been reported in commercial poultry [5], waterfowl [6], and in crows [7]. Evidence of past exposure to H5 virus in nomadic ducks has been reported [8]. A total of eight human cases attributed to the subtype have also been reported since 2008 [9]. Bangladesh reported three mild human cases of H9N2 [10]. An outbreak investigation during 2012–2013 showed that detectable avian influenza viruses (AIV) RNA was found in nasopharyngeal swabs of 4.5% and on arm swabs of 18.5% of 371 asymptomatic poultry workers [11].

The complex nature of the poultry production and marketing systems, limited veterinary capacity, and low level of commitment from the raisers to report mortality to the government favor the persistence of H5N1 [12,13]. Every introduction of AIV into humans poses a risk of coinfection and genetic reassortment with co-circulating human influenza viruses, which could lead to the emergence of a novel influenza viral strain with pandemic potential [14]. There are three prerequisites for the

emergence of a new influenza pandemic: (i) the emergence of a novel virus to which humans are widely susceptible; (ii) the new virus is able to replicate and cause disease in humans; and (iii) the new virus is transmitted efficiently from human-to-human [15]. Although effective human–human transmission of HPAI virus is not evident, the high population density and close contact between humans and animals in Bangladesh poses a pandemic threat [16,17].

In order to combat AIV, the Government of Bangladesh (GoB) adopted the first national preparedness and response plan in 2006 [18]. Since then, there have been numerous efforts to detect, track, and combat AIV from several government and non-government organizations. However, it is yet to be understood how much has changed since the advent of AIV in Bangladesh. This review discusses the history of avian influenza over the past decade in Bangladesh and demonstrates where we are now.

2. Clades of HPAI H5N1 Detected in Bangladesh

Several studies explored the genetic characterization of the HPAI H5N1 virus circulating in Bangladesh. The circulating HPAI H5N1 viruses in Bangladesh clustered with gs/GD clade 2.2.2 from February 2007 until the end of 2010. At the beginning of 2011, new incursions of viruses of clades 2.3.2.1 and 2.3.4.2 were detected in chickens, quails, ducks, crows, and migratory birds [19–21]. According to a phylogenetic analysis of the isolates of 2012 and 2013, all the isolates exclusively belonged to clade 2.3.2.1 [21]. By the end of 2014, circulating Bangladeshi H5N1 viruses exclusively belonged to clade 2.3.2.1a [22,23]. A more recently determined status of circulating AIV in Bangladesh from a surveillance of live bird markets (LBMs) and waterfowl in wetland areas from February 2015 through February 2016 revealed that a new genotype of H5N1 viruses, clade 2.3.2.1a, had become predominant [24]. These newly emerged H5N1 viruses contained the hemagglutinin, neuraminidase, and matrix genes of circulating 2.3.2.1a Bangladeshi H5N1 viruses and five other genes of low pathogenic Eurasian-lineage AIV, some of which were closely related to the genes of the strains isolated from ducks and wild birds from northeastern Bangladesh [24].

3. Surveillance

3.1. Poultry Surveillance

Since HPAI represents an important threat to human health, it is essential to characterize the different strains of AIV that are circulating in poultry. As part of the influenza preparedness and response plan, the Department of Livestock Services (DLS), in collaboration with other partners and donor organizations, strengthened the existing passive surveillance system and initiated an active surveillance program to rapidly detect HPAI H5N1 outbreaks in both commercial and backyard poultry in 2008 (Table 1). Through active surveillance, DLS supported the monitoring of 306 high-risk sub-districts out of 487 in Bangladesh, with support from Sweden, the United States Agency for International Development (USAID), World Bank, and Food and Agriculture Organization (FAO) [25,26]. Community Animal Health Workers (CAHWs), additional veterinary surgeons (AVSs), and Upazila Livestock Officers (ULOs) were trained to collect data and report on morbidity and mortality in poultry using a short message service (SMS) gateway system (i.e., a method of sending and receiving messages between computers and mobile phones) at the end of each working day. A central surveillance team at the DLS reviewed the internet-based SMS outputs to monitor trends in disease, morbidity, and mortality in poultry. This real-time reporting using SMS identified and contained 550 HPAI H5N1 outbreaks, entailing the culling of a total of 3.46 million poultry, and destruction of 2.97 million eggs belonging to 822 farmers. The system facilitated the reduction of the outbreak response time from 4.8 days to 1.4 days and captured 86% of the outbreaks [25]. The initiative continued until 2013 [26].

Table 1. Surveillance for poultry and human infections with avian influenza viruses.

Types of Surveillance	Species	Duration	Type of Samples Collected	Laboratory Tests Used	References
Poultry surveillance [icddr,b]	Waterfowl, commercial chickens, backyard chickens, market environment	2007–till date	Cloacal swabs, swabs from freshly laid feces, tracheal swabs, environmental pooled swabs	rRT-PCR for typing and subtyping of influenza A viruses	[27]
Poultry surveillance [DLS-FAO-ECTAD]	Waterfowl, commercial chickens, backyard chickens	2008–2013	Cloacal swabs, swabs from freshly laid feces, tracheal swabs	rRT-PCR for typing and subtyping of influenza A viruses	Personal communication, DLS
Sink surveillance [DLS-FAO-ECTAD]	Market environment	2016–till date	Environmental pooled swabs	rRT-PCR for typing and subtyping of influenza A viruses	Personal communication, DLS
Poultry worker's surveillance [icddr,b]	Humans	2012–2017	Nasopharyngeal and throat swab (respiratory swabs), acute and convalescent blood specimens	Respiratory swabs: rRT-PCR for influenza A and B viruses and subtyping for influenza A Serum: haemagglutination inhibition (HI) and microneutralization (MN) assay	[28]
Hospital-based Influenza Surveillance (HBIS) [icddr,b]	Humans	2007–till date	Nasopharyngeal and throat swab	rRT-PCR for influenza A and B viruses and subtyping for influenza A	[29]
National Influenza Surveillance, Bangladesh (NISB) [IEDCR]	Humans	2010–till date	Nasopharyngeal and throat swab	rRT-PCR for influenza A and B viruses and subtyping for influenza A	[29]

To strengthen the government surveillance system, the icddr,b, with funding and technical support from the US Centers for Disease Control and Prevention (CDC), has also been performing an LBM-based sentinel surveillance for AIV in poultry since 2007, in collaboration with the DLS, which included specimen and data collection, diagnosis, training, and research on AIV (Table 1). The primary objective of the surveillance is to identify AIV strains that are circulating in the LBMs and domestic poultry within Bangladesh. Initially one sub-district of Netrokona district was selected for sampling and data collection from poultry, based on the presence of mixed populations of domestic and wild birds. The surveillance was expanded to other sites, including Dhaka, Gazipur, Rajshahi, Dinajpur, and Chittagong. The surveillance program is still ongoing, with consistent funding support from the CDC. From 2007–2018, the surveillance has reported year-round detection of AIV, including HPAI H5N1, in waterfowl, commercial chickens, backyard chickens, and pool environmental swabs [27].

In 2016, the animal and human health services of the GoB, in collaboration with FAO, developed a method called 'sink surveillance' to detect AIV using pooled environmental samples in the LBMs of Dhaka and Chittagong (Table 1). LBMs are identified as the pathogen sink area, i.e., common locations where HPAI and LPAI viruses accumulate from various sources (poultry farms and backyards) from different parts of the country. The sink surveillance aims to eliminate the need to find the pathogens at source farms or for farmers to report suspected outbreaks. The surveillance was later expanded to other cities in Bangladesh. A joint team of animal health and human health government officials visited

106 LBMs on a monthly basis to collect environmental specimens. From the 708 pooled environmental samples from 33 LBMs of Dhaka, the surveillance identified 87.9% of the LBMs positive for influenza A, 39.4% positive for H5, and 21.2% positive for H9 [30]. This surveillance is presently ongoing [31].

There have been some efforts to track AIV in wild birds as well. The US Geological Survey, in coordination with FAO and icddr,b, conducted a wild bird survey in 2011 [26]. During 2010–2012, icddr,b, in collaboration with EcoHealth Alliance, conducted a survey of wild birds and domestic ducks in freshwater wetlands in northern Bangladesh and coastal areas of the Bay of Bengals to assess the prevalence of AIV, quantify flight distances, and trace the migratory routes of influenza virus-infected waterfowl [32]. Findings of the survey suggest that both migratory wild birds and domestic ducks in Bangladesh can harbor and shed influenza A viruses and the migratory waterfowl routes connect Bangladesh with other regions in south and central Asia. Another study conducted during 2012–2015 assessed the prevalence of AIV and antibodies against the virus among wild and domestic birds. The study found a higher AIV antibody prevalence in domestic birds than in wild birds, suggesting that domestic birds may be an important reservoir of the virus in Bangladesh, potentially exceeding the role of wild birds [33].

3.2. Surveillance for Human Infection with AIVs

LBMs are the primary hub for poultry marketing across Bangladesh [17], and also serve as a place of human–bird interactions. Studies have identified LBMs as the reservoir of both LPAI and HPAI H5N1 and an important source of transmission [34,35]. Since Bangladeshi LBM workers are at risk of AIV infection due to the ongoing circulation of these viruses among poultry in markets and their occupational exposure to poultry, the icddr,b, in collaboration with the Institute of Epidemiology, Disease Control, and Research (IEDCR) and DLS, initiated an active influenza surveillance among LBM workers and their household members in 16 LBMs in Dhaka in 2012 (Table 1) [28,36]. These markets were selected because they served as sentinel sites for existing AIV surveillance in poultry, and hence served as a 'One Health' platform to monitor the circulation of AIV both in poultry and in market workers. The objectives of the LBM workers' surveillance were to identify human cases of AIV infection, to detect circulating AIV, and to assess serological evidence of AIV infections. This surveillance reported an annual incidence of 24 AIV RNA detections per 1000 LBM workers. Approximately 2% (9/404) of workers at LBMs in Dhaka were found to have seroconverted to H5N1 [28]. Three of the eight H5N1 cases and one of the two H9 cases reported to the World Health Organization (WHO) were detected through this surveillance. However, all H5 and H9 cases identified had mild illness [36]. This poultry worker component of this surveillance has been discontinued since 2017 due to lack of funding.

In 2007, icddr,b, in collaboration with the IEDCR and supported by the US CDC, established a hospital-based influenza surveillance (HBIS) in 12 tertiary care hospitals across Bangladesh to identify individuals and clusters of people with life-threatening infections with influenza virus and to characterize the diversity of strains circulating in Bangladesh [29,37]. The surveillance is currently operational in nine sites—seven government and two private hospitals. One human H5N1 case has been detected through this surveillance. The platform of National Influenza Surveillance, Bangladesh (NISB) was initiated by IEDCR in 2010 [29,37]. The primary objective of this surveillance is to identify strains of the influenza virus circulating in Bangladesh. Patients who meet the case definition of influenza-like illness (ILI) and severe acute respiratory illness (SARI) were enrolled. Currently, NISB is being carried out in 10 sentinel sites, all of which are district hospitals, except Dhaka Medical College Hospital (DMCH). No H5 subtype was detected though this surveillance. From both HBIS and NISB, epidemiological data are shared to FluID and virological data are provided to FluNet through the National Influenza Center (NIC) of the Global Influenza Surveillance and Response System (GISRS). Monthly routine surveillance reports are generated and shared with the collaborating hospitals and institutes, US-CDC, and WHO.

4. Biosecurity

Biosecurity measures can play an important role in preventing AIV in poultry and thus reduce the risk of potential zoonotic transmission to humans [38,39]. FAO defines biosecurity as the "implementation of practices that create barriers in order to reduce the risk of the introduction and spread of disease agents"; biosecurity in poultry farming requires "the adoption of a set of attitudes and behaviors by people to reduce risk in all activities involving domestic, captive exotic, and wild birds and their products" [40]. According to FAO, three principle elements of biosecurity are segregation, cleaning, and disinfection [40].

4.1. Backyard Poultry Sector

In Bangladesh, 64% of the population live in rural villages [41], and approximately 71% of rural households raise backyard poultry (Figure 1) [42]. These backyard poultry raisers come into frequent close contact with poultry through their daily rearing practices, including putting poultry into sheds, feeding sick poultry by hand, and slaughtering sick poultry [43]. Given their limited resources and free scavenging method of raising, even the very basic biosecurity recommendations, such as controlling movement and traffic, separating sick poultry, maintaining regular cleaning, safe disposal and disinfection, are rarely feasible for backyard raisers [43–46], as observed in other similar settings [47]. Their close living arrangements with poultry put them at a heightened risk of zoonotic transmission. Several studies have identified a low awareness of AIV among the backyard poultry raisers, and biosecurity measures are seldom observed [42,48,49].

(**a**) (**b**)

Figure 1. Backyard poultry farming: (**a**) backyard poultry shed; (**b**) backyard poultry kept under the bed.

In the backyard sector, efforts have focused on raising awareness about AIV and measures to be followed to prevent zoonotic transmission [25,26]. The GoB, development partners, private sectors and non-governmental organizations (NGO), were involved in building awareness among communities with respect to biosecurity and HPAI (Table 2) [26,44,46,50–54].

Table 2. Initiatives to improve biosecurity in different poultry sectors.

Programs	Description	Results
Nationwide mass media campaigns *Duration:* 2007–2008 *Implemented by:* GoB, WHO, FAO, OIE, UNICEF, BRAC, CARE, USAID, AI.COMM, icddr,b, other NGOs *Targeted for:* All poultry sectors	Safe behaviors, 10-step recommendations (including basic hygiene messages, e.g., using masks, handwashing, and not touching sick poultry) were disseminated through radio, television, newspapers, public meetings, folk songs and plays, rickshaws and vans equipped with megaphones, posters, training manuals [46,50,51]	70% backyard and 90% commercial poultry farmers and 65% live bird handlers were aware of good biosecurity; 80% targeted journalists accepted good reporting practices; however, adoption of recommended practices remained poor in all

Table 2. *Cont.*

Programs	Description	Results
Avian Influenza Preparedness and Response Project *Duration:* 2007–2012 *Implemented by:* DLS, Department of Mass Communications, Ministry of Fisheries and Livestock (MoFL), FAO *Targeted for:* All poultry sectors	Public awareness and risk communication campaigns conducted in 20 sub-districts in 20 districts using film shows, folk songs, school programs, distribution of leaflets, posters and banners; DLS trained poultry farmers, veterinarians, paraprofessionals, community health workers, media persons, news reporters, and students; piloted Biosecure Poultry Market Chains (BPMC) in 9 LBMs, 18 broiler and layer farms, among 324 poultry farmers, 180 LBM workers, 90 middlemen/transporters, and 1260 poultry chain stakeholders in 9 of the districts at highest risk of HPAI, to establish good biosecurity practices along the entire poultry value chain [26]	sectors; 84% of HPAI outbreaks involving commercial farms indicated a disconnect between the KAP and practice as well as persisting weak biosecurity BPMC: some improvements in the structural biosecurity of the LBM and the farms under intervention was reported, however, operational biosecurity was poor for both the markets and the farms, and biosafety practices were almost absent [26,48,49,55–57]
Teacher training program for AI outbreak reporting *Duration:* 2009 *Implemented by:* FAO, DLS *Targeted for:* All poultry sectors	One-day workshops conducted in three selected sub-districts involving school and madrassa teachers on disease reporting and the risks and prevention of HPAI [52]	Not available
Behavior change pilot intervention *Duration:* 2009–2010 *Implemented by:* icddr,b *Targeted for:* Backyard poultry raisers	Context-appropriate behavior change recommendations piloted among the rural raisers in one community in each of the two districts [44]	Awareness increased but behavior remained unchanged; reasons for non-compliance: perceived absence of AIV in raisers' flocks, low-risk of AIV, cost, inconvenience, personal discomfort, fear of being rebuked or ridiculed, and doubt about the necessity of the intervention [44]
Safe poultry slaughter pilot intervention *Duration:* 2014 *Implemented by:* icddr,b *Targeted for:* Rural communities	A safe poultry slaughtering method piloted in two rural communities in a district in order to reduce human exposure to airborne virus by performing poultry slaughtering in a closed container [53,58]	The recommendations were found to be acceptable and feasible for the villagers with minor modification [53]
Upazila-to-Community (U2C) *Duration:* 2017–till date *Implemented by:* DLS, FAO *Targeted for:* Backyard and commercial poultry sectors	Targeted to cover 496 sub-districts; avails veterinary services to rural communities to improve livestock production and disease control, increasing resilience to emerging disease events [54]	The program is still ongoing, no evaluation/result available
Program on farm biosecurity *Duration:* 2005–2006 *Implemented by:* GoB, DLS, BRAC and other NGOs *Targeted for:* Commercial poultry sector	Training on farm biosecurity (i.e., the prevention and control of AIV) provided along with gloves and disinfectants to 33 breeders/hatchery farm managers and 340 large commercial farms; 150,000 small-scale farmers trained across the country [46]	Not available
Stamping Out Pandemic and Avian Influenza (STOP AI) *Duration:* 2008–2010 *Implemented by:* USAID, FAO, city corporation, DLS *Targeted for:* Commercial poultry and LBM sectors	Different sectors were mobilized to improve biosecurity; biosecurity training implemented for veterinarians and livestock science graduates; 7 LBM training programs implemented in 5 divisions; cleaning and disinfection activities piloted in 2 LBMs; biosecurity improvement models (infrastructure improvements, e.g., farm boundary, footbath, biogas and compost plants) implemented in 12 commercial farms in a district and 2 LBMs in 2 districts; cleaning and disinfection activities implemented in 24 LBMs within and outside Dhaka through training, technical support, financial assistance for infrastructure renovations, renovation of the water supply, the addition of a biogas facility for proper waste disposal, and a slaughter house [54,59–61]	Awareness and precautionary practices increased; substantially fewer HPAI outbreaks were reported; no clusters of infection were found in the intervention farms/LBMs; the effect of the intervention on the incidence of disease was limited to a few months after completion—indicating the challenges of sustaining the progress; despite increased biosecurity, no significant reduction in virus circulation was found in the FAO-intervened markets compared to the non-intervened ones [60,62]

Table 2. *Cont.*

Programs	Description	Results
Community-engaged biosecurity (CEB) model *Duration:* 2016–2018 *Implemented by:* Bangladesh Agricultural University (BAU) *Targeted for:* Commercial poultry sector	From each of the two sub-districts, training of trainers (ToT) was provided to 50 lead farmers, who trained their fellow farmers; regular farm visits by community animal health workers were made to monitor compliance [63]	The program is still ongoing, no evaluation/result available
Biosecurity program in the LBMs *Duration:* 2007–2008 *Implemented by:* BRAC, IFC, SEDF *Targeted for:* LBM sector	A series of trainings and practical demonstrations on biosecurity and the use of personal protective equipment (PPE), along with gloves, masks, disinfectants, and small spray machines, were provided in retail and wholesale shops from 38 LBMs of Dhaka [46]	Not available
The LBM C4D initiative *Duration:* 2012–2013 *Implemented by:* UNICEF, GoB *Targeted for:* LBM sector	Intervention implemented in 16 LBMs to improve the knowledge and threat perception of AIV, as well as the bio-security practices of the poultry workers [56]	Despite an improved knowledge level, no significant change observed in biosecurity measures after the intervention; major barriers: lack of proper infrastructure to adopt the recommendations, concern of negative financial impact, lack of self-risk perception [56]
Piloting workstations for poultry workers *Duration:* 2008–2012 *Implemented by:* icddr,b *Targeted for:* LBM sector	Portable workstations (including a worktop and handwashing facility with soapy water) were designed and piloted in 13 shops in a LBM to reduce the risk of environmental contamination and improve handwashing practices [64,65]	The workstations were acceptable, functional, improved handwashing practices and the use of clean water; soapy water was effective in removing influenza viruses from poultry workers' hands; however, handwashing decreased over time; major barriers: the difficulty to manage the increased cost for water and detergent by shops and the inability to frequently wash hands during busy hours [64,65]
Use of wooden shelters *Duration:* Not available *Implemented by:* BRAC *Targeted for:* Backyard poultry sector	Moveable wooden poultry shelters were developed and promoted to help the smallholder farmers to maintain bio-security measures at low costs [46]	Not available

Despite all these efforts, no significant improvement in biosecurity has been observed in this sector over time [66]. Two major underlying reasons for this low uptake of the standard recommendations were the low perception of the risk of AIV transmission to humans and concerns related to financial benefit or loss [43,44,48].

4.2. Commercial Poultry Sector

In Bangladesh, both large- and small-scale poultry producers have had to bear enormous losses associated with HPAI H5N1 outbreaks [46]. However, large-scale farms are better equipped to maintain biosecurity recommendations and withstand the financial loss due to sudden outbreaks compared to small-scale farmers. Small-scale commercial poultry farms (i.e., poultry population ≤ 2000) (Figure 2) account for 81% of the total commercial poultry farms [67]. Of the 549 confirmed outbreaks, 44% were among small commercial farms [5]. During 2007–2008, studies often characterized these farms with a low level of biosecurity in terms of the location of the farms, restricting the entry of wild birds and animals, fencing, use of footbaths, etc. [68,69], which match with the findings of another assessment conducted in 16 small commercial farms in 2011–2012 [57]. Environmental contamination was also

reported through the use of untreated poultry feces as fertilizer in agricultural lands or as fish feed in waterbodies [57,70].

(**a**) (**b**)

Figure 2. Commercial poultry farming: (**a**) a small commercial broiler farm; (**b**) a small commercial layer farm.

During 2007–2008, the GoB took a number of initiatives, including massive awareness-raising campaigns through mass media to promote the adoption of basic bio-security measures, and sub-contracting the private sectors, which worked at the grass-root level, to provide HPAI-related extension services in rural areas (Table 2). In 2010, the GoB recommended a set of biosecurity measures to reduce the introduction and spread of infectious diseases, including HPAI, into and from commercial poultry farms [71]. Other sectors, including development partners and NGOs, also joined the force [46,59,60]. There have been some individual efforts as well, for example, promoting community-based biosecurity measures by Upazila Livestock Officers (ULO), Kapasia, which reported to have markedly improved the HPAI outbreak situation in the sub-district [46].

Some improvements in farmers' awareness and in some of the biosecurity conditions of the small commercial farms over the past decade have been reported, for example, maintaining the all-in-all-out system with the same broiler strain and age structure and some farm hygiene recommendations [72], as compared to findings from studies conducted during 2007–2008 [68,69]. However, the improvements are marginal and the overall biosecurity conditions of these small commercial farmers are still poor [72,73]. According to the World Bank report in 2013, weak poultry farm biosecurity and potential seasonal reinfection by the overflying and resting of HPAI-carrying migratory birds remained obstacles to successful control and eradication [26].

An anthropological exploration of 16 small commercial farms by icddr,b attempted to explore some underlying reasons for farmers' low adherence to the standard measures during 2011–2012 [57]. The study showed that financial constraints and inconvenience were major constraints to practicing the recommended biosecurity measures. The study also showed that farmers' practices and perception of biosecurity, transmission, and prevention of AIV were inconsistent with standard definitions, but were consistent with the recommendations and perceptions of the local vendors of chicks, feed, and medicines, indicating that these vendors heavily influenced farmers' decisions [57]. A similar dependency on the local vendors was reported among the backyard poultry raisers in a previous study [74], indicating that this group is a key player for both sectors. These vendors, without any formal training, also prescribed antibiotics for poultry indiscriminately [57,74], contributing to the global concern for antibiotic resistance both for human and animal health [75].

4.3. Live Bird Market Sector

LBMs represents 95% of the poultry meat and egg retail in Bangladesh [76], as refrigeration in the production, transport, and selling facilities is limited. As mentioned, these markets also act as a network 'hub' for poultry trading and potential reservoir of infection for poultry and poultry

traders [17]. Bangladeshi LBMs were characterized as having a low level of biosecurity, lack of infrastructure required to maintain biosecurity, and low awareness of transmission, prevention, and risk perceptions associated with AIV (Figure 3) [56]. Waste from these LBMs also contributed to environmental contamination [56]. Among the eight reported cases of H5N1 in Bangladesh, three were LBM workers [77,78].

(**a**) (**b**)

Figure 3. Live bird markets: (**a**) a live bird market in Dhaka; (**b**) slaughtering arrangements adjacent to poultry shops in a LBM.

LBMs are probably the most targeted area for intervention by key stakeholders in order to prevent and control the spread of AIV. A number of intervention efforts have been made to improve biosecurity conditions over the past decade, including massive infrastructural renovation, the installation of short-term infrastructural solutions, market cleaning and disinfection, supplying personal protective equipment, promoting behavior change, and awareness campaigns (Table 2) [26,46,55,56,60,61,64,65]. Regardless of all the efforts, the biosecurity conditions in the LBMs remained low, despite the increased awareness [35,66,79], and the infection prevailed both in poultry and in the environment [80,81]. Evidence mentioned in the surveillance section above suggest that ongoing efforts for controlling HPAI did not have sufficient impact. Sayeed and colleagues identified housing chickens and ducks together in the stalls, birds kept on floors, and lack of adequate hygienic measures of the stall to be the crucial factors for spreading AIV in the LBMs of Chittagong [81]. Market closure or rest days and disinfection interventions were reported to be effective in disrupting the virus circulation in other settings [82], but could not be successfully implemented in Bangladeshi LBM [56].

5. Vaccination

Vaccination reduces the shedding of viruses. Unvaccinated infected chickens shed much higher concentrations of viruses than vaccinated infected chickens seven days post-vaccination [83,84]. Reduced quantities of virus shed into the environment, in turn, reduces human exposure and the likelihood of zoonotic transmission of the virus and pandemic influenza [85]. In parts of Asia, vaccination programs have been implemented and encouraged as part of an integrated control program for poultry [86]. The GoB and Breeder Association of Bangladesh introduced an avian influenza vaccine for the first time in commercial poultry farms of two districts in 2012. This vaccine was targeted for layers (raised for egg production), broilers (raised for meat production), and breeders, and applied to day-old chicks at hatcheries. The cost for a single dose of the vaccine was approximately BDT 5 (US$0.06) [87]. Since 2014, the Drug Administration Authority of the GoB has allowed restricted use of avian influenza vaccines for commercial poultry [88]. Since then, a vaccine against HPAI H5N1 has been available for use in commercial layers and breeder farms. However, it has been found that the virus can replicate and cause illnesses even in vaccinated birds. Ansari et al. showed that anti-H5 sero-positivity levels were similarly low in vaccinated and unvaccinated chickens, highlighting the need for a reevaluation of the currently available vaccine and the overall vaccination program [89].

6. Other Research

A number of epidemiological studies have been conducted to identify the risk factors associated with HPAI H5N1 in poultry in Bangladesh. Case-control studies conducted in Bangladesh have demonstrated several important risk factors—for backyard chickens: offering slaughter remnants of purchased chickens to backyard chickens, having a nearby water body, and having contact with pigeons [90]; for small commercial farms: the presence of dead crow at or near farms, exchanging egg-trays with market vendors, mortality seen in backyard chickens reared nearby [91], farms accessible to feral and wild animals and a footbath at the entry to the farm/shed [92]; and for layer farms: number of staff, frequency of veterinary visits, presence of village chickens roaming on the farm, and staff trading birds [93].

Studies that analyzed the temporal and spatial patterns of HPAI H5N1 outbreaks identified three significant circular clusters of hotspots located near large cities; the outbreaks were spatially clustered along the country's main highways and principal poultry trading routes, with the central part of the country dominated by commercial production systems and the northwestern part primarily by backyard production systems [2,94,95]. Three significant risk factors associated with HPAI H5N1 virus outbreaks that were identified were the quadratic log-transformation of human population density, the log-transformation of the total commercial poultry population, and the number of roads per sub-district [2]. An ecological study identified migratory birds' staging areas, river network, household density, literacy rate, poultry density, LBMs, and the highway network as ecological determinants significantly associated with the risk of HPAI-H5N1 outbreaks at sub-district level [96].

Efforts have been made to explore poultry workers' and traders' networks. A cross-sectional survey was conducted across 17 different districts of Bangladesh to assess poultry trading practices and networks, which could promote the spread of AIV, and their potential implications for disease control and surveillance [97]. The study showed that broiler chickens were generally sold in markets close to their production areas, whereas ducks and backyard chickens were moved over longer distances, and involved several intermediaries. The poultry trading network was highly connected, however, the removal of only nodes denoting 25 LBMs reduced the network's connectedness, and the maximum size of output and input domains by more than 50%. Such knowledge of the network structure could be used to target control and surveillance interventions to a smaller number of areas, which could also be suitable for the optimum use of limited resources.

7. Avian Influenza Policy

During 2005–2006, the world was on high alert for AIV, and the United Nations (UN) agency encouraged every nation to develop its own avian influenza policies. With technical support from WHO and FAO, the GoB developed and adopted a National Avian Influenza and Human Pandemic Influenza Preparedness and Response Plan, covering the period from 2006–2008 [18] and then the period from 2009–2011 [98]. Both international guidelines and practices and local norms, experience, and evidence were considered while developing these avian influenza policies. A multi-sectoral approach was adopted to develop avian influenza policies in Bangladesh. The sectors that led the initiative from GoB included the Ministry of Health and Family Welfare, Ministry of Fisheries and Livestock, and the Ministry of Environment and Forests. Other stakeholders included international multilateral organizations, national and international NGOs, and trade associations, including breeders, feed millers, egg producers, and poultry farmers. Since then, the GoB has passed several ordinances during different outbreak situations, to be followed by different sectors.

UNICEF assisted GoB in the development of a risk communication strategy and USAID committed funds to finance different aspects of HPAI control. The Department of Mass Communication (DMC) under the Ministry of Information (MoI), in collaboration with DLS, implemented parts of the public awareness and information component. In 481 sub-districts, the DLS and MoHFW established joint rapid reaction teams to conduct the culling of exposed poultry, surveillance, and the administration of prophylaxis to exposed persons, and information sharing to minimize the threat to human health

posed by the disease. The diagnostic capacity of the veterinary diagnostic laboratory system has also been strengthened [26].

Avian influenza has received more funding and attention than other zoonotic diseases, such as rabies and anthrax, which cause much higher mortality. Nevertheless, a trend of decreased attention towards AIV prevention and control efforts has been observed over recent years. After the most recent edition (2009–2011) of the AIV response plan was developed, attempts were made to update it but it is yet to be finalized. The major reasons behind AIV prevention and control interventions currently not being prioritized for policy implementation in Bangladesh could be the reduced number of human cases, low fatality among humans, and a perceived decreased trend in the number of outbreaks, despite under-reporting.

As part of a broader research on the Behavioural Adaptations in Live Poultry Trading and Farming Systems and Zoonoses Control in Bangladesh (BALZAC), funded as part of the Zoonoses and Emerging Livestock Systems (ZELS) project, a Chatham House roundtable was convened in Dhaka, Bangladesh in May 2016 [99]. Participants were convened from the government, international multilateral organizations, non-governmental organizations (NGOs), and trade associations in Bangladesh to discuss future policy options to prevent and control AIV and other poultry-related zoonotic diseases in Bangladesh. In the meeting, the policy options recommended were: (1) developing a broad overarching policy based on the One Health concept to cover a range of zoonotic diseases, with a subsidiary plan for each zoonotic disease; (2) using a bottom-up approach to develop policies considering local norms, experience, and scientific evidence; (3) developing sustainable policy through fostering a sense of ownership among those involved and exploring insurance options; and (4) reviewing and updating policy as necessary, including stocktaking and considering the effectiveness, cost-effectiveness, and acceptability of the policy. In order to consider and conceptualize a future policy environment suitable for developing and implementing such policies, the roundtable concluded that Bangladesh should take into account: (1) a multi-sectoral approach by establishing a One Health Secretariat in order to sustain the collaborative work between different sectors/organizations; (2) clearly defined leadership, roles, and responsibilities for each organization; and (3) the need for a common pool of resources and provision for transferring resources. Steps taken by partners to make progress since 2016 included the formation of the One Health Secretariat, Inter-ministerial Steering Committee on One Health, Technical Advisory Committee, and One Health Coordination Committee; the One Health Strategic Framework 2017–2021 being finalized; a revised National Avian and Pandemic Influenza Preparedness and Response Plan 2018–2022 under development; and a zoonotic disease prioritization workshop held to inform the development of an overall zoonoses policy.

8. Discussion

Over the last decade, Bangladesh has made a tremendous effort to combat avian influenza. However, it is evident that the viruses persistently prevailed in all sectors, caused sporadic infection, and continued to remain a public health problem. The apparently declining trend, based on officially confirmed reports since 2013 [1], does not carry any convincing evidence that the prevalence of the virus is decreasing over time, because an increasing yearly trend of its circulation in LBMs has been confirmed through different surveys and published reports. The persistent changing of clades of circulating H5N1 strains suggests probable mutations that might have occurred over time. Weak biosecurity in all the poultry sectors, linked with limited resources, low risk perception, short-term sporadic efforts, and decreasing attention toward AIV prevention and control among the stakeholders have all contributed negatively to the avian influenza situation in Bangladesh.

Avian influenza surveillances have provided evidence that HPAI H5N1 has become enzootic in Bangladesh. Despite the lack of actual disease reporting at the farm level, with the dominance of H9N2, different subtypes of AIV are being commonly identified at the LBMs [100,101]. The risk of transmission and reassortment of the viruses cannot be ruled out, considering the evidence of viable AIV found in the respiratory passages of the LBM workers [28,102] and the prolonged exposure [56].

To identify future reassortment in Bangladesh, monitoring for both HPAI and LPAI viruses of diverse subtypes will be crucial [100]. Although active surveillance can be expensive and time-consuming and may face difficulties surviving, the intensification of surveillance has been key to early detection and controlling and limiting the spread of HPAI viruses among poultry on national scale [54,103,104]. Active surveillance is also needed to track the likely chain of transmission and the genetic diversity of circulating strains. This will, in turn, contribute towards the standardization of sampling, testing, and reporting methods, bolstering full-genome sequencing efforts and encouraging the sharing of isolates with the scientific community [105,106]. Authorities might also consider exploring the potential value of enhancing surveillance for mild illness from HPAI H5N1 virus infection among humans during the typical AIV season in poultry. Capacity building in conducting whole genome sequencing is also important to predict whether the circulating virus strains have any potential to bind to human receptors.

Despite successive interventions to improve biosecurity conditions in commercial farms and LBMs by the government and the private sectors, risky behaviors remained widespread. It seems to be accepted among the stakeholders that 'nothing can be done' to improve the biosecurity conditions in the backyard poultry sector. On the other hand, there have been continuous efforts, although sporadic and disconcerted, to improve the conditions of the commercial poultry sector and LBMs, logically driven by concerns for larger scale financial investments.

The current biosecurity recommendations for commercial farms by the government includes different biosecurity measures for different types of commercial poultry sectors (e.g., grandparents, parents, layers, and broilers), however, the recommendations mostly include general measures for all farm sizes, which may not be practical for small farms [57,71]. To account for the socioeconomic realities of small-scale commercial farmers, biosecurity recommendations could be tailored [40]. Farmers' dependency on the local vendors needs to be taken into account while developing any intervention for these small farmers [57,74]. Despite being an important contributor to this problem, the duck population has typically been ignored in terms of biosecurity interventions. Further research to develop and evaluate interventions that simultaneously improve duck raisers' profitability and biosecurity should be considered.

LBMs remain a complex setting in terms of biosecurity improvements and to date no single intervention has been proven to be successful in the long term. This situation has instigated two different opinions. One opinion supports a gradual shifting of the poultry markets from selling live birds to marketing processed poultry meat, whereas the other supports retaining and improving the LBMs, considering the cultural preferences of the local people related to checking halal meat. The issue still remains unsettled and requires further behavioral studies and testing of small-scale interventions to identify approaches that can be acceptable, feasible, and support favorable conditions to maintain good biosecurity. At a minimum, interventions should prioritize creating a safer slaughtering environment in terms of disease transmission, and an improvement in sanitation and waste disposal facilities. Formative research could be helpful to explore if and how environmental controls (e.g., handwashing stands, improved ventilation flow), improved poultry handling and slaughtering techniques, and improved personal protective equipment (e.g., more accessible, cost-effective, and better tolerated) could help decrease the risk for AIV transmission at these markets.

Vaccination is another controversial issue. Some stakeholders favored vaccination in order to reduce the amount of circulating virus, which is important for an enzootic country like Bangladesh. However, others argued that vaccinated birds can still become infected and shed viruses with few or no clinical signs of infection [107], and the character of the virus might also change due to mutation. The complex infrastructures of the poultry industries and LBMs of some Asian countries made vaccination campaigns infeasible and HPAI enzootic in vaccinated poultry populations [108]. To prevent future AIV outbreaks in enzootic countries, vaccination campaigns need to be implemented along with biosecurity interventions. If the vaccination program is not properly managed with upgraded biosecurity, the prevention or control of AIV will not be possible [109]. For the high-risk LBM workers, a seasonal influenza vaccination can be considered to minimize the chances of a co-infection

of seasonal and avian viruses and reduce the chances of re-assortment events, as seasonal viruses were also reported among the LBM workers [36]. Nevertheless, there is a strong need for impact evaluation and routine monitoring of vaccination. A Differentiating Infected from Vaccinated Animals (DIVA) program must be put into action to monitor the vaccine efficacy and natural infection.

9. Future Directions

Controlling AIV necessitates a concerted multi-sector One Health approach that includes human health, animal health, and environmental health to manage the health, social, and economic factors of the disease, since it affects poultry, humans, and the environment. In Bangladesh, there has been an increased acceptance of approaches or interventions that are effective against a combination of other zoonoses, such as Salmonella and Campylobacter, or other diseases that farmers are more concerned with, such as Newcastle and infectious bursal diseases [57], instead of AIV alone, among the donors and stakeholders. Responses to avian influenza has led to a longer-term trans-disciplinary One Health movement in many Asian and African countries [110,111], moving towards approaches that simultaneously address a variety of endemic zoonotic infections [110]. Multi-country networks, such as Mekong Basin Disease Surveillance (MBDS), have proven to be effective in regional cooperation and reporting, communicating, and containing disease outbreaks in isolated and economically marginalized border communities [110]. Failure to integrate and sustain One Health in national health policies in India has led to duplicative and weak response systems, failing to trigger investments and inadequate intersectoral action—a lesson for the developing countries with a significant burden of zoonoses, poverty, and a reliance on livestock [112]. Although Bangladesh has made significant progress in institutionalizing One Health, there are still some operationalization challenges which need to be mitigated in order to make it fully functional and sustainable. Multiple efforts are being undertaken by different stakeholders within the same sector in silos. Regular data sharing should be encouraged and maintained across government agencies and institutions, universities, research and multilateral organizations, and NGOs in order to secure the health benefits of all species.

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Short communication

Sales and immunogenicity of commercial vaccines to H9N2 low pathogenic avian influenza virus in Korea from 2007 to 2017

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ABSTRACT

The present study was conducted to monitor sales activity and immunogenicity of commercial H9N2 vaccines produced in Korea from 2007 to 2017. Recorded sales of H9N2 vaccine were around 671 million doses, with 10 million doses sold in 2007, rising to a peak of 93 million doses in 2016, with a slight fall in 2017. Multivalent combined vaccines made up around 90% of all vaccine sales, and around 30% of all vaccines were distributed by regional governments for free. The regional vaccination rate was the highest in Gyeonggi and Chungnam, respectively with proportional to the population of layer and breeder chickens. There have been no cases of field infection since 2009. The mean antibody titer was 5.82 $\log_2$ across the study period. Our results suggest that continuous genetic monitoring of H9N2 viruses circulating in the field and updating the vaccine seed strain periodically are necessary in order to control H9N2 outbreaks.

1. Introduction

The H9N2 low pathogenic avian influenza (LPAI) viruses have been circulating across countries of North Africa, the Middle East, and Asia in multiple avian species, resulting in substantial economic losses as a result of reduced egg production and increased mortality associated with coinfection with other pathogens [1,2]. In several of these countries, vaccines have been deployed for disease control. However, H9N2 infections have become endemic in the poultry industries in a number of countries [3].

Although immunization with inactivated LPAI vaccine cannot completely protect against viral infection and shedding into the environment, it is one of the most promising control measures to date for H9N2 LPAI. Therefore, vaccination against H9N2 LPAI has been used in many of the countries and regions where the virus is endemic, such as China, the Middle East, and Korea [4,5]. In Korea, the first field outbreaks of H9N2 LPAI occurred in 1996. Since 2000, it has been endemic, especially in layer farms. To control H9N2 LPAI outbreaks, the Korean veterinary authorities used measures of stamping-out and compensation between 1996 and 1999, but this policy did not achieve complete eradication of the disease. Since 2007, the Korean government has permitted the use of a single, inactivated H9N2 vaccine strain (A/chicken/Korea/01310/2001(H9N2)). This strain had a high yield through multiple passages in embryonated eggs [6].

On the basis of the results of animal challenge experiments, a requirement for the minimum efficacy of inactivated H9N2 LPAI vaccines was determined in Korea to be a >80% inhibition of the virus recovery rate in cecal tonsils of vaccinated chickens, relative to the virus recovery rate in unvaccinated chickens, at 5 days post-infection [4]. However, avian influenza virus evolves continuously, and a novel strain could emerge at any time and necessitate the selection of a new vaccine candidate. In addition, the current vaccine does not block all viral infection and shedding. The Korean government therefore conditionally issued a vaccine-production license with the requirements that producers record and submit sales activity, and that they deploy and test sentinel birds on vaccinated farms. In the current study, our aim was to monitor sales activity and immunogenicity of H9N2 vaccines sold by five domestic producers for use in vaccination of chickens in farms in Korea from 2007 to 2017.

2. Materials and methods

2.1. Sales of H9N2 vaccines

According to the stipulations of the vaccine-production license, H9N2-vaccine producers have to record all sales activity and submit their records to the Korean Animal and Plant Quarantine

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Agency (APQA) on request. From 2007 to 2017, H9N2 vaccines made by five vaccine producers were analyzed annually for production and sales volumes, vaccine types (monovalent or multivalent combined vaccine), sales channels (farmers or regional governments), poultry types (layer or breeder chickens), and vaccination regions (provinces). All production and sales volumes are reported by dose.

2.2. Immunogenicity of H9N2 vaccines in vaccinated birds

According to the stipulations of the vaccine production license, 20–30 unvaccinated sentinel birds must be deployed on every vaccinated poultry farm and usually co-housed with the vaccinated flock for 3 or 4 weeks. Serum samples from 10% of the vaccinated farms must then be tested along with the sera from the sentinel

Table 1
Annual hemagglutination inhibition titers according to H9N2 vaccine producers.

Year	Number of samples	Hemagglutination inhibition titers (log_2, mean ± SD)[a]					
		Vaccine producers					Total
		A	B	C	D	E	
2007	48	5.52 ± 1.38	3.9 ± 0.62	6.84 ± 0.89	6.76 ± 2.22	3.95 ± 1.17	5.66 ± 1.78
2008	131	6.09 ± 0.84	5.12 ± 0.52	6.61 ± 0.98	5.42 ± 0.74	5.36 ± 0.93	5.88 ± 0.95
2009	160	6.07 ± 0.91	5.59 ± 1.11	6.37 ± 1.56	6.48 ± 1.46	6.83 ± 1.46	6.12 ± 1.11
2010	81	6.47 ± 0.53	4.40 ± 1.61	4.74 ± 2.15	5.95 ± 0.62	8.31 ± 1.43	5.69 ± 1.81
2011	133	6.50 ± 0.42	4.66 ± 1.06	5.83 ± 0.85	5.06 ± 0.78	7.59 ± 0.74	6.11 ± 1.10
2012	133	6.39 ± 0.34	4.47 ± 1.37	5.77 ± 0.79	5.10 ± 0.72	7.66 ± 0.79	5.93 ± 1.20
2013	117	6.31 ± 0.49	4.89 ± 1.55	5.91 ± 0.59	5.33 ± 0.43	6.28 ± 0.75	5.98 ± 0.95
2014	134	6.16 ± 0.29	5.29 ± 1.77	6.05 ± 0.49	3.8 ± 0	5.12 ± 0.60	5.75 ± 1.18
2015	128	5.71 ± 0.25	6.69 ± 0.65	5.97 ± 0.44	5.31 ± 0.88	5.10 ± 0.62	5.71 ± 0.63
2016	129	5.60 ± 0.25	5.73 ± 0.34	6.29 ± 0.19	6.06 ± 0.83	4.59 ± 0.43	5.68 ± 0.57
2017	166	5.55 ± 0.33	5.40 ± 1.80	6.35 ± 0.16	5.86 ± 0.55	4.77 ± 0.99	5.55 ± 0.86
Total	1,360	6.03 ± 0.66	5.10 ± 1.52	6.05 ± 1.06	5.74 ± 1.14	5.88 ± 1.52	5.82 ± 0.19

[a] Mean titers and standard deviation for the tested farms.

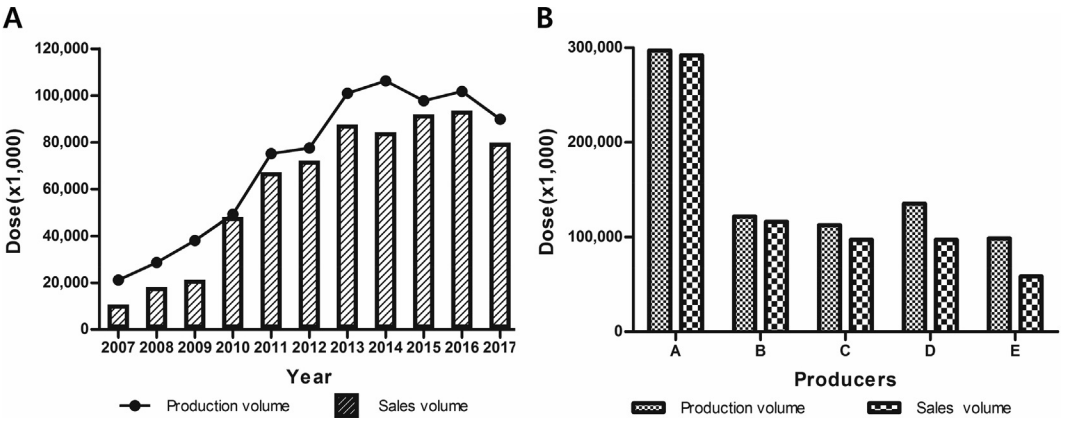

Fig. 1. Production and sales of H9N2 vaccines in Korea from 2007 to 2017. Production and sales of vaccines by five producers of H9N2 vaccines in Korea in each year from 2007 to 2017 (A) and total production and sales of vaccines by each of five H9N2 vaccine producers in Korea from 2007 to 2017 (B).

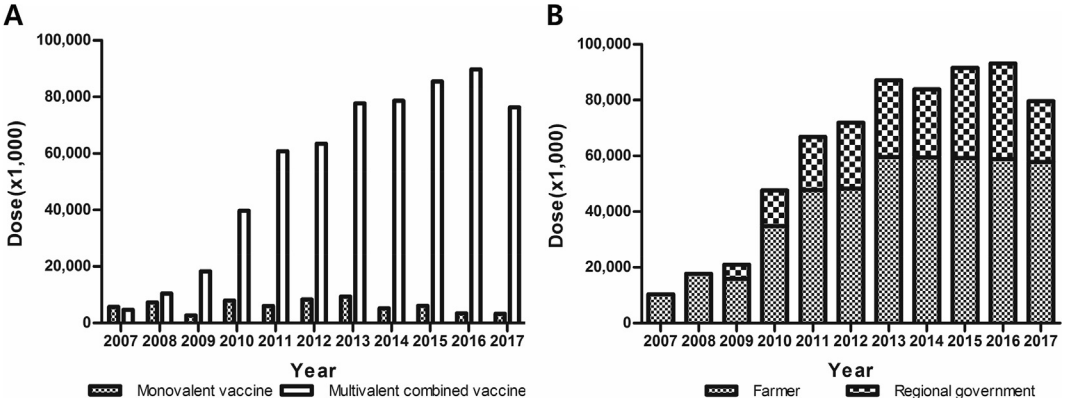

Fig. 2. Sales of monovalent H9N2 low pathogenic avian influenza vaccines and multivalent combined vaccines including inactivated H9N2, by five H9N2 vaccine producers in Korea during each year from 2007 to 2017 (A) and distribution of H9N2 vaccines by farmers and by regional governments in Korea from 2007 to 2017 (B). Numbers of doses of H9N2 low pathogenic avian influenza vaccines that were sold directly to farmers or sold to regional governments for free distribution, in Korea during each year from 2007 to 2017.

birds, to enable monitoring of LPAI infection, with results being submitted to the APQA. All producer's facilities are certified (i.e., they comply with Korea Veterinary Good Manufacturing Practice) by the Ministry of Agriculture, Food and Rural Affairs. From 2007 to 2017, hemagglutination inhibition (HI) antibody titers in 1,360 serum samples were measured in accordance with the OIE's terrestrial manual [7] (Table 1).

3. Results and discussion

We analyzed the sales activity for H9N2 vaccines and immunogenicity in 10% of vaccinated farms in Korea in each year from 2007 to 2017. The registered sales of H9N2 vaccines totaled 671 million doses, increasing from 10 million in 2007 to 80 million in 2017, with a peak of 93 million doses sold in 2016 (Fig. 1A). The fall in

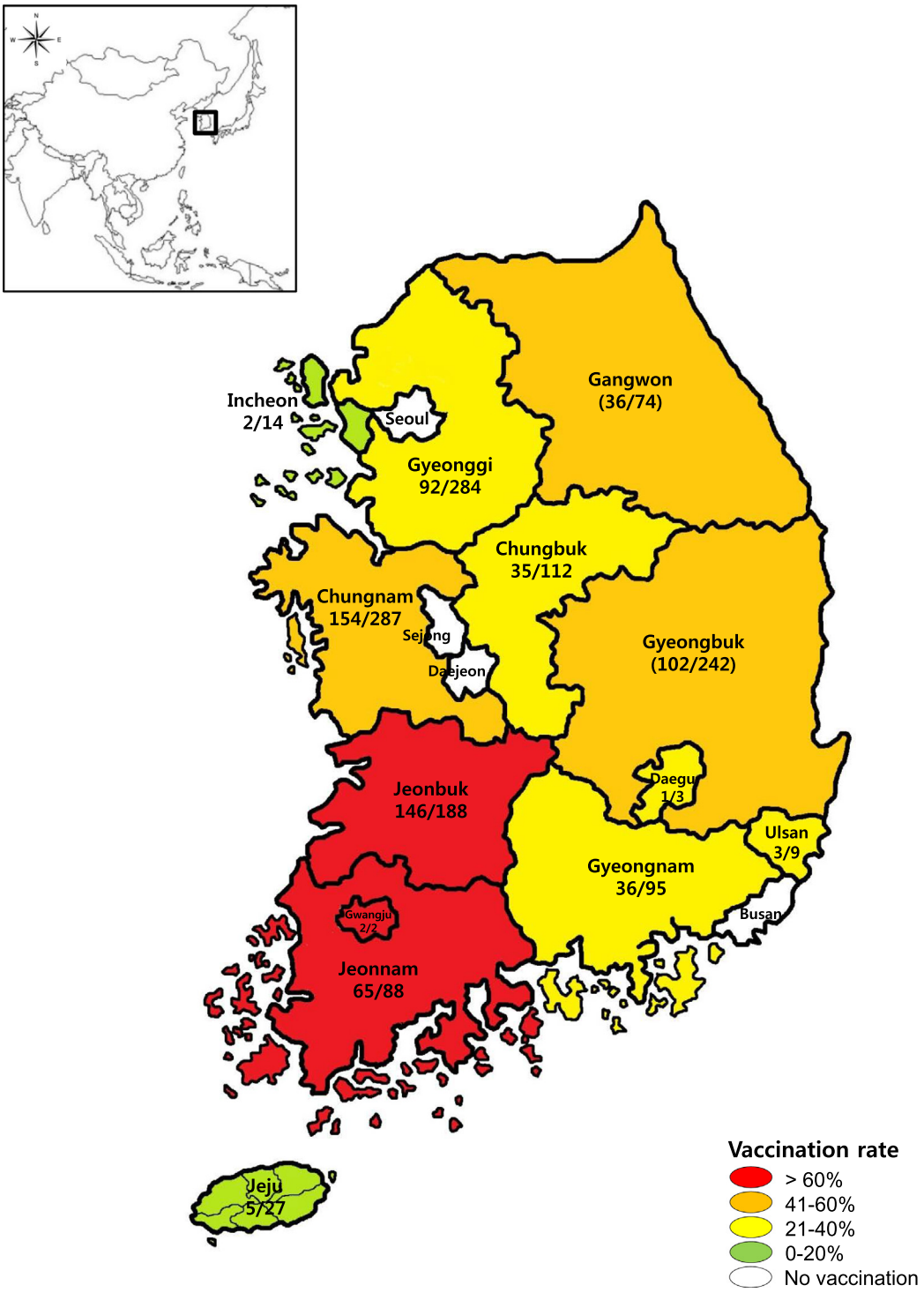

Fig. 3. Regional vaccination rate in Korea in 2017. The H9N2 low pathogenic avian influenza vaccination rate was calculated as follows: the number of vaccinated farms/the total number of layer and breeder chicken farms.

sales in 2017 from the 2016 peak may have been caused by a decrease in H9N2 LPAI outbreaks following enhancement of control measures, and strict biosecurity such as prohibition of livestock transactions in live bird markets (LBM) during the worst highly pathogenic avian influenza outbreaks in Korea in 2016/2017.

During this survey, one producer (A) was responsible for 44.1% of vaccine sales (295.6 million doses), with producer B recording 116.4 million sales, producer C recording 99.8 million, producer D recording 99.2 million, and producer E recording 59.9 million (Fig. 1B). Multivalent combined vaccines (including bivalent, trivalent, and tetravalent vaccines combining inactivated LPAI with Newcastle disease virus, infectious bronchitis virus, and/or egg drop syndrome virus) accounted for 90% of vaccine sales (605 million doses), with monovalent vaccine making up only 10% of sales in Korea in the study period (Fig. 2A). This result suggests that multivalent combined vaccines are preferred in poultry farms in Korea, presumably because they provide reductions in application time and overall costs compared with monovalent vaccine. Our data also show that H9N2 vaccines were purchased either directly by farmers or indirectly by regional governments. Around 30% (201 million doses) of the H9N2 vaccine sales were distributed by regional governments for free in the study period, beginning in 2009 (Fig. 2B).

The number of vaccinated farms in each province was proportional to the population of layer and breeder chickens. In layer chickens, the highest vaccinated farm was Gyeonggi (1,610 farms), followed by Gyeongbuk (1,178 farms) and Chungnam (868 farms). In breeder chickens, the highest vaccinated farm was Chungnam (487 farms), followed by Jeonbuk (364 farms) and Gyeonggi (193 farms). On the basis of 2017 statistics from the Korea Statistical Information Service, 48.4% of the layer chicken farms and 48.5% of the breeder chicken farms in Korea received H9N2 LPAI vaccination [8]. The combined rates of vaccination of both layer and breeder chicken farms in Gwangju, Jeonbuk, and Jeonnam provinces were particularly high (>60%) in 2017 (Fig. 3).

The mean antibody titer (log_2) for 10% of each vaccinated farms, averaged across the survey period, was 5.82, which was close to the criterion for H9N2 LPAI vaccine efficacy (>6.0 (log_2) in specific pathogen free chickens) (Table 1). This result corresponds with that of a previous report that commercial chickens vaccinated in the field had less immunization than in laboratory birds due to maternal antibody, immunosuppressive viruses, used of a reduced vaccine dose, and the time of bleeding post-vaccination [9].

In 2007 and 2008, H9N2 infections occurred in 18% and 9%, respectively, of vaccinated farms, whereas no further H9N2 infections have been reported since 2009. Serum titers in sentinel birds were often reported as 1 or 2 (log_2), which is considered negative in accordance with the OIE's terrestrial manual for positive diagnosis of HI test (>4.0 (log_2) against 4 HAU of antigen) [7]. This result indicates that there was no natural infection of sentinel birds during the 3 or 4 weeks of co-housing. In fact, there have been no reports of H9N2 outbreaks in broiler or breeder farms in Korea more recently. Based on statistics provided by the Korea Animal Health Integrated System for the last three years, only a few cases of H9N2 viruses were isolated from unvaccinated LBM (three cases in 2017 and one case in 2018) in Korea [10]. Taken together, these results suggest that H9N2 vaccination on poultry farms is effective. However, despite the long-term vaccination programs, H9N2 viruses persist, mainly in Korean native chickens of unvaccinated flocks, and in the LBM. In China, inactivated H9N2 has been used in >20 different commercial vaccines, with frequent updating of the vaccine seed, since 1998 [11]. However, H9N2 LPAI viruses persist in chicken populations, even in vaccinated flocks, in China [11].

As vaccination is one of the primary measures used for the prevention of infectious diseases, to maintain optimal protection by vaccination, the efficacy of human influenza vaccines is evaluated annually and the vaccine preparation is updated to include the prevalent strains [12,13]. In China, H9N2 isolates identified between 2009 and 2013 had undergone significant antigenic drift from the vaccine strains isolated in the 1990s [14]. Likewise, Korean H9N2 avian influenza viruses isolated in 2009 are genetically and antigenically different from the vaccine strain; in addition, immunized chickens did not demonstrate inhibition of virus replication and transmission [15]. Moreover, Korean H9N2 viruses in unvaccinated LBM have evolved through antigenic drift and genetic reassortment with other LPAI viruses [2,16,17,18]; this may have resulted in antigenic differences between the vaccine strain and the recent field isolate. In fact, the current vaccine used in Korea is prepared from an isolate that was circulating in 2001. The HA gene of this isolate showed 91–92% similarity with recently isolated viruses in 2015 (unpublished results).

4. Conclusion

Implementation of the H9N2 vaccination program on poultry farms in Korea has been effective at reducing outbreaks of disease, suggesting that there would be considerable benefits to enforcement of vaccination on Korean native chickens in both poultry farm and LBM. Continuous genetic monitoring of H9N2 viruses circulating in the field, as well as monitoring post-vaccination, may be necessary. In addition, periodic updating of the vaccine seed strain should be considered to maintain control of H9N2 LPAI outbreaks.

CRediT authorship contribution statement

Hyun-Kyu Cho: Writing - original draft, Writing - review & editing, Investigation, Formal analysis, Validation. **Yong-Myung Kang:** Investigation, Formal analysis, Validation. **Hyun-Mi Kim:** Resources. **Chi-Ho Lee:** Resources. **Do-Young Kim:** Resources. **Sang-Hyun Choi:** Resources. **Myoung-Heon Lee:** Conceptualization, Funding acquisition. **Hyun-Mi Kang:** Supervision, Conceptualization, Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

All authors have approved the final article.

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Evaluation of vaccination strategies to control an avian influenza outbreak in French poultry production networks using EVACS tool

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ABSTRACT

France recently faced two epizootic waves of highly pathogenic avian influenza (HPAI) in poultry (H5N6 in 2015–2016 and H5N8 in 2016–2017), mainly in the fattening duck production sector. Vaccination against avian influenza (AI) is currently not authorised in France even though its potential benefits were discussed during these epizootic events. The objective of this work was to evaluate the potential efficiency of different vaccination strategies that could be applied against AI in France.

The EVACS tool, which is a decision support tool developed to evaluate vaccination strategies, was applied in several French poultry production sectors: broiler, layer, turkey, duck and guinea fowl. EVACS was used to simulate the performance of vaccination strategies in terms of vaccination coverage, immunity levels and spatial distribution of the immunity level. A cost-benefit analysis was then applied based on EVACS results to identify the most efficient strategy. For each sector, vaccination protocols were tested according to the production type (breeders/production, indoor/outdoor), the integration level (integrated/independent) and the type of vaccine (hatchery vaccination using a recombinant vaccine/farm vaccination using an inactivated vaccine). The most efficient protocols for each sector were then combined to test different overall vaccination strategies at the national level. Even if it was not possible to compare vaccination protocols with the two vaccines types in "foie gras" duck, meat duck and guinea fowl production sectors as no hatchery vaccine currently exist for these species, these production sectors were also described and included in this simulation.

Both types of vaccination (at hatchery and farm level) enabled protective immunity levels for the control of AI, but higher poultry population immunity level was reached (including independent farms) using hatchery vaccination. We also showed that hatchery vaccination was more efficient (higher benefit-cost ratio) than farm vaccination. Sufficient and homogeneously spatially distributed protective levels were reached in the overall poultry population with vaccination strategies targeting breeders, chicken layers and broilers and turkeys, without the need to include ducks and guinea fowls. However, vaccination strategies involving the highest number of species and production types were the most efficient in terms of cost-benefit.

This study provides critical information on the efficiency of different vaccination strategies to support future decision making in case vaccination was applied to prevent and control HPAI in France.

1. Introduction

France was hit with two epizootic waves of highly pathogenic avian influenza (HPAI) during the winters 2015–16 and 2016–17 (Briand et al., 2017; Napp et al., 2018). In both outbreaks, the viruses mainly circulated within the duck production network, the majority producing "foie gras" - a delicacy made from duck liver (Bronner et al., 2017; Le Bouquin et al., 2016). The duck production processes were identified as the main reason for the spread of HPAI viruses in the south-eastern region of France (Guinat et al., 2019). To control the spread of the disease, surveillance was increased and birds in infected farms were systematically culled. During the second outbreak, given the rapid and extensive spread of the disease, preventive culling was also performed in areas around confirmed outbreaks. In 2016–17, about 6.8 million birds were

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culled (Guinat et al., 2018). Culling caused huge economic losses not only for farmers but also for the whole French poultry industry. Total French and European compensation reached 137 million euro in 2015–16 and 123 million euros in 2016–17 (partial estimation) (Lalaurette and Hercule, 2019). The psychological impact on the farmers due to the suspension of their activity, the massive culling of their birds and the intense media focus on the epizootic was also very high (CIFOG, 2017). Even if vaccination was applied in the duck production sector in 2006 during the H5N1 epizootic (Capua et al., 2009), no vaccination was conducted during the both 2015–2016 and 2016–2017 waves. Vaccination against AI is currently not authorised in France, mainly due to the trade restrictions on exports. During the second outbreak (2016–17), because of the very large number of birds culled, some farmers and the media raised the issue of the use of vaccination if there was to be a similar event in the future.

Two main types of avian influenza (AI) vaccines exist: inactivated whole AI virus vaccine and live vector vaccines (Peyre et al., 2009). Sub-unit and virus-likes particle vaccines have been commercialised more recently (Beato et al., 2013) but less widely used. Inactivated vaccines can be homologous (based on strains with the same haemagglutinin (HA) and neuraminidase (NA) as the circulating field virus) or heterologous (based on strains with the same HA but different NA from the circulating field virus). In the case of HPAI strains, reverse genetics is often applied to the HA gene to make the virus strain low pathogenic for vaccine production. Vector vaccines are based on the insertion of an AI gene of interest (HA) into a carrier vector (non-pathogenic virus). Different types of recombinant vector vaccines exist for poultry: fowlpox recombinant vaccine (Swayne et al., 2000), Newcastle disease recombinant vaccine (Veits et al., 2006) and Herpes virus of turkey's (HVT) recombinant vaccine (Kapczynski et al., 2015). Inactivated vaccines require several applications (boosters) to maintain protection in the long run while recombinant vaccines provide long term protection with a single application, mostly at the hatchery (Peyre et al., 2009). As of today, the HVT vaccine is the main vector vaccine used for HPAI vaccination. It is currently applied in routine in Mexico, Bangladesh, Egypt and Viet Nam. To date no study has compared the efficiency of vaccination strategies using these two different types of vaccines in the French poultry production sector.

EVACS (Evaluation tool of VACcination Strategies) is one of the few existing decision support tools that has been developed to compare vaccination strategies (Peyre et al., 2016). The objective of this study was to apply the EVACs tool to identify the most effective and economically efficient vaccination strategy, using different types of vaccines (inactivated farm vaccines and/or recombinant hatchery vaccines) and risk-based approach to protect each French poultry production sector and the whole poultry production from a new HPAI epizootic wave. These results will support future decision making on the use of vaccination to prevent and control HPAI in France.

2. Materials and methods

2.1. Description of the EVACS tool

The EVACS tool was used to evaluate the performances of different AI vaccination strategies in France in different poultry production networks. This tool has been previously described as part of its application in Egypt (Peyre et al., 2016). The tool allows to evaluate the effectiveness and efficiency of different vaccination strategies within poultry production networks by estimating for each production type: i) the vaccination coverage (percentage of vaccinated birds versus total bird population), ii) the immunity level (percentage of birds with seroconversion, i.e. hemagglutinin inhibition level >4Log2); iii) the duration of immunity (proportion of weeks where more than 70 % of birds had a protective seroconversion level)); iv) the spatial distribution of the immunity level (the density of sero-positive birds) and v) the cost-benefit analysis of each strategy (efficiency) (Fig. 1). Only vaccination strategies and no other type of control strategies (i.e. culling, biosecurity, movement restriction, etc.) are compared. The implementation of the tool requires five steps: 1) modelling of the poultry production networks, 2) definition of vaccination strategies to be tested based on the poultry production networks; 3) simulation of vaccination strategies within the networks to generate the outputs in terms of vaccination coverage, immunity levels and duration of immunity; 4) spatial analysis of the immunity level distribution and 5) comparative cost-benefit analysis of the different strategies. Steps 1–4 are performed using specific scripts built in the EVACS "RStudio" project previously developed using the "RStudio" software version 1.1 ("R" version 3.5.1); step 5 is performed using the EVACs "cost-benefit analysis" Excel spread sheet (Microsoft Excel 2007). A description on how the tool applied to the evaluation of AI vaccination strategies in France is presented here.

2.2. Data requirement and collection

In order to model the poultry production networks in France, data on the poultry production organisation and census were collected for each production sector (layers or meat) and species (chicken, ducks ….) including: the number of birds and farm per type of production (grandparents, breeders, free-range production, indoor production); the level of integration (integrated with or without hatchery or independent); the type and volume of movements of birds, eggs or day-old birds between production types. Data were collected both from a public database for the national poultry census per production sector and production type (Agreste, 2018) and from a private database for day-old bird flows from hatcheries (Ceva Poultry database). In addition, interviews with representatives of most French poultry production sectors were performed. After this data collection and collation phase, a participatory workshop was organised with these representatives to

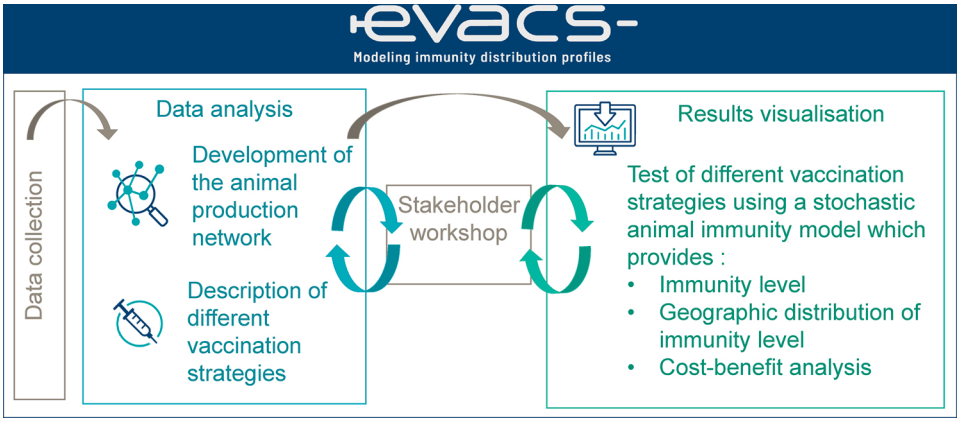

Fig. 1. Schematic representation on how the EVACS tool works.

validate the modelled networks.

To model the immunity within the poultry production networks, data were collected on: the type of vaccines used, the vaccination coverage per production type, the efficacy of the vaccine used (in terms of seroconversion and duration of protection), the number of vaccine doses administered and vaccination schedule (time interval between doses). To perform the spatial analysis the number of heads of the different poultry production types and sectors (grandparents, breeders, indoor production, free-range production) per region were collected (Agreste, 2018). To perform the cost-benefit analysis, data on the vaccination cost (i.e. cost of vaccine dose, vaccination implementation costs) and on the production values (i.e. sale price of eggs, meat birds, day-old birds, adult breeders and grand-parents) were collected.

2.3. Data analysis

All the data collected were entered in a database developed with Excel software (Microsoft Excel 2007).The EVACs tool was applied using "RStudio" software version 1.1 ("R" version 3.5.1). The network script is using "igraph" and "sna" packages (Butts, 2016). The immunity modelling script is a stochastic simulation model using gamma distribution and sensitivity analysis, and "igraph" and "MASS" packages (Gábor, 2018; Ripley et al., 2018). The spatial analysis script also uses "raster" and "rgeos" packages to generate maps (Bivand et al., 2018; Hijmans et al., 2017). The cost-benefit analysis uses an Excel spreadsheet (Microsoft Excel 2007).

2.4. Step 1: poultry production network modelling

The "network modelling" R script of the EVACs tool was used to conduct the network analysis (Peyre et al., 2016). The aim of the network modelling step is to characterise the poultry production networks and to identify the main type of farms (i.e. nodes of the social network analysis) and bird flow between the farms (e.g. day-old birds). Production network models were developed for each of the major French poultry production sectors in France (i.e. broiler, layer, fattening duck, meat duck, turkey and guinea fowl). Attribute tables were used to generate networks based on: the type of production (grandparents, breeders, free-range production, indoor production) for farms and hatcheries, the integration level (integrated with or without hatchery or independent), and the number of birds (heads) on the farms. Backyard flocks (i.e. flocks under 250 birds) were not included in this description as they had a limited role in the spread of H5N8 HPAI during the 2016–2017 epizootic (Souvestre et al., 2019). The different types of poultry production and integration levels were represented by the different nodes in the network. The movement of hatching eggs (between breeder farms and hatcheries) or of day-old birds (chicks, turkeys, ducklings or guinea fowls) between hatcheries and farms were represented by the directed links in the network, i.e. showing the direction of movements between the nodes. The volume of exchange of day-old birds between nodes was considered using directed-weighted matrices.

2.5. Step 2: vaccination strategies identification

Vaccination strategies were defined and tested at both sector and total poultry population level.

2.5.1. Vaccination protocols per sector

Vaccination protocols were defined following the network organisation for each production type and sector (Table 1 and Supplementary file 1). The first vaccination protocols focused on the bird population at higher risk (i.e. free-range) when the following protocols progressively include other production types (indoor, integrated and independent) while combining inactivated farm vaccine and recombinant hatchery vaccine. All protocols were tested in broiler and turkey production sector. The same protocols were tested in layer sector except protocol 3, as there is no hatchery integrated with production farms in this sector. Only protocols using inactivated vaccines were tested for duck and guinea fowl sectors (Table 1 P1, P5 and P6), as no recombinant vaccines are commercially available for these species yet. For all protocols, all grandparent and breeder farms of the concerned sector are vaccinated with inactivated farm vaccines.

2.5.2. Vaccination strategies for the total poultry population

The most efficient vaccination protocol per sector (i.e. resulting in the highest benefit cost ratio above 1) was selected to define the vaccination strategies at the total poultry population level, using a risk-based approach i.e. targeting the higher risk production type to start with i.e. layers and free-range production and then adding on more production types (Table 2). The risk level categorisation was retrieved from previous studies (Barnes et al., 2019; Elbers and Gonzales, 2019; Singh et al., 2018).

2.6. Step 3: Estimation of the efficacy of the vaccination strategies

The "immunity modelling" R script of the EVACs tool was used to estimate the efficacy of the different vaccination strategies for each type of production (network nodes) in terms of: vaccination coverage (proportion of birds in the entire poultry population which have been vaccinated); immunity level (proportion of birds with a protective seroconversion level) and duration of the immunity (proportion of weeks where more than 70 % of birds had a protective seroconversion level) (Peyre et al., 2016).

The parameters used for the model are described in Table 3. As the vaccination would be mandatory if applied in France, the vaccination coverage at farm level (% of vaccinated farms) was considered maximum (100 %). Due to practical aspects, the vaccination coverage at bird level (% of vaccinated birds in a vaccinated farm) was considered better with hatchery vaccination (mean = 98 %, IC 95 %=[95, 99]%) as compared with farm vaccination (mean = 95 %, IC 95 %=[90, 98]%)

Table 1
Vaccination protocols tested.

Vaccination protocol [a]	Production type				
	Grandparents and breeders	Free-range		Indoor	
Farm integration level	Not applicable	Integrated	Independent	Integrated	Independent
P 1	I[b]	I	–	–	–
P 2	I	R	R	–	–
P 3	I	R	R	R Int H	R Int H
P 4	I	R	R	I	–
P 5	I	I	–	I	–
P 6	I	I	I	I	I
P 7	I	R	R	R	R

[a] P: Protocol.
[b] I: Inactivated farm vaccine, R: Recombinant hatchery vaccine, R Int H: Recombinant hatchery vaccine in integrated hatcheries, - : No vaccination.

Table 2
Vaccination strategies tested at the total poultry production level.

Vaccination strategy[a]	Sectors					
	Grandparents and breeders (all sectors)	Broiler	Layer	Turkey	Duck (meat and fattening)	Guinea fowl
S 1	I All (as for all protocols)[b]	R FR (P2)	R All (P7)	R FR (P2)	I Int FR (P2)	I Int FR (P2)
S 2	I All (as for all protocols)	R FR (P2)	R All (P7)	R All (P7)	I Int (P5)	I Int (P5)
S 3	I All (as for all protocols)	R Indoor (P7 only for indoor)	–	–	–	–
S 4	I All (as for all protocols)	R All (P7)	R All (P7)	R All (P7)	–	–
S 5	I All (as for all protocols)	R All (P7)	R All (P7)	R All (P7)	I Int (P5)	–
S 6	I All (as for all protocols)	R All (P7)	R All (P7)	R All (P7)	I Int (P5)	I Int (P5)
S 7	I All (as for all protocols)	R All (P7)	R All (P7)	R All (P7)	I All (P6)	I All (P6)

[a] S: Strategy.
[b] I All: Inactivated farm vaccine in all farms; I Int FR: Inactivated farm vaccine in integrated free-range farms; I Int: Inactivated farm vaccine in all integrated farms; R All: Recombinant hatchery vaccine in all day-old birds; R FR: Recombinant hatchery vaccine in all free-range day-old birds; R Indoor: Recombinant hatchery vaccine of all indoor day-old birds; - : No vaccination; P2−7: Vaccination protocol at the sector level.

Table 3
Inputs parameters for the immunity modelling.

Vaccine type	Production type	% of vaccination coverage		Vaccine efficacy (% of seroconversion)
		% of farms vaccinated	% of birds vaccinated	
Inactivated vaccines (farm)	Grandparents and breeders	100 %	98 % [95−99]	92 % [90–95]
	Layers, broilers, turkeys, ducks and guinea fowls		95 % [90–98]	
Recombinant vaccines (hatchery)	Layers, broilers and turkeys	100 %	98 % [95−99]	92 % [90–95]

(Peyre et al., 2016). As no AI vaccination is currently performed in France, data on vaccine efficacy were collected from the literature. The same vaccine efficacy was applied in the model for both vaccination types based on literature data (Peyre et al., 2016).

The vaccination coverage was considered sufficient above 80 % of the entire targeted population (Bouma et al., 2009). The immunity level was considered to be protective above 60 % based on the R0 estimations previously reported (Fine et al., 2011; Garske et al., 2007; Tiensin et al., 2007).

2.7. Step 4: spatial analysis section

The "spatial analysis" R script of the EVACs tool was used to map the distribution of the immunity levels according to the different vaccination strategies (Peyre et al., 2016). Poultry census data at the region level (Agreste, 2018) were used for the spatial analysis. Data were aggregated according to the production types (grandparents, breeders, indoor production, free-range production) and production sectors.

2.8. Step 5: Cost-benefit and break-even analysis

The "cost-benefit analysis" Excel spreadsheet of the EVACs tool was used to identify the most efficient vaccination strategy, i.e. which offers the highest benefit/cost ratio (BCR) (Peyre et al., 2016). The costs were defined as the vaccination costs (i.e. cost per vaccine dose and vaccination implementation costs) and the value of the losses in the non-vaccinated population As there is currently no vaccination against HPAI in France, the estimates of the vaccination costs for Newcastle disease vaccination in France were used. These costs include the cost of the vaccine but also the cost of its application for each type of vaccine (farm or hatchery application).

The benefits were limited to the value of the avoided production losses in the vaccinated population and calculated for a disease cumulated incidence of 2.5 % (level observed in France during the 2016–2017 H5N6 epizootics from surveillance data) (Bronner et al., 2017). This incidence level was considered to be fixed and equal for all poultry production types and sectors. The production losses due to AI infection were estimated as a function of the risk of infection at a certain point of time (disease cumulated incidence level) and the vaccine efficacy in terms of immunity rate and duration of protection. The parameters used in the cost-benefit analysis (CBA) are presented in Supplementary file 2.

A break-even analysis was conducted on the most efficient vaccination protocol for each sector (i.e. which provided an immunity level above 60 % for the total population) to estimate the level of disease cumulated incidence where vaccination would no longer be efficient (BCR < 1). A sensitivity analysis was also performed on the parameters used for the CBA: cost of vaccination, value of birds, cumulated incidence and level of immunity.

2.9. Stakeholder validation workshop

A participatory stakeholder workshop including poultry producers, vaccine producers and distributors, veterinary services and laboratory experts (both from public and private sectors) was conducted to validate the poultry production network models and the parameters used in the immunity simulation model, to present the results of the evaluation and to discuss on the recommendations.

3. Results

3.1. Network analysis of the French poultry production network

In 2018, almost 810 million commercial broilers (680 million indoor and 130 million free-range), 47 million layers, 42 million turkeys, 40 million meat ducks, 35 million fattening ducks (for "foie gras" production) and 30 million guinea fowl were produced in France. In France, most farms are integrated in a farmer association, which often includes a feed manufacturer as a horizontal integration system. Some farmers associations have one or several breeder hatcheries in a vertical integration system. In the layer, turkey, duck and guinea fowl production sectors, some breeder hatcheries are integrated with selection, i.e. grandparent hatchery (vertical integration with selection), but some hatcheries are independent. In France, no sector is fully vertically integrated i.e. all type of farms from grandparent farms to breeder farms and to production farms are integrated within the same company. Moreover, a few production farms do not belong to a farmer association and are considered as independent. These farms are mostly small farms with on-farm sales of their products (on-farm slaughter or with an individual contract with a slaughterhouse). Based on these observations, the level of integration makes it possible to divide production farms into three groups: farms integrated in a farmers' association with a hatchery, farms integrated in a farmers' association with no hatchery, and independent

farms. The level of integration concerns all production sectors except layers (no hatcheries are integrated with production). No distinction was made between farms integrated in a farmer association with a hatchery and farms integrated in a farmer association with no hatchery because of the limited number of hatcheries and producers in these sectors compared to the broiler sector. This structure was validated by representatives of the turkey, meat duck and guinea fowl sectors. The network analysis conducted in broiler production sector is presented in Fig. 2. The network analysis conducted in the other French poultry production sectors are presented in Supplementary file 3. The spatial distribution of poultry density for each production sector is also provided in Supplementary file 4.

3.2. Evaluation of vaccination protocols for each sector

3.2.1. Immunity distribution profile

For all sectors, the model predicted that targeted integrated production farms (indoor and free-range) with vaccination protocols using inactivated farm and/or recombinant hatchery vaccines were enough to provide a protective vaccination coverage and immunity level for the entire poultry population (more than 80 % and 60 % respectively). The vaccination of higher risk population only (free-range) is not enough to reach a protective immunity level (< 60 %). For broiler, layer and turkey sectors, hatchery vaccination seems to lead to a higher number of vaccinated farms (independent farms included).

3.2.2. Spatial distribution of the immunity level

For broiler, turkey, duck and guinea fowl sectors, vaccination protocols including at least integrated production farms (indoor and free-range) allowed to provide a geographically homogeneous immunity level above 60 % of the total sector population. For layer, only vaccination protocols including all farms allowed to reach this geographically homogeneous level.

3.2.3. Cost-benefit analysis

For all sectors, except the broiler sector, all vaccination protocols tested (immunity level > 60 %) were efficient (BCR > 1) (Table 4). For layer and turkey sector, vaccination protocol including hatchery vaccination of all day old birds (P 7) was the most efficient. For duck and guinea fowl sector, all protocols tested were equivalent in terms of cost-benefit. For broiler sector, none of the tested vaccination protocols was efficient (BCR < 1) but the protocol including hatchery vaccination of all day-old birds was the one with the highest ratio (Table 4). The break-even analysis showed that vaccination is efficient for short lifespan birds (i.e. broilers) when the cumulated incidence is high, while vaccination can be efficient even when the cumulated incidence is low for long lifespan birds (i.e. layer, turkey and duck). Hatchery vaccination ensure a positive BCR at a lower cumulated incidence than farm vaccination. The results of the sensitivity analysis are presented in Supplementary file 5.

3.2.4. Conclusion on the most efficient vaccination protocol for each sector

The vaccination protocol including hatchery vaccination for all day-old birds was considered as the most efficient protocol for broiler, layer and turkey sectors (Table 5). Both vaccination protocols including farm vaccination in all integrated farms (P5) and all farms (P6) were efficient protocols for duck and guinea fowl sectors.

3.3. Evaluation of vaccination strategies at the national level

Vaccination strategies tested at the national poultry production level combined the most efficient vaccination protocols identified for each individual sector with the risk level of each production type (free-range and/or long production life) (Tables 2 and 5). For broiler, layer and turkey sectors, the selected vaccination protocol was hatchery vaccination applied in all hatcheries. For duck and guinea fowl sectors, the most realistic protocol (farm vaccination in all integrated farms, P5) was used in the vaccination strategies 5 and 6 while the most idealistic protocol (farm vaccination in all farms, including independent ones, P6) was used in the vaccination strategy 7.

3.3.1. Immunity distribution profile

The vaccination of layer and free-range production (S 1) did not allow to reach a protective vaccination coverage and immunity level for the entire poultry population (more than 80 % and 60 % respectively) (Fig. 3, S 1). While the vaccination of all sectors except indoor broilers was not enough to reach an immunity level above 60 % (Fig. 3, S 2), the vaccination of indoor broiler production only was enough to reach this level (Fig. 3, S 3). The vaccination including at least all farms in layer, broiler and turkey sectors, without duck and guinea fowl sectors, was sufficient to reach a national vaccination coverage and an immunity level above 80 % of the entire poultry population (Figs. 3, S 4, 5, 6 and 7).

3.3.2. Spatial distribution of the immunity level

A protective immunity level (> 60 %) was reached in the area at

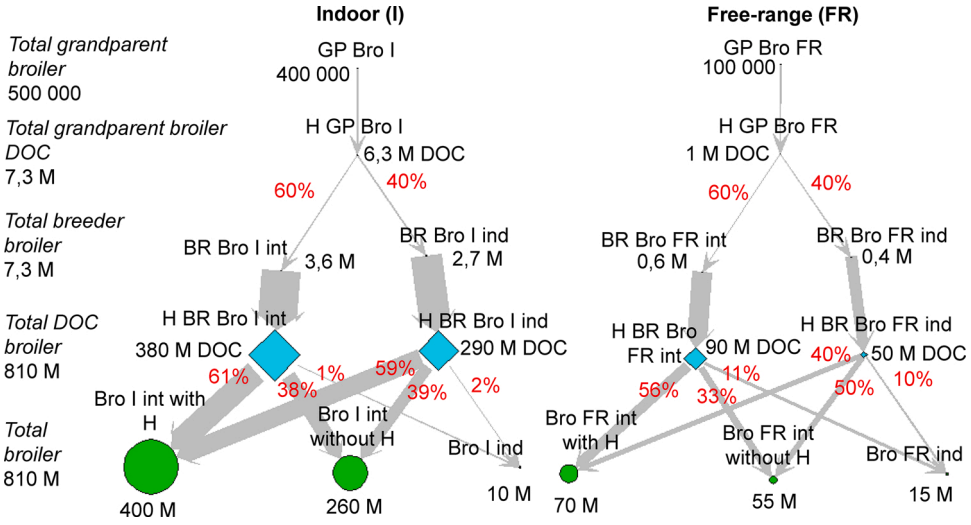

Fig. 2. French broiler production network. The type of nodes represents the different types of production (indoor (I) or free-range (FR); integrated (int) or independent (ind)): grandparents (GP) and breeders (BR) (point), hatcheries (H) (diamond), commercial broilers (Bro) (circle). (DOC: day-old chicks, M: million).

Table 4
Cost-benefit analysis of the different vaccination protocols.

Sector	Vaccination protocols [a]	Immunity level (%)	Cost		Benefit (million euro)	Benefit/cost ratio
			Vaccination cost (million euro)	Losses cost (million euro)		
Layer	P 4	80	8.5	3.1	14.5	1.2
	P 5	76	9.2	3.8	13.8	1.1
	P 6	88	10.7	2.1	15.5	1.2
	P 7	**90**	**2.5**	**1.8**	**15.8**	**3.7**
Turkey	P 4	88	4	1.8	14.9	2.6
	P 5	86	4	2	14.7	2.5
	P 6	88	4	1.9	14.8	2.5
	P 7	**90**	**2.4**	**1.7**	**15**	**3.7**
Duck	**P 5**	**82**	**6.7**	**4**	**22.2**	**2.1**
	P 6	**88**	**7.2**	**3**	**23.2**	**2.3**
Guinea fowl	**P 5**	**77**	**2.5**	**0.6**	**3.3**	**1.1**
	P 6	**88**	**2.8**	**0.4**	**3.5**	**1.1**
Broiler	P 4	87	68.2	5	38	0.5
	P 5	85	72.4	6.3	36.9	0.5
	P 6	88	74.7	5	38.2	0.5
	P 7	**90**	**42.3**	**4.3**	**38.8**	**0.8**

P 4 (broiler, layer and turkey): farm vaccination of breeders and grandparents and integrated indoor farms and hatchery vaccination of all day-old-birds for free-range production.
P 5 (all): farm vaccination in grandparent and breeder farms and in all integrated farms (indoor and free-range).
P 6 (all): farm vaccination in all farms (breeders and indoor and free-range).
P 7 (broiler, layer and turkey): farm vaccination in grandparent and breeder farms and hatchery vaccination of all day-old-birds (indoor and free-range).
[a] P: Protocol.

Table 5
List of the selected protocol per sector.

Sector	Selected protocol [a]	Justification
Broiler	P 7	Highest BCR [b]
Layer	P 7	Highest BCR
Turkey	P 7	Highest BCR
Duck	P 5 and P 6	Equivalent BCR
Guinea fowl	P 5 and P 6	Equivalent BCR

[a] P: Protocol.
[b] BCR: Benefit-cost ratio.

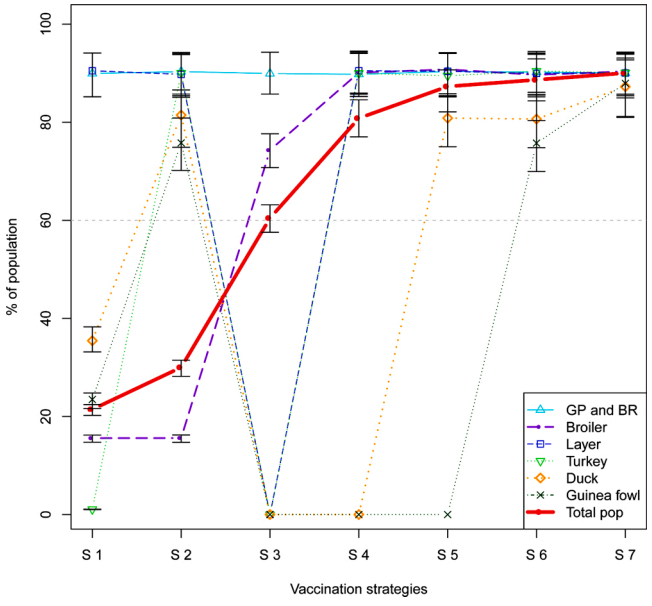

Fig. 3. Overall immunity level per production type (total population (total pop), grand-parent (GP) and breeder (BR) of all sectors, broiler, layer, turkey, duck and guinea fowl) according to the different vaccination strategies at national level (S1 to S7).

higher risk (linked to the highest population density (Shapiro and Stewart-Brown, 2009), located in West of France) when vaccination strategies included at least layers, broilers and turkeys sectors (indoor and free-range productions included) (Figs. 4, S4–S7). Vaccination strategies also including at least integrated duck farms led to a very good immunity level (> 80 %) that was spatially uniform at the national level (Figs. 4, S5, 6 and 7). Indeed, indoor meat poultry productions (broiler, turkey, meat duck) are localised in West of France and fattening duck production is mainly localised in South West of France while free-range productions are mainly localised in South of France. A vaccination strategy focused on high risk populations (layer production and free-range broiler, turkey, duck and guinea fowl productions) and breeders did not provide a protective immunity level (> 60 %) (Figs. 4, S 1).

3.3.3. *Cost-benefit analysis*

All tested vaccination strategies had good BCR (BCR > 1) at the disease cumulated incidence level of the previous epizootic event (2.5 %) except the strategy including only indoor broiler (Tables 6, S 3). Vaccination strategies including at least integrated duck farms (Tables 6, S 5, 6 and 7) offered the highest BCR.

4. Discussion

This study demonstrated the added value of the EVACS evaluation tool for comparing potential vaccination strategies for avian influenza (AI) in French poultry production networks. The best efficiency was obtained with vaccination strategies deploying hatchery vaccination with a recombinant vector vaccine in all species for which such vaccine is commercially available (i.e. broilers, layers and turkeys) and for the other species (i.e. ducks and guinea fowls) on-farm vaccination with a inactivated vaccine on all integrated farms. This work is the first to provide the evidence decision makers need to design a vaccination strategy against AI customized to the capacity and needs of French poultry production networks.

A vaccination strategy limited to the high-risk population (i.e. layers and free-range production in all sectors) and to breeders did not ensure protective immunity level at the sector level, except in fattening ducks, or at the whole poultry population level. Free-range production is often considered more at risk of AI than indoor production mainly because of

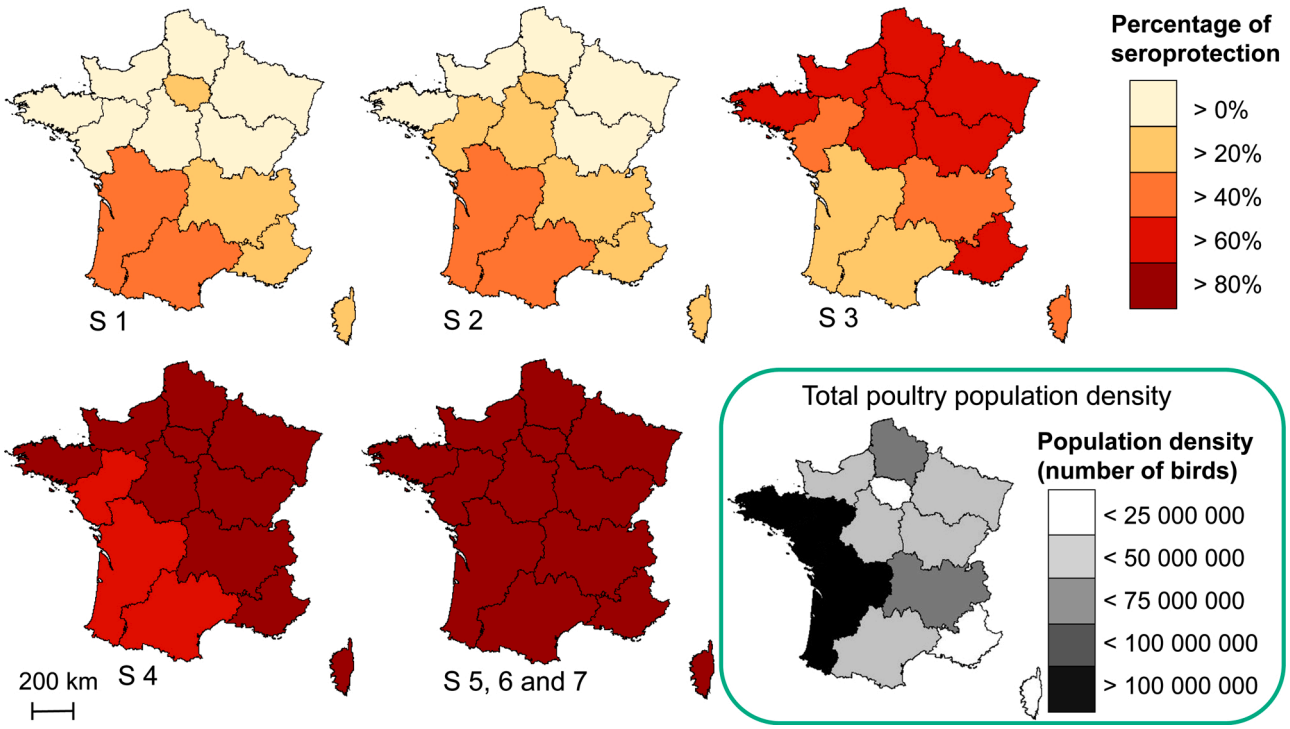

Fig. 4. Spatial distribution of the poultry population immunity against AI according to the different vaccination strategies (S) tested in the model.

Table 6
Cost-benefit analysis of the different vaccination strategies.

Vaccination strategy	Immunity level (%)	Cost		Benefit (million euro)	Benefit/ cost ratio
		Vaccination cost (million euro)	Losses cost (million euro)		
S 3	60	36	53	54	0.6
S 4	81	48	23	84	1.2
S 5	86	54	14	94	1.4
S 6	89	56	12	95	1.4
S 7	90	57	11	96	1.4

S: Strategy.
For all strategies: farm vaccination of all breeder and grandparent farms.
S 3: hatchery vaccination of all indoor broiler day old chicks.
S 4: hatchery vaccination of all day old bird broilers, layers and turkeys.
S 5: S4 + farm vaccination of integrated duck farms.
S 6: S 5 + farm vaccination of integrated guinea fowl farms.
S 7: S6 + farm vaccination of independent duck and guinea fowl farms.

the higher risk of contact with infected wild birds (Elbers and Gonzales, 2019; Singh et al., 2018). Free-range production represents only 20 % of French poultry production. The fattening duck sector is the exception as the whole production is free-range at least during the grow-out stage (Delpont et al., 2018). Moreover, the risk of mutation of low-pathogenic avian influenza virus into an HPAI virus increases with the duration of the productive life of the birds. As the productive life of layers is longer than in other poultry sectors, layers are considered more at risk of inducing this mutation than other types of poultry production (Barnes et al., 2019; Singh et al., 2018). A vaccination strategy focusing on duck production sector, like the one conducted in 2006 (Capua et al., 2009), would not be sufficient to provide a protective immunity level for the whole French poultry production. If a similar choice were made in the future, vaccination of all the animals in the duck production sector would offer the highest level of immunity possible in the sector. But a vaccination protocol focusing only on integrated duck farms would be equally efficient (i.e. BCR). Previous studies recommended focusing vaccination strategies on the most at-risk population (Spackman and Pantin-Jackwood, 2014; Swayne et al., 2014). This option was implemented in some countries to prevent the introduction of the disease or to protect specific bird populations such as zoo birds (Peyre et al., 2009; Swayne et al., 2011). Nonetheless, the risk of large outbreaks is high as this strategy does not provide protective immunity level for the whole poultry population (Bouma et al., 2009; Iwami et al., 2009). Should the choice be made to target only the most at-risk population (e.g. free-range) for vaccination, strict biosecurity measures and a high level of surveillance in the other populations would be required (Peyre et al., 2009; Swayne et al., 2014).

The absence of vaccination of indoor broilers has led to a low immunity level nation-wide (<60 % of the whole poultry population), as broilers represent the largest part of birds produced in France yearly. Vaccination of short lifespan birds like broilers is rarely recommended mostly due the low price of broilers compared to the cost of vaccination (Spackman and Pantin-Jackwood, 2014). Indeed, in our study, even if the vaccination strategy including only broilers raised indoor offered a good level of immunity, this strategy was not efficient (BCR < 1). In this study, only the avoided production losses were included in the CBA. The real cost of HPAI outbreaks is often higher due to the broader impact of the disease on the poultry industry as a whole and a drop in poultry consumption, with a resulting demand shock on the price of poultry (McLeod, 2009). Moreover, trade bans would increase the impact of the disease on costs (live birds but also meat and egg products), especially when the country is a large exporting country (Wieck et al., 2012). The objective of the CBA in the EVACS tool is to compare the efficiency of different vaccination strategies to provide information on the best one to implement but not to provide an exhaustive economic analysis of the impact of the disease. As the wider impacts would be the same for any vaccination strategy, they were not included in this study. This implies that the benefit of vaccination would have been under-estimated. Anyhow, the sensitivity analysis showed that vaccination would still be efficient even if there was a drop in the price of meat or egg (up to a 70 % drop in prices for the layer and turkey sectors for a protocol using hatchery vaccination).

A vaccination protocol based on hatchery vaccination systematically

provided the highest BCR compared to the same protocol based on farm vaccination in the sectors in which hatchery vaccination is available (i.e. broilers, layers and turkeys). However, the exact price of an AI vaccine to be applied in France is currently not known. Under the hypothesis used in this study, the vaccination strategies which provided the highest immunity level (S5, S6 and S7) would be efficient if the vaccination costs (including vaccine application and the number of application) were less than 2% of the value of birds. As fewer applications is are needed for recombinant hatchery vaccines than for inactivated farm vaccines (1 versus 2–5), inactivated farm vaccines would need to be cheaper than recombinant hatchery vaccines to reach an equivalent BCR. Furthermore, implementation of the vaccination is often considered as the critical aspect in reaching protective immunity level (Swayne et al., 2011). Hatchery vaccination makes it possible to reduce the number of applications thereby limiting vaccination implementation constraints and hence the impact on vaccination coverage compared to vaccination at farm level.

As AI vaccination is currently not authorised in France, no data are available on AI vaccine application in the French context. To get round the lack of information, two hypotheses were used in a context of a mandatory vaccination: 1) the vaccination coverage to be reached would be optimal and 2) the applied vaccines would be effective. Inactivated farm vaccines and recombinant hatchery vaccines were considered to have a good and comparable level of efficacy based on the literature (Table 3). As a result, the immunity level and the BCR simulated in this work were mostly differentiated by the vaccine protocol (on-farm, at the hatchery, application frequency) rather than by the type of vaccine. But, the limits of the vaccination strategy used in France in 2006 were not vaccine application but poor response in duck to the vaccine, especially when vaccinated at an early age (Capua et al., 2009). The effectiveness of current AI vaccines in duck is thus questionable (Cha et al., 2013; Pantin-Jackwood et al., 2015; Pfeiffer et al., 2010). Limited studies have been conducted in guinea fowl (Bertelsen et al., 2007). Our study shows that the vaccination strategy targeting other poultry production sectors than duck and guinea fowl (i.e. the broiler, layer and turkey sectors) was sufficient to induce protective immunity level in the whole poultry production. As the previous AI epizootic waves mainly concerned ducks farms in France, an effective vaccine is needed to protect these important production sectors in France. Promising vaccine solutions exist for ducks (Niqueux et al., 2018; Tatár-Kis et al., 2019) but the absence of a secure vaccine market does not encourage vaccine manufacturing companies to invest in vaccine registration costs. Break-even analysis showed that for long lifespan birds (i.e. layer, turkey and duck sectors), vaccination protocols were efficient (BCR $>$ 1) even at low cumulated incidence level (up to 2% for farm vaccination protocol (P6) and to 0.5 % for hatchery vaccination protocol (P7) in the layer sector). These cumulated incidence levels are below the cumulated incidence rate observed during the 2016–2017 epizootic. For the broiler sector, the break-even analysis showed that a vaccination protocol with hatchery vaccination (P 7) or farm vaccination (P 6) would be efficient if the cumulated incidence level was above 3% or 5.5 % respectively. This was under the cumulated incidence level actually observed in the most affected area in 2016–2017 epizootic which was 15 % in the Landes administrative department (Bronner et al., 2017). The difference in cumulated incidence rates at local scale underlines the importance of regionalised vaccination, a choice made by some countries (Swayne et al., 2011).

The impact of AI vaccination on international trade, particularly on exports, would be high due to export ban. The OIE code states that if a country can prove that the exported birds are free of the disease using an effective surveillance system, the epidemiological status of the country should not be linked to a ban on exports (OIE, 2018). As exports account for a large proportion of the French poultry production revenues, this decision would be taken only in the case of extensive uncontrolled spread and with an effective vaccine. During the stakeholders' workshop implemented as part of this study, participants considered that if a vaccination policy were applied in France, it would only be deployed in the case of an emergency, with only the geographical area where the outbreaks occurred being targeted. The EVACS tool has initially been developed to compare preventive vaccination strategies and not emergency vaccination strategies. This is more relevant in countries where the disease is endemic (Peyre et al., 2016). The application of the tool in France allowed to identify some critical aspect that should be considered when defining vaccination strategies even in an emergency context. The results of our study could also be applied in the case of an emergency vaccination strategy. We have shown that vaccination of free-range production would not provide a protective level of immunity for the whole production. In the case of an HPAI outbreak in a geographically limited production sector such as the fattening duck sector, the use of vaccination in this specific sector as a complementary tool to culling and increased biosecurity is an efficient option to protect the specific production network while limiting the economic and psychological impact of culling for the farmers. If an emergency vaccination strategy was to be applied, the questions relating to management of vaccinated birds (culling or slaughter for consumption) should be clearly defined.

The vaccination of grandparents and breeders included in all strategies tested was also discussed during the stakeholders' workshop. The participants considered that these productions represent a low risk of HPAI introduction due to the high level of biosecurity on these farms. Moreover, as selection companies export the majority of their production, vaccinating their flocks would actually prevent them from exporting. Compartmentalisation is one possible option to focus vaccination policies on production stages while allowing breeding companies to continue business-as-usual (Hagenaars et al., 2018). Compartmentalisation is also recommended by the OIE for an infected country to continue exports of live birds (OIE, 2018).The development of an epidemiological model linked to the EVACS tool will make it possible to include these levels of biosecurity in the evaluation of vaccination strategies.

The effectiveness of the vaccination applied in 2006 in the duck sector could not been assessed due to the absence of outbreaks in the area where vaccination took place (Capua et al., 2009). Nonetheless, if no vaccination had been applied, the situation in France could have evolved like in Hungary, where there were 29 outbreaks of HPAI H5N1 in the duck and geese production sector (Capua et al., 2009). Interestingly, during the H5N8 epizootic wave in 2016–2017, France and Hungary were the two countries with the highest number of reported HPAI outbreaks, mainly in the duck production sector (Napp et al., 2018). Vaccination was not implemented in either country. Even if the poultry production system has increased in both countries since the 2006 H5N1 wave (FAO, 2016) and the virus strains implicated in the epizootic waves were not the same, this observation should encourage reconsidering vaccination as a valuable option combined with surveillance and other control strategies such as culling and biosecurity, to control a future epizootic. As previously highlighted by Swayne et al. "there is no one AI control solution for all countries; each AI strategy must be specific to the country and production sectors concerned" (Swayne et al., 2011). The EVACS tool is able to support decision makers in defining a vaccination strategy specific to their country and their production sectors.

5. Conclusion

In our study, we have used the EVACS tool to compare multiple national strategies based on the use of two main types of vaccination (farm versus hatchery) and targeting different production sectors. Our study has shown that vaccination of only high-risk poultry productions (free-range, layer) did not produce protective immunity level and that the vaccination strategies including the highest number of birds were the most efficient. Moreover, vaccination protocol based on hatchery vaccination with a recombinant vaccine were most efficient than the same protocol based on farm vaccination with an inactivated vaccine,

for the sectors in which hatchery vaccination is available (i.e. broilers, layers and turkeys). Such approach can support decision makers to compare the expected efficiency of these strategies. At this stage, the tool provides evidence in terms of vaccination coverage, immunity level, spatial distribution of this immunity level and benefit cost ratio. Combining EVACS with an epidemiological model will add information on the expected effectiveness of the strategies tested to control HPAI. This work is the first one to provide the evidence decision makers need to design the most efficient AI vaccination strategy in France.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prevetmed.2020.105129.

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Review

Status and Challenges for Vaccination against Avian H9N2 Influenza Virus in China

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Abstract: In China, H9N2 avian influenza virus (AIV) has become widely prevalent in poultry, causing huge economic losses after secondary infection with other pathogens. Importantly, H9N2 AIV continuously infects humans, and its six internal genes frequently reassort with other influenza viruses to generate novel influenza viruses that infect humans, threatening public health. Inactivated whole-virus vaccines have been used to control H9N2 AIV in China for more than 20 years, and they can alleviate clinical symptoms after immunization, greatly reducing economic losses. However, H9N2 AIVs can still be isolated from immunized chickens and have recently become the main epidemic subtype. A more effective vaccine prevention strategy might be able to address the current situation. Herein, we analyze the current status and vaccination strategy against H9N2 AIV and summarize the progress in vaccine development to provide insight for better H9N2 prevention and control.

Keywords: influenza virus; H9N2 AIV; vaccine; antigenicity

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1. Introduction

Avian influenza virus (AIV) affiliates with the genus of type A influenza virus in the *Orthomyxoviridae* family, packaged with eight negative-sense and single-strand RNA segments encoding 10 core proteins and a variable number of accessory proteins. Influenza A viruses are commonly characterized by their combinations of hemagglutinin (HA) and neuraminidase (NA), giving rise to a multitude of different subtypes, such as H5N1, H7N9 or H9N2. According to their intravenous pathogenicity index (IVPI) in chickens, AIVs are classified into high pathogenicity avian influenza viruses (HPAIVs) and low pathogenicity avian influenza viruses (LPAIVs).

H9N2 AIV was first isolated from turkeys in Wisconsin, USA, in 1966 [1]. Since then, the presence of H9N2 AIV in poultry flocks has been reported in various countries worldwide [2–4]. The first isolate of H9N2 AIV in China was collected from chickens in Guangdong province in 1994 [5]. Compared to H5 and H7 HPAIVs, H9N2 LPAIV infection did not induce obvious clinical signs or death in chickens. However, H9N2 infections in poultry increased their susceptibility to secondary infections with other pathogens that could cause high mortality, leading to huge economic losses [6]. Moreover, a recent study showed that co-infection of H9N2 with infectious laryngotracheitis virus live-attenuated vaccine caused enhanced pathogenicity and immunosuppression, suggesting that we need to be more concerned about H9N2 infection during vaccination [7]. In China, which is regarded as an epicenter of avian influenza viruses, the H9N2 virus has been detected in multiple avian species [8], including chickens, domestic waterfowl and pigeons [9]. Notably, H9N2 AIVs in poultry populations, especially in China, have already acquired the ability to cross species barriers and can directly infect mammals, even humans, without a need for intermediate hosts. H9N2 AIV in poultry has been transmitted to pigs [10]

and dogs [11], generating variants with novel antigenic and genetic characteristics. The enlarged host range of the H9N2 virus substantially increases its possibility of transmission to mammals. Importantly, human infections with H9N2 AIV have been sporadically reported worldwide [12]. In addition, the six internal genes of H9N2 constitute a relatively stable community to be transferred into other emerging reassortants, such as the human-infecting H7N9 [13,14], H10N8 [15] and clade 2.3.4.4 human-lethal H5N6 viruses [16], which significantly threaten public health.

Influenza vaccines are one of the best tools currently available to reduce the risk of influenza infection and its associated complications, which has been proved in a variety of animals, including humans and birds. Humoral immunity, mainly based on HA and NA, utilizes secretory IgA and IgM to provide protection against the establishment of initial infection, while IgG acts to neutralize newly replicating viruses [17]. HA antibody levels have been shown to correlate with protection against infection by influenza; antibodies against NA may also correlate with protection against infection as well as causing a reduced severity of illness [18,19]. Cell-mediated immunity, on the other hand, as elicited by major histocompatibility complex (MHC) class I-restricted CD8+ cytotoxic T lymphocytes (CTLs), plays a central role in controlling influenza-virus infection [20,21]. The major antigenic targets of these cross-reactive T cells are epitopes in the highly conserved internal proteins of influenza, particularly polymerase basic protein 1 (PB1), matrix protein 1 (M1) and nucleoprotein (NP) [22]. Recently, there has been increasing consensus on the importance of T cells being present locally in the airway or parenchyma of the respiratory tract to protect against influenza. The presence of mucosal immune responses in the respiratory tract or lung is particularly important, because most severe influenza symptoms are due to lung infection [23–25].

Controlling the prevalence of H9N2 virus, especially in poultry, will decrease the incidence of H9N2 human infection and reduce the production of new reassortants, thus minimizing the risk of influenza pandemics caused by H9N2 AIV. We note, however, that the prevalence of H9N2 in immunized flocks indicates that the effectiveness of the H9N2 vaccine is facing a great challenge. Therefore, this review focuses on analyzing the current status of H9N2 under vaccination programs in Chinese poultry, summarizing the progress of vaccine development, and looking forward to the control of H9N2 in China in the future.

2. Prevalence and Antigenic Drift of H9N2 AIVs under Vaccination in China

2.1. H9N2 AIV in Avian and Human

China, supplying over 70% of the H9N2 isolates in the database (data from GISAID), is considered the epidemic center of AIV. Since it was first reported in 1994, H9N2 AIVs have continued to circulate in China [5]. A number of reports have confirmed the widespread prevalence of H9N2 in poultry flocks in China [26–29]. Genetic evolution analysis has shown that the vast majority of H9 genes in China belong to the BJ/94-like lineage [30] (also known as the Y280-like lineage), and multiple sublineages of this lineage are currently prevalent (Figure 1). When considering all eight genes, H9N2 AIV continued to reassort and evolve to produce novel genotypes during continued circulation [31–34]. G57, a novel H9N2 genotype, was generated in 2007, triggering a widespread epidemic in poultry flocks in China, with exaggerated spread advantages [35–37]. The latest live-poultry-market surveillance results showed that the isolation rate of H9N2 in chickens from 2014–2019 was persistently high (~11%) and that the H9N2 subtype had gradually surpassed other subtypes to become the most predominant subtype in chicken, duck and pigeon flocks, making it the top problem in the poultry industry [38,39]. It is worth noting that such a status has occurred in the context of continuous vaccination since 1998 [33].

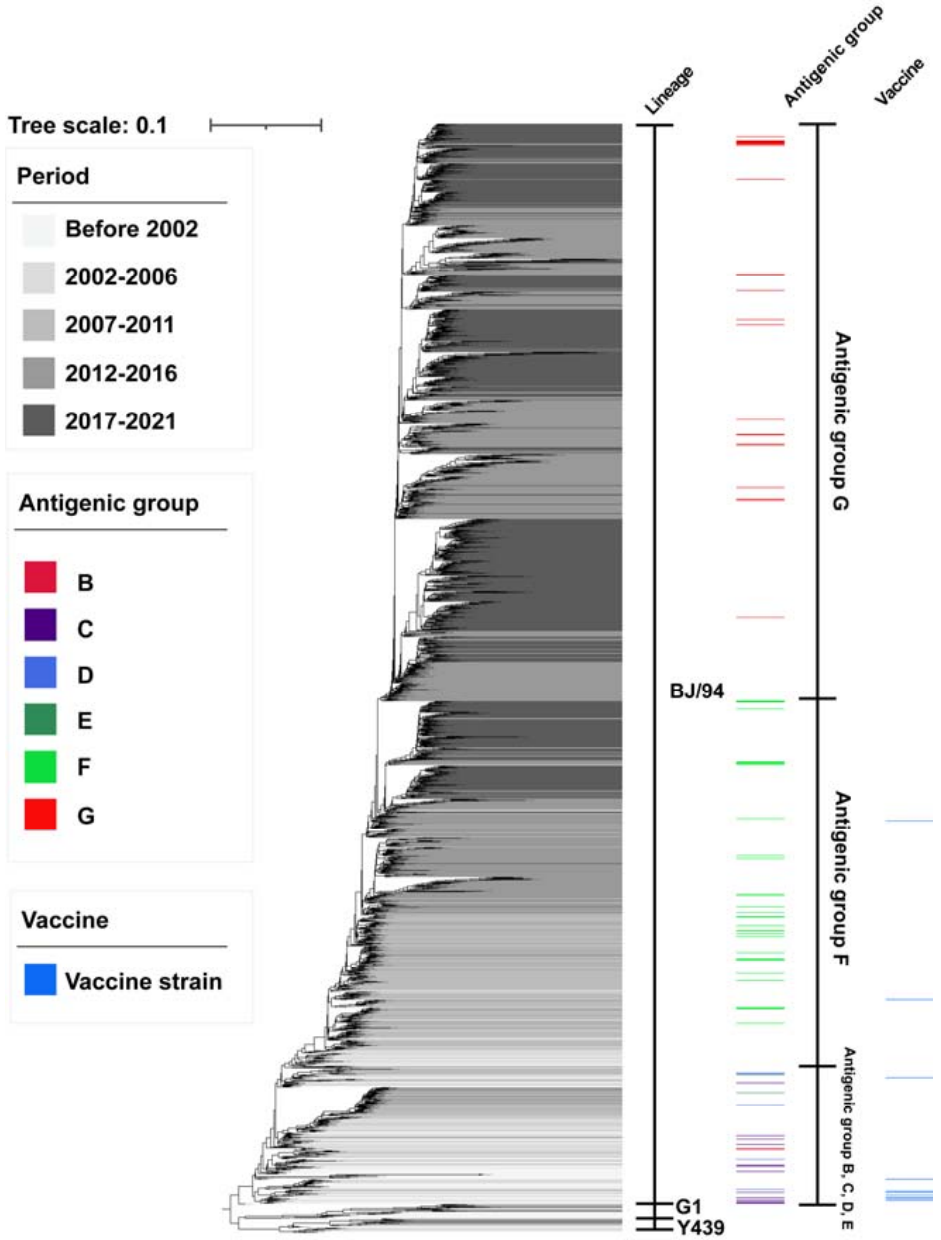

Figure 1. Genetic evolution of H9N2 virus in China. All available nucleotide sequences of the HA genes of H9N2 influenza virus in China as of 31 December 2021 from GISAID (www.gisaid.org) were downloaded. Duplicated sequences and sequences with 99% identity were further removed, and a total of 1923 sequences were finally gutted. Following this, the MAFFT tool was used to perform a global multiple alignment of the sequences, and the MUSCLE tool was used to perform a partial alignment to correct part of the alignment errors and manually correct some of the frameshift errors. The H9N2 virus HA gene sequences were used to construct a phylogenetic tree using IQtree software based on the maximum likelihood method. Different colors in the phylogenetic tree represent virus strains isolated during different periods; the darker the color is, the more recent the isolation time. Adobe Illustrator 2021 and Interactive Tree Of Life (iTOL) were used to annotate the strains with antigen groups and vaccines.

The widespread prevalence of H9N2 AIV in chickens increases the risk of host spillover and has public health implications. The world's first case of human infection with H9N2 AIV was reported in Guangdong, China, in 1998 [40]. As of December 2021, global reports of laboratory-confirmed human H9N2 AIV cases had reached 95 cases, of which 32 of the 33 cases reported in 2020–2021 were from China, confirming the high risk of host spillover

from the H9N2 AIV chicken epidemic [41]. Most chickens infected with H9N2 AIV do not die directly, preserving the opportunity for secondary infection with other subtypes of influenza viruses, which promotes virus reassortment. H9N2 avian influenza viruses have served as "donor viruses", providing multiple internal genes to various subtypes of viruses, including H5N1, H5N6, H7N9, H10N8 and H10N3 [35,38,42–44]. Such reassortment events occurred intensively with the increased isolation rate of the G57 genotype H9N2 AIV. These novel viruses could present as regional epidemics in avian populations, or even pose a significant threat to humans [45], so controlling H9N2 AIV epidemics in avian populations is vitally important to human influenza prevention and control.

2.2. Antigenic Drift of H9N2 AIV under Vaccination in China

The continuous variability of H9N2 AIV antigenicity is greatly conducive to its prevalence in immunized flocks. The antigenic epitopes of HA proteins are mainly located in the head region of the trimeric protein structure, which also performs the key function of cell receptor binding [46,47]. There are four potential antigenic sites in H9-subtype hemagglutinin: Site I, Site II, H9-A, and H9-B, defined in previous studies [48–52]. The continued evolution of the BJ/94-like lineage of H9N2 in China has gradually produced a number of antigenic mutations, resulting in the formation of different antigenic groups.

The first commercially available H9N2 vaccine was introduced in China in 1998, 4 years after H9N2 AIV was confirmed in chicken flocks [33]. The use of inactivated vaccines was considered to be very effective in the early stages, at least for maintaining production performance. However, most H9N2 AIVs isolated from 1997–2002 showed antigenic drift from the representative vaccine strain SD/6/96 [33]. Sun et al. classified representative H9N2 strains from 1994–2008 into five antigenic groups, groups A–E, based on their antigenic distance [30]. The distribution of antigenic groups showed an obvious correlation with the year of isolation, suggesting that H9N2 AIV was undergoing continuous antigenic changes. However, the renewal of vaccine strains is slow and lags behind the changes in antigenic groups. It usually takes approximately 5 years for a new vaccine strain to come into use. Recent findings suggest that H9N2 in China has evolved two antigenic groups with HI cross-titer differences ranging from 8- to 32-fold, with vastly different antigenicity, which can be referred to as antigenic groups F and G [37,53–56]. At present, isolates with different antigenicity are cocirculating, resulting in a mismatch between the vaccine strains and epidemic strains, which makes it difficult to effectively inhibit the spread of H9N2 AIV.

3. Vaccination Strategy for H9N2 AIV in China

Mass vaccination strategies, mainly using inactivated vaccines, have been implemented in China and have demonstrated satisfactory effects in controlling HPAIV [57]. H5 outbreaks have declined significantly since a mass vaccination program was initiated in 2005. HPAIV of H5 clade 7.2, which was widely circulating in northern China from 2006 to 2013 [58], has been largely eliminated by mass vaccination. In addition, mass vaccination against the zoonotic H7N9 subtype was implemented in 2017, and the isolation rate of H7N9 AIV in poultry decreased significantly. Coincidently, human cases of the sixth wave declined by 99.6% compared with the fifth wave [57]. The control strategy of H9 AIV was different from that of the H5 and H7 HPAIVs. Compulsory vaccination was not carried out against H9N2 AIV, but almost all chicken flocks were immunized with the H9 vaccine to reduce potential economic losses. According to the China National Veterinary Drug Basic Information Database, there have been approximately 50 biologic companies nationwide that have manufactured monovalent or multivalent H9N2 vaccines within the last 5 years. Among the certified H9 vaccine-related products, there were as many as 25 vaccine strains, and all of them were inactivated whole-virus vaccines (IWVs) (Table 1). Research and clinical data show that H9N2 vaccination can provide effective protection for immunized flocks by reducing clinical signs caused by virus infection. However, the frequent antigenic drift of H9N2 is one of the challenges of current vaccination against H9N2. IWV does not provide sufficient protection when there are differences in antigenicity between the H9N2

vaccine strain and the circulating strain [35,59]. The H9N2 vaccine needs to be optimized to fit the current co-epidemic status of multiple antigenic groups. Importantly, the inactivated H9N2 vaccine mainly induces humoral immunity, which makes it difficult to interrupt virus infection and shedding in the chicken upper respiratory tract. H9N2 AIV strains are capable of efficient chicken-to-chicken aerosol transmission, even among vaccinated chickens [60], increasing the difficulty of virus prevention and control. Therefore, 'preventing shedding' has become a new criterion and another challenge for the development of novel H9N2 vaccines. There is an urgent need to develop more effective vaccines to prevent and control H9N2, such as improving existing vaccines at the levels of cellular and mucosal immunity and developing universal vaccines to control the presence of multiple antigenic groups.

Table 1. Commercial vaccines against H9 subtype avian influenza in China.

Vaccine Strain Abbreviation	Commercial Vaccine	Announcement No.	Announcement Date	Notes
F strain	AI (H9 subtype) IV (F/98 strain)	N/A	N/A	A/Chicken/Shanghai/F/98
	ND and AI (H9 subtype) combined IV (La Sota strain + F strain)	N/A	N/A	
HN03 strain	AI (H9 subtype) IV (HN03 strain)	2534	23 May 2017	N/A
HN106 strain	AI (H9 subtype) IV (HN106 strain)	2306	8 October 2015	A/chicken/Henan/01/2006
	ND and AI (H9 subtype) combined IV (La Sota strain + HN106 strain)	2390	15 April 2016	
	ND, IB and AI (H9 subtype) triple IV (La Sota strain + M41 strain + HN106 strain)	1489	26 November 2010	
	ND, IB, ED and AI (H9 subtype) quadruple IV (La Sota strain + M41 strain + HE02 strain + HN106 strain)	1883	7 January 2013	
LG1 strain	AI (H9 subtype) IV (LG1 strain)	N/A	N/A	A/Chicken/Shandong/LG1/2000
	ND and AI (H9 subtype) combined IV (La Sota strain + LG1 strain)	1322	11 January 2010	
	ND, IB, and AI (H9 subtype) triple IV (La Sota strain + M41 strain + LG1 strain)	1507	30 November 2010	
NJ01 strain	AI (H9 subtype) IV (NJ01 strain)	1938	6 May 2013	A/chicken/Nanjing/01/99
SD696 strain	AI (H9 subtype) IV (SD696 strain)	N/A	N/A	A/Chicken/Shandong/6/96
SS strain	AI (H9 subtype) IV (SS/94 strain)	N/A	N/A	A/Chicken/Guangdong/SS/94
	ND and AI (H9 subtype) combined IV ((La Sota strain + SS/94 strain)	N/A	N/A	
	ND, IB and AI (H9 subtype) triple IV (LaSota strain + M41 strain + SS/94 strain)	1530	19 January 2011	
	ND, IB, ED and AI (H9 subtype) quadruple IV (La Sota strain + M41 strain + K-11 strain + SS/94 strain)	2083	26 March 2014	
SY strain	AI IV (H9 subtype, SY strain)	N/A	N/A	A/chicken/Shaanxi/SY/97
	ND and AI (H9 subtype) combined IV (La Sota strain + SY strain)	1821	22 August 2012	
	ND, IB and AI (H9 subtype) triple IV (La Sota strain + M41 strain + SY strain)	1779	4 June 2012	
SZ strain	AI (H9 subtype) IV (SZ strain)	2270	24 June 2015	A/chicken/Shandong/SZ/2008
	ND and AI (H9 subtype) combined IV (La Sota strain + SZ strain)	2506	14 March 2017	
	ND, AI (H9 subtype) and IBD triple IV (La Sota strain + SZ strain + rVP2 protein)	2525	3 May 2017	
	ND, IB, AI (H9 subtype) and IBD quadruple IV (La Sota strain + M41 strain + SZ strain + rVP2 protein)	2400	5 May 2016	
HL strain	ND and AI (H9 subtype) double IV (La Sota strain + HL strain)	N/A	N/A	HL strain isolated from clinical cases in Luoyang, Henan, China in 2001
	ND, IB and AI (H9 subtype) triple IV (La Sota strain + M41 strain + HL strain)	N/A	N/A	
	ND, IB, ED and AI (H9 subtype) quadruple IV (La Sota strain + M41 strain + AV127 strain + HL strain)	N/A	N/A	

Table 1. *Cont.*

Vaccine Strain Abbreviation	Commercial Vaccine	Announcement No.	Announcement Date	Notes
HP strain	ND and AI (H9 subtype) combined IV (La Sota strain + HP strain)	N/A	N/A	HP strain was isolated from diseased chicken flocks in Puyang, Henan, China in 1998
	ND, IB and AI (H9 subtype) triple IV (La Sota strain + M41 strain + HP strain)	1335	1 February 2010	
	ND, IB, ED and AI (H9 subtype) quadruple IV (La Sota strain + M41 strain + Z16 strain + HP strain)	N/A	N/A	
JD strain	ND and AI (H9 subtype) combined IV (La Sota strain + JD strain)	2577	31 August 2017	N/A
WD strain	ND and AI (H9 subtype) combined IV (La Sota strain + WD strain)	136	1 February 2019	WD strain was isolated from Wangdu, Hebei, China in 1998
	ND, IB and AI (H9 subtype) triple IV (La Sota strain + M41 strain + WD strain)	1489	26 November 2010	
	ND, IBD and AI (H9 subtype) triple IV (La Sota strain + BJQ902 strain + WD strain)	2557	2 August 2017	
	ND, IB, ED and AI (H9 subtype) quadruple IV (La Sota strain + M41 strain + HSH23 strain + WD strain)	2268	17 June 2015	
G strain	ND and AI (H9 subtype) combined IV (aSG10 strain + G strain)	164	16 April 2019	A/chicken/Hebei/G/2012
WJ57 strain	ND and AI (H9 subtype) combined IV (A-VII strain + WJ57 strain)	registered	15 October 2018	A/chicken/Jiangsu/WJ57/2012
D1 strain	DP and AI (H9 subtype) double IV (AV1221 strain + D1 strain)	2557	2 August 2017	N/A
HZ strain	ND, IB and AI (H9 subtype) triple IV (La Sota strain + M41 strain + HZ strain)	1556	13 April 2011	N/A
	ND, IB, ED and AI (H9 subtype) quadruple IV (La Sota strain + M41 strain + HS25 strain + HZ strain)	2106	28 May 2014	
JY strain	ND and AI (H9 subtype) combined IV (La Sota strain + JY strain)	1507	30 November 2010	A/chicken/Jiangsu/JY/99
L strain	ND, IB and AI (H9 subtype) triple IV (La Sota strain + M41 strain + L strain)	N/A	N/A	N/A
NJ02 strain	ND, IB and AI (H9 subtype) triple IV (La Sota strain + M41 strain + NJ02 strain)	1448	27 August 2010	A/chicken/Nanjing/02/2001
	ND, IB, ED and AI (H9 subtype) quadruple IV (La Sota strain + M41 strain + AV127 strain + NJ02 strain)	1548	4 March 2011	
Re9 strain (HuN33 strain)	ND, IB and AI (H9 subtype) triple IV (La Sota strain + M41 strain + Re9 strain)	2324	18 November 2015	A/chicken/Hunan/33/2008
	ND, AI (H9 subtype) and ED triple IV (La Sota strain + Re9 strain + Jing 911 strain)	164	16 April 2019	
YBF003 strain	ND, IB and AI (H9 subtype) triple IV (La Sota strain + M41 strain + YBF003 strain)	N/A	N/A	N/A
	ND, IB, ED and AI (H9 subtype) quadruple IV (La Sota strain + M41 strain + NE4 strain + YBF003 strain)	1908	1 March 2013	
	ND, AI (H9 subtype) and IBD triple IV (La Sota strain + YBF003 strain + VP2 protein)	1865	3 December 2012	
	ND, IB, AI (H9 subtype) and IBD quadruple IV (La Sota strain + M41 strain + YBF003 strain + SVP-2 protein)	1532	21 January 2011	
S2 strain	ND, IB, ED and AI (H9 subtype) quadruple IV (La Sota strain + M41 strain + AV-127 strain + S2 strain)	1532	21 January 2011	A/chicken/Shandong/S2/2005
YT strain	ND, AI and IC triple IV (La Sota strain + YT strain + DY2 strain)	582	24 July 2022	N/A
	ND, AI and IC triple IV (La Sota strain + YT strain + QD strain)	463	29 August 2021	
YBF13	ND, AI and IC triple IV (La Sota strain + YBF13 strain + YBAV-4 strain)	441	29 June 2021	N/A

Data source: China Institute of Veterinary Drug Control. N/A indicates that data are not available. Abbreviations: AI: avian influenza; ND: Newcastle disease; IB: infectious bronchitis; ED: egg-drop syndrome; DP: duck plague; IBD: infectious bursal disease; IC: infectious coryza; AA: avian adenovirus disease; IV: inactivated vaccine.

4. Vaccine Development against H9N2-Subtype Avian Influenza

4.1. Inactivated Whole-Virus Vaccines

As the most widely used type of influenza-virus vaccine in both humans and birds, IWV's clinical effectiveness is unquestionable. IWV offers some advantages compared to other types of vaccines, including ease of production and its lack of ability to revert to a virulent state. Vaccine candidate strains determine the immunogenicity of the vaccine. The compatibility between HA and NA, the effect of HA deglycosylation and protective antigenic epitopes should be considered to screen better candidate strains (Table 2) [61].

Given that multiple H9N2 antigenic groups were prevalent in China, rapid preparation of an antigen-matched vaccine or effective prediction of antigen variation would be crucial for the control of H9N2. Antigenicity prediction models based on HA sequences show good potential [62]. Strains with broad-spectrum cross-protection may exist among the naturally prevalent strains, as reported for H7N9 subtypes of AIV [63]. An inactivated vaccine modified based on mosaic vaccine design ideas for the H9 gene was successfully prepared, but its broad-spectrum protection still needs to be further explored [64]. Epigraph, a graph-based vaccine-design algorithm, has been applied and demonstrated its broad protection against H3 subtypes [65,66]. In addition, a chimeric H9/H5N2 recombinant vaccine that expressed the whole HA1 region of H9N2 and the HA2 region of H5N8 viruses protected immunized chickens against lethal challenge by HPAI H5N8 viruses and significantly attenuated virus shedding after infection by both H9N2 and HPAI H5N8 viruses [67].

However, the H9N2 inactivated vaccine could not prevent immunized chickens from reinfection with H9N2 AIV and shedding virus. Many studies have tried to improve the immune protection of inactivated vaccines from multiple aspects. The virus used for producing inactivated vaccine is the main factor in inducing vaccine immunity. The seed virus used for vaccine preparation needs to be screened to better meet the immunogenicity of the current epidemic strain, which has cross-immune protection characteristics. For production purposes, vaccine candidate strains should replicate effectively in embryonated chicken eggs. At present, the construction of H9N2 vaccine candidate strains mostly uses PR8 (H1N1) as the backbone and recombines the HA and NA genes of epidemic viruses. This PR8 backbone has also been shown to improve titers in embryonated chicken eggs, a common propagation system for influenza viruses [68]. Alternatively, some scholars used the H9N2 virus to directly produce vaccine virus by screening the H9N2 virus, optimizing the virus sequence and increasing the virus load and its antigenicity [69,70].

The IWV is mainly prepared from the formaldehyde inactivation of virus-containing allantoic fluids from infected chicken embryos. Recently, several virus inactivation methods for producing influenza IWVs, including formaldehyde, treatment with beta-propiolactone (BPL) and the application of gamma radiation, have been analyzed for their immune protection effects. A study showed that antibody-mediated immune responses were increased in chickens that received BPL and gamma IWVs compared to formaldehyde IWV against H9N2 AIV [71]. Inactivated vaccines mainly induce humoral immunity. Adjuvants could improve the cellular immunity or mucosal immunity induced by IWV. CpG oligodeoxynucleotides assist the whole inactivated H9N2 influenza virus in crossing the intestinal epithelial barriers via transepithelial uptake of dendritic cell dendrites [72,73]. Similarly, bursopentine [74], bursin-like epitope peptide [75], poly I:C [76], bursal peptides [77], chitosan [78], *Bacillus subtilis* spores [79] and polyethyleneimine-coated PLGA nanoparticles [80] as adjuvants induce antigen-specific antibody and T-cell responses in poultry against H9N2 AIV. However, these adjuvants have not been used in the clinic so far.

Table 2. Development and application of various types of vaccines.

Type of Vaccine	Development and Application	Notes	References
Inactivated whole-virus vaccines	Change vaccine strains	Enhanced vaccine compatibility	[61]
	Universal vaccine	Provides cross-protection	[63–67]
	Recombinant with PR8 virus	Improved production efficiency	[68]
	Gene modification	Modified HA sequence	[69,70]
	Inactivation method	Antibody-mediated immune responses were increased in chickens that received the BPL and gamma IWVs compared to the formaldehyde IWV	[71]
	Development of new adjuvants	CpG oligodeoxynucleotides, bursopentine, bursin-like epitope peptide, poly I:C, bursal peptides, chitosan, *Bacillus subtilis* spores, polyethylenimine-coated PLGA nanoparticles	[72–80]
Vector vaccine	Fowlpox virus	Affected by preexisting immunity	[81,82]
	Fowl adenoviruses	Application in IBD, not yet developed in influenza	[83]
	Marek's disease virus	Could not induce robust local mucosal immunity in the respiratory tract	[84,85]
	Newcastle-disease virus	Interference from maternal antibodies greatly hinders clinical application	[86–89]
	APMV-2	Conferred complete immune protection	[90]
Live-attenuated vaccine	Attenuated cold-adapted live H9N2-subtype AIV vaccine	Provides better protection, but carries a biological risk	[91–93]
	Recombinant influenza virus with modified, truncated or absent NS1	Effectively reduced viral replication	[94,95]
	Codon-pair bias	Effectively reduced viral replication	[96–98]
DNA and mRNA vaccine	DNA vaccines	Conferred complete immune protection	[99]
	mRNA vaccine	Inability to induce strong immunity and unsuitable for mass vaccination	[100]
Virus-like particle vaccine	H9N2 VLP	Reduced biosecurity threats and costs	[101]
	Universal vaccination	Provides cross protection	[102,103]
Recombinant protein vaccine	conjugated to anti-chicken Dec205 monoclonal antibody	Strong immune protection 14 days after initial immunization	[104]
	H9 HA1-fliC	Promotes superior protective immune responses	[105]
	Chickens vaccinated with CD83 scFv targeted H9 HA	Effectively reduced viral replication	[106]

4.2. Vector Vaccine

Advances in molecular biology have allowed a number of different vectored vaccines to be developed and licensed for use in the control of avian influenza (Table 2) [107]. Vector vaccines can induce additional cellular immunity and mucosal immunity through infection to provide good immunity. Recently, a variety of vectors have been used for H9 vaccine development, including fowlpox virus (FPV), fowl adenoviruses (FAdVs), Marek's disease virus (MDV) and NDV. In addition to viral vectors, *Lactobacillus* [108–111] and *Eimeria*

acervuline [112] are also used as vectors to prepare vaccines to prevent and control H9N2 avian influenza. However, overcoming maternal antibody interference is a major challenge.

Attenuated fowlpox virus (FPV) strains have been used as vaccines for decades to prevent wild-type virus infection. Recombinant FPVs have been developed and evaluated to prevent viral and mycoplasma infections in birds, and some have been licensed for their commercialization. Recombinant fowlpox virus (rFPV-HA) expressing the HA gene of H9N2 AIV-vaccinated groups could prevent virus shedding and replication in multiple organs in response to H9N2, and coexpression of IL18 enhanced the inhibition of viruses compared with the rFPV-HA-vaccinated group [81]. However, the efficacy of recombinant FPV-based vaccines can be affected by preexisting immunity [82].

Fowl adenoviruses (FAdVs), with a linear, 26–45 kb, double-stranded DNA molecule, can also be used as virus vectors. By inserting the nucleotide sequence encoding the VP2 protein of IBDV into rFAdV, rFAdV-VP2 was generated [83]. These authors evaluated the protection induced by rFAdV-VP2 in SPF chickens and found that rFAdV-VP2 vaccination induced good immune protection, suggesting its potential in the vector vaccine development of H9N2-subtype avian influenza virus. Currently, none of the recombinant FAdVs are being commercialized as vectored vaccines. Preexisting immunities hamper the application of FAdV vectors in vaccine development.

Marek's disease virus (MDV) belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, members of the *genus Mardivirus*, which are divided into three species, *gallid* herpesvirus 2, *gallid* herpesvirus 3 and *meleagrid* herpesvirus 1, formally named MDV serotype 1 (MDV-1), MDV serotype 2 (MDV-2) and MDV serotype 3 (MDV-3), respectively. Turkey herpesvirus (HVT), belonging to the meleagrid herpesvirus 1 family, has been extensively used as a vaccine against Marek's disease for over 40 years. Attenuated MDV-1 strains and HVT have several characteristics that make them appropriate for the development of recombinant vector-based vaccines for poultry diseases. The attenuated CVI988/Rispens MDV-1 strain has been used to express the S1 glycoprotein of IBV [113] and the VP2 of IBDV [114]. Coding sequences for protective antigens of IBDV [115], avian leukosis virus [116] and AIV [117] were also inserted into the genome of the avirulent 814 MDV-1 strain. The efficacy of the recombinant MDV-1 strain 814 expressing AIV-H5 glycoprotein (rMDV-H5) and an HVT expressing the same antigen (rHVT-H5) was compared by challenge with the virulent MDV-1 (J-1) and AIV (HPAI H5N1 A/Goose/HLJ/QFY/03) strains [117]. The results showed that protection against AIV in chickens vaccinated with rHVT-H5 and rMDV-H5 was 66.7% and 80%, respectively. MDV-1 was also used to express HA of H9N2 AIVs (MDV-H9). Chickens vaccinated with MDV-H9 induced less than 50% protection [84]. The vector vaccine candidate HVT-H9 could induce robust humoral and cellular immunity in chickens. In a challenge study, no chicken shed H9N2 virus from the oropharynx and cloaca, and no H9N2 virus was found in the viscera in the vaccination groups when challenged with homologous virus, suggesting that HVT-H9 provides effective protection against H9N2 AIV in chickens [85].

Recombinant HVTs (rHVTs) encoding proteins of infectious laryngotracheitis virus (ILTV), IBDV, AIV and NDV have been commercialized as dual vaccines to control MDV and each of those pathogens. Vaccines based on HVT or MDV can be produced on mass by in ovo inoculation of the embryos or a subcutaneous route in one-day-old chickens. As these viruses are cell-associated, that is, display cell-to-cell transmission, they are not susceptible to maternal antibodies. In addition, MDV and HVT persistently infect their host, inducing lifelong immunity. Thus, HVT or MDV will be an important virus vector for H9N2 vaccine development. To date, HVT-H5 has been used for more than ten years, but there is no avian-influenza vector vaccine based on MDV-1 on the market. Similarly, duck-enteritis virus (DEV) was also used as a virus vector to construct a vector vaccine for the prevention and control of H9N2 virus in ducks, and DEV-H9 vaccination completely prevented the oropharyngeal shedding of H9N2 AIV [118]. This vector vaccine delivery was mainly through subcutaneous injection in the neck, which could not induce robust local mucosal immunity in the respiratory tract or provide better immune protection for AIVs.

NDV belongs to the family *Paramyxovidae* and genus *Avulavirus*. Its genome is a non-segmented, single-stranded, negative-sense RNA. In the poultry industry, naturally attenuated NDV strains are widely used to control NDV. It has been over 20 years since NDV was first used as a vector [119]. NDV-based viral vectors expressing the influenza NA and HA glycoproteins [86,120] have been obtained and evaluated as immunogens for chickens. Recombinant NDV expressing H9 HA protects SPF chickens against heterologous avian influenza H9N2 virus challenge [86–89]. However, interference from maternally derived antibodies greatly hinders the clinical application of these vaccines. APMV-2 belongs to the same genus as NDV, distantly related to NDV in the phylogenetic tree, based on the sequences of the fusion (F) and hemagglutinin-neuraminidase (HN) genes, and has low cross-reactivity with anti-NDV antisera. APMV-H9 conferred complete immune protection to prevent viral shedding in oropharyngeal and cloacal swabs from chickens challenged with H9N2 virus [90].

4.3. Live-Attenuated Vaccine

Live-attenuated AIV vaccines have been demonstrated to provide cross-protection against different influenza viruses (Table 2) [71]. In addition to inducing strong humoral immunity, it also elicited robust cellular immunity and mucosal immunity. Live-attenuated vaccines for humans are considered better than inactivated vaccines [121,122]. In poultry, attenuated cold-adapted live H9N2-subtype AIV vaccine strains have been shown to provide better protective efficacy [91–93]. Therefore, a live-attenuated vaccine has more advantages than an inactivated vaccine. However, there has been concern about the risks associated with reassortment events between vaccine strains and circulating wild-type viruses.

In recent years, many new technologies have been applied to develop novel live-attenuated vaccines. NS1 is the main viral protein responsible for counteracting the antiviral response, and it acts as an interferon (IFN) antagonist to suppress type-I IFN production while promoting viral replication [123]. Hence, recombinant influenza viruses with modified, truncated or absent NS1 are likely to be reasonable alternatives to generate live-attenuated influenza viruses, since they are attenuated in IFN-competent hosts [124]. Live-attenuated H9N2 vaccine produced by truncating the NS1 gene could also protect chickens against homologous and heterologous H9N2 AIV challenge [94,95]. In addition, reorganized PR8 viruses were constructed by splitting the overlapping open-reading frames of M1 and M2. Importantly, PR8 viruses that contained the M-split segment were highly attenuated in vivo, and they protected mice from a lethal homologous challenge with WT PR8 [125].

Codon-pair bias refers to the fact that some pairs of codons occur more frequently than others, and this frequency differs between species [126]. Using codon combinations that are less represented in the genetic code of the host could alter the expression of viral proteins and affect viral spread, thus generating live-attenuated influenza virus [127,128]. Synthetic attenuated-virus engineering was used to recode and synthesize the viral genome of PR8 [127] and A/California/7/2009 pandemic H1N1 (pH1N1) viruses [129] in a way that preserved the WT amino acid sequence but created a suboptimal arrangement of codon pairs. There were no significant differences in the growth kinetics or plaque phenotype of mutant deoptimized viruses compared to wild-type virus. In vivo analysis showed that the deoptimized viruses were remarkably attenuated in mice. Based on the premature termination codon (PTC), live-attenuated influenza viruses were also generated. PTC-harboring viruses exerted full infectivity but could not replicate in conventional cells. Vaccination with PTC viruses elicited robust humoral, mucosal, and T-cell-mediated immunity against antigenically distinct influenza viruses and even neutralized existing infecting strains [96]. In addition, single-cycle infectious IAVs can be generated by the mutation, deletion or substitution of viral components using molecular biology techniques. These viruses can be defective in viral genome synthesis, assembly or the release of viral particles and thus lack the ability to spread after initial infection [97]. A PR8 virus without the PB2 gene was

shown to be safe in mice, and it was also immunogenic and protected mice from lethal challenge with PR8 [98], suggesting that this platform can be used to develop both monovalent and/or bivalent vaccines against influenza strains or different respiratory pathogens.

4.4. DNA and mRNA Vaccines

DNA and mRNA vaccines can use target sequences of clinical isolates as soon as they are available (Table 2). DNA or mRNA vaccines are also an effective vehicle for universal influenza vaccines. Based on highly conserved T-cell epitope studies, multiepitope DNA vaccines for the NP and M genes have been reported to be effective in protecting against multiple subtypes of influenza viruses in mice [130].

In combination with improved adjuvants to enhance humoral immune responses [131,132], DNA vaccines have been shown to be highly immunogenic and efficacious in poultry [99,133]. Chickens immunized with a DNA vaccine developed high levels of HI and NT antibodies and were completely protected from lethal H5 virus challenge. Importantly, the tri-clade DNA vaccine encoding HAs of clades 0, 2.3.2.1 and 7.2 elicited broadly neutralizing antibody responses against all H5 clades and subclades and protected mice against high-lethal-dose heterologous H5N1 challenge [133]. For chickens immunized with the H9N2 DNA vaccine, T lymphocytes were activated and proliferated, the numbers of CD3+, CD4+ and CD4+/CD8+ cells increased, and the chickens were completely protected against H9N2 AIV challenge [99]. Recently, DNA vaccines have been approved for the prevention of avian H5N1 in the USA and China.

mRNA-based vaccine platforms have been used to develop vaccines against infectious diseases, such as respiratory syncytial virus (RSV) [134], Zika virus [135], SARS-CoV-2 [136], Ebola virus [137] and HIV [138]. Importantly, mRNA-based vaccines have been licensed for commercial use against SARS-CoV-2. mRNA influenza vaccine constructs encoding the hemagglutinin of the H10N8 and H7N9 influenza strains formulated in a lipid nanoparticle delivery system induced HI and microneutralization titers when inoculated intramuscularly, although a significant T-cell response to vaccination was not found [100]. Although DNA or mRNA vaccines offer advantages, setbacks, including the inability to induce strong immunity and the fact that they are not currently applicable for mass vaccination, impede their use in the poultry industry.

4.5. Virus-like Particle Vaccine

Virus-like particles (VLPs) have a similar morphology to natural viruses but they lack any pathogenicity or infectivity (Table 2). With highly ordered epitope repeats, VLPs have excellent immunogenicity and can induce strong cellular and humoral immune responses [139]. VLP vaccines have mainly employed baculovirus-insect cell systems for production. Importantly, VLPs can avoid the biosafety threat caused by live viruses during the process of vaccine production. In addition, they can greatly reduce the costs due to the improvement of the production process. Experimentally, many different AIV VLP vaccines have been developed and shown to be highly immunogenic in chickens, including an H9N2 VLP [101], an H5N1 VLP [140], an H6N1 VLP [141] and an H7N9 VLP [142]. A single injection of the VLP vaccine induced high levels of HI antibodies and lowered the frequencies of virus isolation after wild-type virus challenge. VLPs are also widely used to develop universal vaccines. Triple-subtype VLPs that colocalized H5, H7 and H9 antigens derived from H5N1, H7N3 and H9N2 viruses were prepared and provided complete protection for H5N2 and H7N3 HPAIVs. The immune response was also detectable after challenge with H9N2 LPAIV [102,103]. The HA stalk and M2e are two potentially effective broad-spectrum immunogens against influenza, and displaying either on the surface using VLP technology can fulfill their protective potential as a universal vaccine against avian influenza viruses [143,144].

4.6. Recombinant Protein Vaccine

Since recombinant protein vaccines are nonreplicating and lack any of the infectious components, they are considered a safer approach than vaccines derived from live viruses (Table 2). Baculovirus-insect cell systems or *E. coli* expression systems have been widely used to produce recombinant protein vaccines, which greatly reduces the production cost. However, a weak cellular immune response and no mucosal immune response prevent them from providing good protection. A recombinant protein vaccine can enhance the protective effect by mixing the main immune antigens with proteins that stimulate immune cells. The complete HA protein of the H5N2 virus was chemically conjugated to an anti-chicken Dec205 monoclonal antibody, and a single dose of this vaccine was shown to be sufficient to elicit a strong antibody response in chickens as early as fourteen days after initial immunization [104]. Furthermore, targeting a synthetic peptide antigen to the chicken CD40 receptor showed accelerated and enhanced antibody responses against the peptide antigen compared to untargeted peptide [145].

In addition, pattern recognition receptors targeting recombinant vaccines have also been investigated in chickens. An H7HA influenza subunit vaccine recombinantly fused to *Salmonella typhimurium* flagellin (H7HA-fliC) was generated. The immunization of chickens with H7HA-fliC showed robust antibody responses leading to a significant reduction in viral loads compared to the chickens receiving only H7HA [146]. Similarly, recombinant H9HA1-fliC enhances adherence to respiratory epithelial cells and promotes superior protective immune responses against H9N2 influenza virus in chickens [105]. CD83 is thought to play important roles during interactions between cells of the immune system and in B-cell function for antibody production in response to influenza A virus infection [106]. Recently, the H9N2 avian influenza virus hemagglutinin (HA) antigen was targeted by fusing it to single-chain fragment-variable (scFv) antibodies specific to the CD83 receptor expressed on chicken APCs. Following this, the vaccine-induced cellular and humoral immunity in chickens was compared to untargeted H9HA. Chickens vaccinated with CD83 scFv-targeted H9HA showed reduced mortality from an H9N2 challenge virus [106]. It can induce strong humoral immunity due to the high concentration of antigen. In addition, conserved proteins or conserved fragments of functional proteins have been used to develop universal vaccines. M2e is poorly immunogenic because of its small size, but its protective effect can be greatly enhanced by embedding it with antibodies that target specific immune cells, resulting in a universal vaccine [147,148]. To improve its immunogenicity while maintaining its original stability, chimeric HA and hyperglycosylated HA concepts have been applied to induce targeted HA-stalk immunity. This modified HA protein achieves a wide range of protective effects against at least one subtype [149–151].

5. Conclusions and Perspective

Currently, vaccination is still one of the principal strategies to control H9N2 avian influenza in China. However, a variety of antigenic H9N2 viruses are prevalent in China, and the vaccine development speed lags behind the speed of virus mutation, leading to a challenge for effective vaccination. Therefore, the development of a universal vaccine with broad-spectrum neutralizing activity is of great significance for the control of H9N2. Inactivated vaccine immunization can effectively reduce the clinical symptoms after H9N2 virus infection, greatly reducing economic losses. However, another challenge is that immunization with IWV cannot block H9N2-AIV reinfection or virus shedding. Live-attenuated vaccines can provide comprehensive immune protection, mainly because induced mucosal immunity plays an important role, indicating that vaccine-induced local mucosal immune responses, especially tissue resident T lymphocytes, play an important role in influenza virus immune protection in humans and mice. Many vaccines designed based on the mucosal immune response induce good mucosal immune effects and provide good immune protection in humans [152–154]. It is necessary to develop novel vaccines, especially those that can induce cellular immunity and local mucosal immunity, to control the prevalence of H9N2 AIV. In China, the traditional livestock-raising systems, including free-ranging

and polyculture, continue to maintain their vital status. However, the main development direction of the Chinese poultry industry is moving toward intensive confinement and feeding. In addition to enhancing the mucosal immune response, improving the immune response to H9N2 in double or multiple combined vaccines also needs to be considered along with simplifying the immune procedure to reach an ideal goal of "one injection preventing multiple diseases". To date, a large number of genetically engineered avian influenza vaccines have been designed in China. However, most of them have not been applied clinically. Developing multivalent live-vector vaccines or improving the protective effect of H9N2-multivalent inactivated vaccines will significantly contribute to the prevention and control of H9N2.

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REVIEW

REVISED Emerging threats and vaccination strategies of H9N2 viruses in poultry in Indonesia: A review [version 2; peer review: 2 approved]

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Abstract

Avian influenza virus subtype H9N2 was first documented in Indonesia in 2017. It has become prevalent in chickens in many provinces of Indonesia as a result of reassortment in live bird markets. Low pathogenic avian influenza subtype H9N2 virus-infected poultry provides a new direction for the influenza virus. According to the latest research, the Indonesian H9N2 viruses may have developed through antigenic drift into a new genotype, posing a significant hazard to poultry and public health. The latest proof of interspecies transmission proposes that the next human pandemic variant will be the avian influenza virus subtype H9N2. Manipulation and elimination of H9N2 viruses in Indonesia, constant surveillance of viral mutation, and vaccine updates are required to achieve effectiveness. The current review examines should be investigates/assesses/report on the development and evolution of newly identified H9N2 viruses in Indonesia and their vaccination strategy.

Keywords

avian influenza, public health, emergence, vaccination, Indonesia

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Any reports and responses or comments on the article can be found at the end of the article.

This article is included in the Emerging Diseases and Outbreaks gateway.

This article is included in the Pathogens gateway.

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REVISED **Amendments from Version 1**

We have changed the title from threat to threats to further emphasized the generality of our findings. We slightly modify the intro to insist the breadth of our work and add 4 lines for more Justification of the study. We have corrected the minor typos, and grammar and we want to thanks the reviewers for their valuable comments which improved the quality of manuscript. We have added the important risk factors of AIV.

Any further responses from the reviewers can be found at the end of the article

Introduction

The avian influenza virus subtype H9N2 is a LPAIV widely circulated in Asian poultry.[1] In the future, the LPAI H9N2 virus-like H5N1 could pose a serious zoonotic threat[2] because they have been isolated from backyard and wild bird species. It was discovered in a variety of avian species throughout Eurasia, the poultry industry has suffered significant financial losses as a result of this.[3] The H9N2 virus has gained much attention due to its rapid dispersion between native birds.[4] This low pathogenic virus survives in chicks and transmit to unaffected birds via the fecal-oral routes despite causing extreme clinical signs.[5] The avian influenza virus subtype H9N2 cause severe respiratory illness in immuno-compromised chickens. It causes an increase in early chick mortality as well as a considerable decline in egg production in laying chickens, resulting in financial loss.[6] When this virus is co-infected with other pathogens, the intensity of clinical symptoms, death rates, and viral replication can increase.[7,8] Based on their genetic and antigenic properties H9N2 viruses prevalent in Asia have been classified into three genotypes: A/Quail/Hong Kong/G1/97-like (G1-like); the Y280 lineage, represented by A/Chicken/Hong Kong/Y280/97-like (Y280-like); and the Korean lineage, represented by A/Chicken/Korea/38349-p96323/96 (Korean-like).[9] The G1 prototype virus (A/Quail/Hong Kong/G1/97) is common in southern Chinese quail. It may have been the source of internal genes for the highly pathogenic avian influenza (HPAI) subtype H5N1 that hit Hong Kong in 1997. H9N2 viruses with G1 lineages have been found in field epidemics of influenza in poultry in the Middle East and the Indian subcontinent since 1997. Since the early 1990s, H9N2 has evolved to create a more diversified genotype in grassland poultry birds by acquiring gene fragments from other viruses. The genomes of newly isolated avian influenza (H9N2) viruses showed significant genetic recombination in HPAI viruses.[10–12]

A novel H9N2 genotype, expressed by A/chicken/West Java/BBLitvet-RI/2017, A/chicken/East Java/Spg147/2018, A/chicken/East Java/BLi25Ut/2018, and A/chicken/Central Java/SLO.105/2018 was isolated from Indonesian poultry birds, and these replaced by Y280 or G1 Lineage.[13,14] Inter- and Intra-subtype genotype genomic recombination between LPAIV subtype H9N2 (G1-like), HPAIV subtype H5N1 (clade 2.2), and H7N9 viruses resulted in these novel reassortants (Figure 1). A novel H9N2 genotype in Indonesia represented 98% sequence identity with that of (A/Muscovy duck/Vietnam/LBM719/2014(H9N2) was isolated from chicken in a study conducted by Melina Jonas.[15] Co-circulation of the LPAI virus subtype H9N2 has been reported in Egypt with H5N1 since 2011 infecting the same hosts. Subsequently, H9N2 has established an endemic status in the poultry sector. Human infections with both H7N9 and H10N8 viruses highlighted that H9N2 has an emerging state of the new human infecting virus.[16]

In Indonesia, the circulation of the H9N2 and H5N1 viruses and the possibility of reassortment between the two viruses have resulted in various virus control situations.[17] The LP avian influenza subtype H9N2 virus raises a public health risk. It has human-like receptor specificity[2,18,19] that might surpass the species barrier.[20,21]

In 1999, the LPAIV subtype H9N2 was first discovered in a human patient in Hong Kong.[22,23] This discovery raises concerns about the H9N2 pandemic potential alongside the H5N1 virus.[24,25] The recent isolation of AI H9N2 from a patient in Bangladesh and poultry workers in China has heightened public health concerns about LP avian influenza.[26–28] Bangladesh, Pakistan, and Egypt have all reported further cases.[29–31] Even though low pathogenic avian influenza H9N2 viruses could harm humans, the significance of low pathogenic H9N2 viruses has been surpassed by HPAI H5N1 viruses.[32] A further indication of the significance of the H9N2 subtype of the low pathogenic avian influenza virus is discovering of two other subtypes (H10N8 and H7N9) with internal genomes comparable to those of H9N2.[33] In the Western Pacific Region, 72 cases of avian influenza A(H9N2) infection have been reported to WHO since December 2015, including two deaths (both due to underlying diseases).[34]

Oil-based inactivated H9N2 LPAI vaccines were used in the poultry sector in many countries to avoid H9N2 infection owing to the extensive essence of H9N2 viruses and their zoonotic potential.[7,35–37] However, because the nature of HA antigenic epitopes is constantly changing, influenza vaccines must be updated each year to make sure strain-specific immunity, posing a significantly challenging task to vaccine manufacturers. As a result, a global flu vaccine with broad protection against conserved influenza protein regions is required.

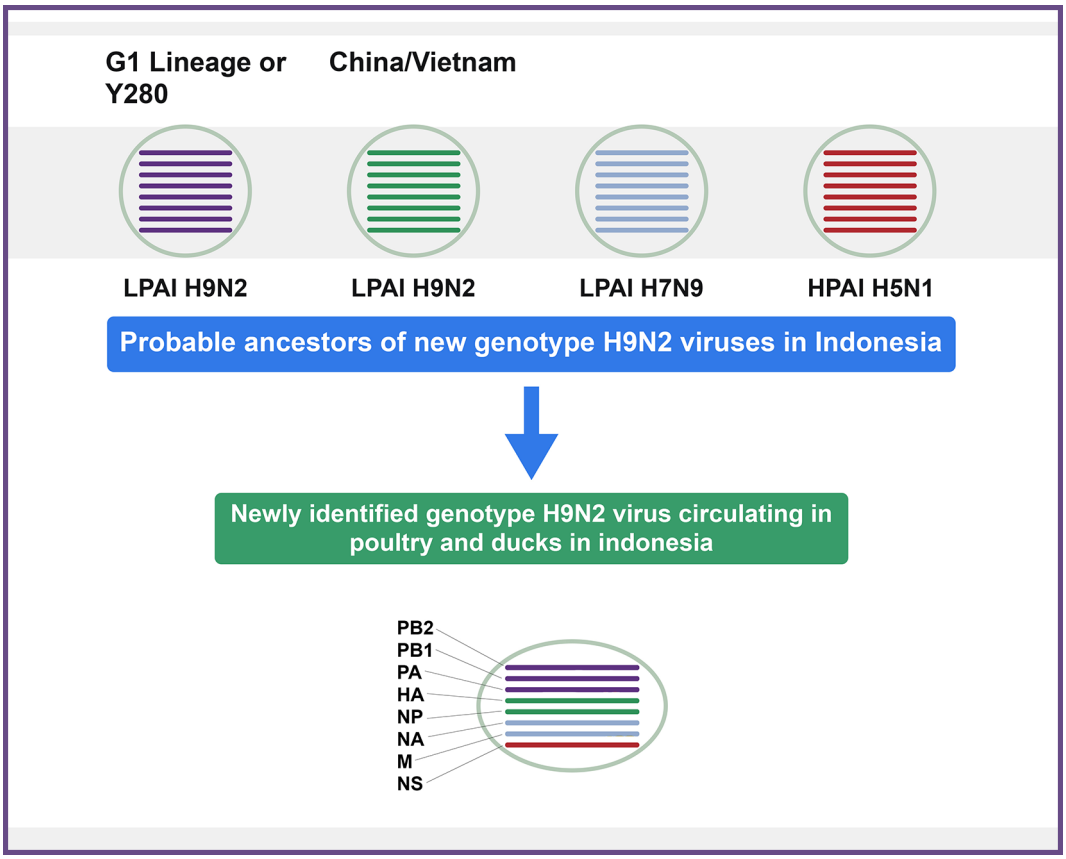

Figure 1. Representation of recently emerged H9N2 virus genotypes in poultry in Indonesia.[86]

The poultry industry, as well as public health, are both affected by the avian influenza virus. This is a huge issue all around the world. Keeping that in view, this study was designed to investigate emerging threats and vaccination strategies for H9N2 viruses in poultry in Indonesia.

In the Indonesian poultry industry, this review addresses critical issues concerning the evolution of AI viruses and vaccination strategies. Vaccination against the LPAI H9N2 virus is also discussed, including recent advances and challenges.

The emergence and evolution of the LPAI H9N2 virus

A brief history of avian influenza in Indonesia

To date, the poultry industry in Indonesia has faced a serious threat from highly pathogenic avian influenza (HPAI). The H5N1 virus has rapidly spread across most provinces since its initial report in 2003–2004, eventually subsiding by the end of 2007 after killing over 16 million chickens.[38,39] A second epidemic was recorded in Gorontalo in April 2011, leaving only one province disease-free.[40] A phylogenetic assessment of the Indonesian 2.1. clade virus revealed a direct relationship to viruses of genotype Z discovered in Hunan Province, China, in 2002, indicating that they were likely introduced together. However, the propagation and transmission of the virus from Hunan to Indonesia remained unknown.[41,42] All Indonesian H5N1 viruses were categorized as clade 2.1 up until 2008, with three virus sub-lineages: 2.1.1, 2.1.2, and 2.1.3. During the outbreaks between 2003 and 2005, the viruses of clade 2.1.1 were mostly isolated from HPAI-infected poultry. Clade 2.1.2 viruses with avian and human origins were primarily detected in Sumatra between 2004 and 2007, whereas clade 2.1.3 viruses were detected in 2004 and isolated from either birds or humans. Surprisingly, when clade 2.1.3 viruses became more prevalent, the number of clade 2.1.1 and 2.1.2 isolates began to fall. Even though 2.1.3 viruses have spread throughout Indonesia and grown endemic in several areas, a new sub-lineage virus has arisen since 2004. In September 2012, AIV H5 subtype mortality was detected at several duck farms in Central Java. The HA genes of the duck's isolates did not match those of long-established Indonesian clade 2.1 isolates, but they were surprisingly comparable to clade 2.3.2.1 viruses found lately in Vietnam, China, and Hong Kong.[43] Although Bali is thought to be an excellent environment for influenza re-assortment because of its world-renowned tourism destination,

suckling pigs, and fighting cocks' history, until 2017, the island had reported only one human death from avian influenza. Between 2009 and 2011, surveillance of AI (H5N1) viruses in Bali revealed that the circulating A(H5N1) viruses belonged to clade 2.1.[44–46]

Avian influenza subtype H9N2

In early 2017, I Ketut Diarmita, Director General of Livestock and Animal Health at the Ministry of Agriculture, Indonesia announced that the newly emerging AIV subtype H9N2 was discovered during surveillance by the Ministry of Agriculture' Veterinary Center in South Sulawesi, West Java, Bali, Central Java, and Yogyakarta. As a result of these incidents, the egg supply has decreased by the end of 2017.[47]

According to Drh. Ni Made Ria Isriyanti, Ph.D., Head of Sub-Supervision of Veterinary Medicine, Directorate General of Livestock and Animal Health, Indonesia, the current state of the H9 virus is its proliferation in several provinces in Indonesia, including Java, Sumatera, Kalimantan, Sulawesi, and Bali. The number of H9N2 positive samples amounted to 49. Infected chickens are typically 30–60 weeks old. Although mortality is normally modest, one indication of the H9 virus is a decrease in egg production of up to 40–60% of normal, resulting in significant economic losses for farmers.[48] The LPAI virus subtype H9N2 has been circulating in poultry and ducks in Indonesia, causing significant financial losses. It was also happening because of high mortality and decreased production, particularly in broiler and layer chickens.[15] Since 2003, the HPAIV subtype H5N1 has been found in Indonesia,[49] with human cases resulting from H5N1 viruses being transmitted cross-species.

A study conducted by Muflihanah *et al.* (2017) in Sidrap Regency, South Sulawesi found that the occurrence of AIV disease occurs within 3–14 days, with an average mortality rate of less than 5% and a 50–80 percent decline in egg production. The genetic similarity of three isolates A/Chicken/Sidrap/07161511-1/2016, A/Chicken/Sidrap/07161511-61/2016, and A/Chicken/Sidrap/07170094-44OA/2017 is 98 percent H9N2. The phylogenetic tree results suggest that the tested sample appears to be from the Asian group or lineage Y280-H9N2.[50]

In another study conducted by Nugroho *et al.* (2018) in layer chicken on Java island, 13 of the 33 virus isolates were VAI subtype H9N2 and belonged to the Y280 lineage, clade h9.4.2.5, and had a genetic closeness with Chinese isolates in 2013 and Vietnam in 2014, with a nucleotide homology percentage of 96.9 percent–98.8 percent.[51]

According to a study conducted by Wibawa H, *et al.* (2020) phylogenetic analysis of the H9N2 virus HA9 gene (Bt/1291-OP/16) was found to be part of the China-Vietnam-Indonesia linage (CVI lineage).[52]

It indicated a close relationship with H9N2 viruses prevalent in China and Vietnam. That is why it was classified with the H9N2 viruses of the China Vietnam-Indonesia (CVI) lineage. Vietnam H9N2 viruses (H7F-LC4-51/14, H7F-LC4-26/14, and H7F 14 BN4 423/14) had already been recognized as members of the Y280-like group.[53] The probable transmission paths of the AIV subtype H9N2 from Hong Kong to Indonesia (Figure 2).

Live and Wet bird markets play an essential role in the ecology of HPAI subtype H5N1 and LPAI subtype H9N2 in Indonesia (Figure 3) and are a critical factor in the disease prevalence and endemicity.[54] The co-circulation of H5N1 and H9N2 viruses in poultry farming and live bird markets have raised the danger of human infection, complicating the epidemiological picture and heightening fears of a new influenza A virus pandemic.[55]

In Indonesia, primarily poultry (layer, backyard, and broiler) and duck are raised in conventional methods on small outdoor farms with poor management and are primarily sold through Wet and LBMs. Ducks, commercial and domestic poultry, pigeons, starlings, quails, and other species of fancy birds are among the avian species found in Wet and LBMs.[56,57]

According to Joerg Henning *et al.*, live bird markets in Indonesia are critical for the prevalence and endemicity of AIV. A total of 22 risk factors potentially influencing HPAI H5 virus prevalence were identified from survey data, including chicken cages, stacking systems, display table materials, and slaughter surfaces. Other risk factors were the density of poultry, human density, environmental factors, road density, percentage of paddy field, and percentage of water sources had a statistically significant relationship with the AIV prevalence.[54]

Some farmers have begun to grow chickens and ducks in semi-intensive or intense ways. In Indonesia, conventional farming involves herding ducks and poultry onto open rice fields after harvest to consume leftover rice, other grains, and insects.[58] H9N2 and high pathogenic avian influenza focused on continuing avian influenza surveillance. The subtype H5N1 was found in commercial chicken farms and backyard chickens traded in LBMs.[15] This previously confirmed

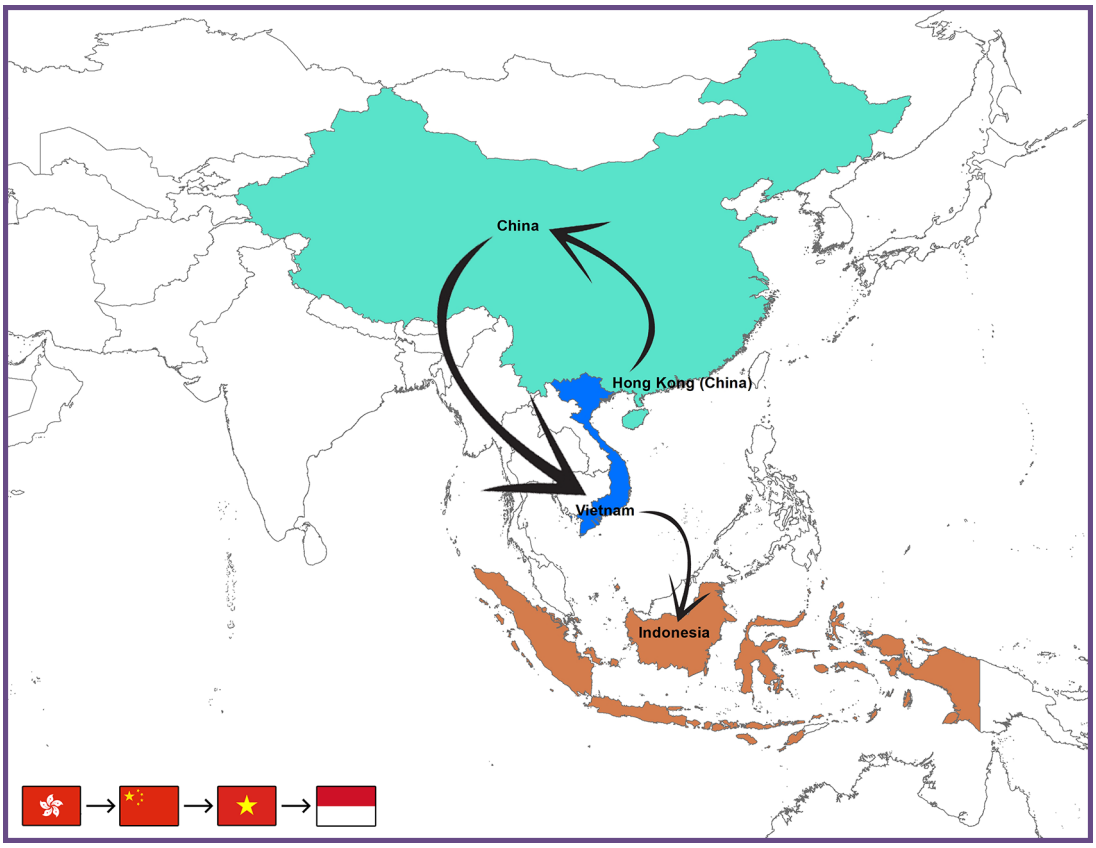

Figure 2. **Map depicts the proposed and hypothesized pathways of the avian influenza H9N2 virus taken from Hong Kong to Indonesia.**

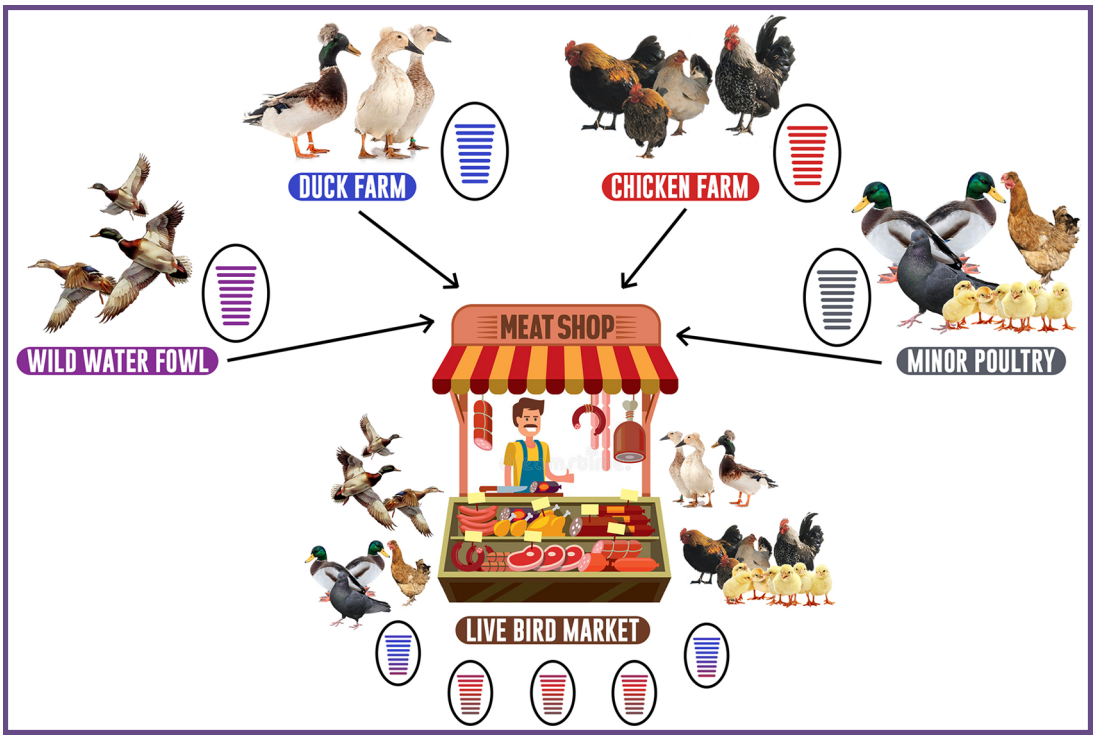

Figure 3. **Role of live bird markets assisting in the evolution of LPAI virus H9N2 in Indonesia.**

findings reported that the trade of poultry, ducks, and other birds in live bird markets (LBMs) played a crucial role in discovering a new AIV.[59,60] According to research, H9N2 viruses may operate as "new ventures" or "implementers" for human-infecting wild-bird influenza viruses (H7N9, H10N8).[61,62]

Moreover, any reassortment of LPAI H9N2 viruses with high pathogenic avian influenza viruses may result in a more remarkable ability to cause human infection.[63] Together with the tropical temperature in this region, these features allow long-term survival, multiplication, and spread among various chicken species, as well as transfer from chickens to humans. These variables also provide enough possibilities for existing influenza viruses, such as H9N2 and H5N1, to rejoin and form newer viruses with different host specificity. In Indonesia' wet and live bird markets, the broad co-circulation of H9N2, H7N9, and high pathogenic H5N1 acts as a perfect mixing vessel for forming novel influenza subtypes. It is making the country a hotspot for the AI epidemic. Comprehensive vaccination programs have been implemented to mitigate the effects of H5, particularly newly emerging H9 subtype viruses spreading in Indonesia.[14]

Prospects for AI vaccination in the future

In Indonesia, vaccination is one of the most effective ways to combat the spread of avian influenza (AI) viruses. The vaccine master seed used in the field must be updated to keep up with the variety of circulating viruses and their potential to change. A vaccination strain (LPAI H9N2) virus isolated in 2017 (A/chicken/West Java/BBLitvet-RI/2017) vaccine (Patent IDP000056903)[64] and BLi25Ut/18 virus were chosen in Indonesia based on their pathogenic, antigenic, and genetic features. Inactivated bivalent and monovalent H9N2 influenza vaccinations can induce an antibody response. It can lower mortality and virus shedding caused by reassortant H9N2 virus infection.[17] With the help of FAO/OFFLU, the Indonesian government has built an effective vaccination strategy against H5N1 and H9N2 strains. Influenza Virus Monitoring (IVM online) is a web-based animal health laboratory system. This system manages antigenic and genomic data for circulating HPAI and LPAI viruses in Indonesia.[65] Animal Disease Investigation Centers (DICs), private companies, and universities collaborate to monitor and collect isolates. The data is then submitted to IVM Online, which provides an up-to-date map of circulating HPAI and LPAI viruses throughout Indonesia, allowing the optimal AI vaccine to be prescribed. In backyard farms, HPAI vaccines are commonly used to prevent LPAI using homologous (H5N1) or mixed with H9N2 strains.

In Indonesia, oil-based inactivated bivalent and monovalent vaccinations produce detectable antibody titers for all structural proteins, especially nucleoprotein and matrix protein. Antigens for antibody testing can be one or both of these proteins. As a result, using this method, vaccinated birds cannot be discriminated from naturally sick birds. The inability to conduct surveillance has been a critical impediment to vaccination to combat avian influenza. There has been a lot of effort put into matching the vaccination to the field variations. This is partly because immunization with any H9 virus appears to protect against clinical illness from a low pathogenic avian influenza exposure of the same subtype, irrespective of genetic variations. Oil-based inactivated bivalent and monovalent vaccinations produce many serum antibodies. The heterogeneity between vaccine and field strain can be estimated by comparing genomic information in the HA gene. However, when the vaccine is utilized as a control tool, both clinical safety and virus replication are concerns. According to experimental research, the closer the vaccine is to the field strain, the less virus is released in exposed birds.[37] Genetic variation is a significant issue with avian influenza vaccines, as it reduces immunization effectiveness. Antigenic drift is considered to unfold when the field virus changes in response to the host's antibodies. This method could be owing to vaccination or natural infection. However, in any scenario, the virus is under evolutionary changes to elude the body's immunity, allowing multiplication at more significant titers in the host. There is a higher probability that a strain of the virus will spread to new hosts if the proliferative phase is better managed.

Virus detection has decreased in Indonesia following vaccination programs against HPAI H5N1, showing that HPAI H5N1 is now under control. While the LPAI H9N2 virus is a new subtype, recent research has shown that monovalent and bivalent vaccines can protect chickens against reassortants H9N2 virus infections. It could lower mortality and virus shedding in chickens.[17] Active surveillance of chicken farms and live bird markets is essential for further identifying new variants of the LPAI H9N2 virus in Indonesia. In order to prevent future epidemics, suitable vaccine seed viruses should be evaluated. Differentiation Infection in Vaccinated Animals (DIVA), a vaccine strategy, could be useful in assuring trading partners of the safety of poultry and poultry products. It has enhanced surveillance to detect virus infections.[66] West Java has tested a proposed DIVA technique involving sentinel chickens.[67,68] In Indonesia, the DIVA approach has not been widely accepted. Several different ways of employing viral protein as a marker in chickens, such as HA2,[69] NS1,[70] and M2e[71] have been developed. New prospects for developing novel concept vaccines arise due to better molecular virology and the accessibility of genetic data on avian influenza. VLPs (Virus-like particles) have been proposed as a new generation of non-egg-based vaccinations with potential safety profiles for some viral illnesses.[72–74] VLP is structurally and morphologically similar to infectious virus particles. Various antigenic epitopes are particularly effective, owing to their ability to induce a wide spectrum of immune responses in the host.[75,76] Insect or mammalian cells

can easily create virus-like particles (VLP) vaccines incorporating influenza hemagglutinin (HA) and neuraminidase (NA) antigens by expressing HA and NA proteins together with a viral core protein, such as influenza M1.

The majority of influenza VLPs were created using viral nucleic acid expression methods. Their safety and immunogenicity were tested in various animal models.[76,77] The H5N3 avian influenza virus-like particles (VLP) vaccine was studied in ducks. This study has demonstrated that the VLP vaccination may be administered safely in poultry.[78] In a specified pathogen-free (SPF) chicken model, a VLP vaccination including the HA and M1 proteins was designed and tested against H9N2 LPAIV.[32,35] The pure VLP protein solution can be emulsified with Montanide ISA70 oil adjuvant (Seppic, Paris, France) to make a VLP vaccine. A single dose of H9 VLP vaccination resulted in significant antibody titers and reduced expulsion and release of virus progeny from the respiratory and gastrointestinal tracts in chickens. Furthermore, it enabled ELISA-based discrimination of avian influenza-infected poultry from vaccinated poultry utilizing a nucleocapsid antigen, availed DIVA approach.[79] On the other hand, vaccination cost is regarded to be a major factor influencing the efficacy of synthetic subunit vaccines, such as VLP for poultry. Two subunits make up the influenza virus haemagglutinin (HA). The current influenza vaccine largely produces antibodies against the HA1 component, which is continually developing unexpectedly. The other component, HA2, is more stable, but the HA head region protects it. As a result, increasing the immunological response to HA2 may elicit broadly inhibiting antibodies.[80,81] For the activation of protecting immune responses against infectious diseases, DNA vaccination has emerged as a viable alternative to standard protein-based vaccines. DNA vaccines have many advantages over traditional vaccinations, including greater stability, quick and low-cost manufacture, and the capacity to create vaccines for a broad range of infectious diseases. After being inoculated directly into mouse muscle for the first time in 1990, it was discovered that plasmid DNA vaccines could be made for the first time.[81–83] These DNA vaccines are capable of encoding a chimeric DNA molecule of numerous antigenic sequences, which decreases production time and costs when compared to the traditional vaccinations we now use, without carrying the illnesses related to live attenuated vaccines. These vaccines based on plasmids can trigger both immune responses (humoral and cellular) while expressing high amounts of proteins of interest in cells. They can also neutralize antibodies produced by the mother.[84,85]

Conclusion

In Indonesia, the avian influenza subtype H5N1 is still endemic. In 2017, the newly developing subtype LPAI H9N2 was reported for the first time in Indonesia, on the island of Java. According to a previous study, H9N2 viruses have experienced significant genetic reassortment in recent years, resulting in novel genotypes of H9N2 viruses in Indonesia. H9N2 virus genotypes that have recently emerged could play a vital role in the disease transmission in poultry and ducks. To detect future evolution and potential adaption of the LPAI H9N2 virus to humans and other mammalian species, active surveillance of these viruses is required in Indonesia. The widespread use of AI vaccinations in populations of animals may raise immunological selection pressure and mutation rates, which can lead to fast antigenic drift at antigenic locations. Better vaccination procedures and regular updating of vaccine seed variants are needed to boost immunogenicity and protective efficacy on poultry and duck farms. These techniques might be involved in selecting highly immunogenic vaccine seed strains, using efficient adjuvants for chickens and ducks, and utilizing innovative technology. In Indonesia, the co-circulation of H9N2 and H5N1 viruses in the field and live bird markets will increase the chances of gene reassortment between the viruses. Continued intensive monitoring of chicken farms and live bird markets for new variant low pathogenic H9N2 viruses and investigation of relevant vaccine seed viruses should be explored for future prevention. In Indonesia, inactivated bivalent and monovalent vaccinations have been utilized, and numerous new technology vaccines have been proposed to create low-cost, high-immunogenic vaccines. Together with efficient adjuvants, these novel vaccinations will undoubtedly lead to improved immunity against low pathogenic avian influenza subtype H9N2. In Indonesia, vaccination must be included in a complete, integrated disease-control strategy. National monitoring must be maintained at all times, as well as agricultural biosecurity and the DIVA strategy. In Indonesia, the eradication of these viruses could only be accomplished if all components of the control are implemented.

Data availability

No data are associated with this article.

Acknowledgments

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Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 04 July 2022

https://doi.org/10.5256/f1000research.135273.r142576

✔ **Asghar Abbas**
Faculty of Veterinary and Animal Sciences, Muhammad Nawaz Shareef University of Agriculture, Multan, Pakistan

I have reviewed the revised version of the manuscript, come to conclusion that authors have addressed my previous minor comments, and that it contains valuable information on Avian Influenza subtype H9N2. I believe the article is acceptable for indexing in its present form. Thanks.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Pathobiology Research

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 01 July 2022

https://doi.org/10.5256/f1000research.135273.r142575

✔ **Agumah Nnabuife Bernard**
Department of Applied Microbiology, Faculty of Science, Ebonyi State University, Abakaliki, Nigeria

I have no further comments to make. I affirm my earlier stance on approving the article for publication.
Thank you

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: I am a medical Microbiologist with specialties in Fungi of both medical and veterinary importance. I am also a certified Medical Laboratory Scientist with specialties in Medical Parasitology and entomology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 16 June 2022

https://doi.org/10.5256/f1000research.130513.r138445

Agumah Nnabuife Bernard
Department of Applied Microbiology, Faculty of Science, Ebonyi State University, Abakaliki, Nigeria

I have gone through the review article. The author did wonderfully well as the facts and figures were aptly captured with most categorical statements backed up with appropriate citations.

The author did well in:

1. Capturing the history and the first documentation of the H9N2 Avian Influenza in Indonesia.
2. Noting the fact that the contemporary low pathogenic nature exhibited by the ethiologic agent and its prospects of becoming highly pathogenic in the nearest future. This is also a good headway.
3. It is worthy of note that the author threw good light to the prospects of antigenic drift and zoonotic transmission.
4. Though only the Hongkong and the Korean genotypes were explained by way of history; the author was able to fully highlight how Indonesia currently has a very high distribution of H9N2 in Indonesian poultry Industry.
5. It is also worthy of note that the author highlighted how the conventional methods used in raising birds has contributed in the endemicity of the H9N2 Avian Influenza in Indonesia. Poor management and sales in wet and live birds market are well cited as contributing factors in the spread of Avian influenza.
6. The author was able to highlight various subtypes especially the H5N1 which caused the Bird flu pandemic that lasted from 2003-2007.
7. The most novel information from the authors review is that it is highly likely that the next form of Influenza to be contracted by humans in Indonesia is the H9N2 Avian influenza. Hence adequate surveillance and analysis of the genetic flexibility of the virus opens up a huge prospect for vaccine development should the H9N2 virus infect humans.

A little correction I may suggest is with the topic. It should read Emerging threats and not threat. . . . as there may be many variables with respect to the medical importance of the H9N2 virus in Poultry in Indonesia.

Is the topic of the review discussed comprehensively in the context of the current literature?
Yes

Are all factual statements correct and adequately supported by citations?
Yes

Is the review written in accessible language?
Yes

Are the conclusions drawn appropriate in the context of the current research literature?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: I am a medical Microbiologist with specialties in Fungi of both medical and veterinary importance. I am also a certified Medical Laboratory Scientist with specialties in Medical Parasitology and entomology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 24 Jun 2022

Saifur Rehman, Universitas Airlangga, Surabaya, Indonesia

2nd Reviewer comments

1. A minor correction I may suggest is with the topic. It should read Emerging threats and not threat. as there may be many variables with respect to the medical importance of the H9N2 virus in Poultry in Indonesia.

   Response: We have changed the Title according to your suggestions as “Emerging threats and vaccination strategies of H9N2 viruses in poultry in Indonesia: A review".

Competing Interests: All authors declare no competing interests

Reviewer Report 08 June 2022

https://doi.org/10.5256/f1000research.130513.r138444

?

Asghar Abbas
Faculty of Veterinary and Animal Sciences, Muhammad Nawaz Shareef University of Agriculture, Multan, Pakistan

Dear Editor/Authors,

I have review of the manuscript "Emerging threat and vaccination strategies of H9N2 viruses in poultry in Indonesia: A review". The Article is well written and contains valuable information on Vaccination against Avian influenza Disease. However the manuscript needs minor revisions and improvement as per following comments

1. English Language of whole manuscript should be revised and improved.
2. Justification (4-5 lines) of study should be added in introduction with proper latest references.
3. Kindly add Associated risk factors of Avian influenza disease in Indonesia.

I recommend manuscript be accept after minor revision.

Is the topic of the review discussed comprehensively in the context of the current literature?
Yes

Are all factual statements correct and adequately supported by citations?
Yes

Is the review written in accessible language?
Yes

Are the conclusions drawn appropriate in the context of the current research literature?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Pathobiology Research

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 24 Jun 2022

Saifur Rehman, Universitas Airlangga, Surabaya, Indonesia

Dear Reviewer,

Thanks for your valuable comments and suggestions on the manuscript entitled:

"Emerging threats and vaccination strategies of H9N2 viruses in poultry in Indonesia: A review"

We welcome feedback. We have made modifications to the study on the following points:

- English Language of the whole manuscript should be revised and improved

  Response: We have revised the English language of the whole manuscript.

- Justification (4-5 lines) of study should be added in the introduction with proper latest references.

  Response: We have added the Justification of the study in the Introduction section.

- Kindly add the Associated risk factors of Avian influenza disease in Indonesia.

  Response: We have added the associated risk factors of AIVs which showed association with AIV in Indonesia

Competing Interests: All authors declare no competing interests

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Journal of Veterinary Science

Review
Zoonotic Disease

Check for updates

Current situation and control strategies of H9N2 avian influenza in South Korea

Mingeun Sagong [1,2], **Kwang-Nyeong Lee** [1], **Eun-Kyoung Lee** [1], **Hyunmi Kang** [1], **Young Ki Choi** [2,*], **Youn-Jeong Lee** [1,*]

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ABSTRACT

The H9N2 avian influenza (AI) has become endemic in poultry in many countries since the 1990s, which has caused considerable economic losses in the poultry industry. Considering the long history of the low pathogenicity H9N2 AI in many countries, once H9N2 AI is introduced, it is more difficult to eradicate than high pathogenicity AI. Various preventive measures and strategies, including vaccination and active national surveillance, have been used to control the Y439 lineage of H9N2 AI in South Korea, but it took a long time for the H9N2 virus to disappear from the fields. By contrast, the novel Y280 lineage of H9N2 AI was introduced in June 2020 and has spread nationwide. This study reviews the history, genetic and pathogenic characteristics, and control strategies for Korean H9N2 AI. This review may provide some clues for establishing control strategies for endemic AIV and a newly introduced Y280 lineage of H9N2 AI in South Korea.

Keywords: Avian influenza; H9N2 virus; history; pathogenicity; vaccine

INTRODUCTION

H9N2 avian influenza viruses (AIVs) have spilled over from wild birds, their natural host, to domestic poultry. These viruses have become endemic in poultry in many countries since the 1990s. H9N2 AIVs can be broadly categorized into two major lineages: Eurasian and American. Eurasian H9N2 AIVs, in particular, have circulated in poultry and are classified further into several lineages: G1 (represented by A/quail/Hong Kong/G1/1997), Y280 (represented by A/duck/Hong Kong/Y280/1997; also known as the BJ94 or G9 lineage) and Y439 (represented by A/duck/Hong Kong/Y439/1997; also known as the Korean lineage) lineage [1-3].

The Y439 lineage of H9N2 AIV is a group originating from Eurasian wild birds, and it has been reported in many regions, including Europe and Asia [4]. In South Korea, the Y439 lineage of H9N2 AIV was first reported in chicken farms in 1996 and has since spread in poultry and become endemic since 2000s [5-7]. Outbreaks of the Y439 lineage of H9N2 AI decreased after vaccinating layer and broiler breeders since 2007. However, even after vaccination, the virus has continued to circulate mainly in Korean native chicken farms and live bird markets (LBM), there have been no reports since it was last detected in 2018 [8-10].

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Conflict of Interest

The authors declare no conflicts of interest.

Funding

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The G1 lineage of H9N2 AIV is the most widely distributed H9N2 AIV group in Asia, the Middle East, and Africa [11,12]. The lineage is divided into two sublineages according to the geographical distribution and genetic association: "G1-Eastern" and "G1-Western" [3,13,14]. Among them, the G1-Eastern lineage is endemic to poultry in southern China and neighboring Southeast Asian countries, Vietnam and Cambodia. On the other hand, the G1-Western is distributed across a wide range of regions, from Asia, including Bangladesh and India, to the Middle East and Africa [14].

The Y280 lineage of H9N2 AIV has become the dominant lineage in China since the mid-1990s and has evolved into sublineages (presented as BJ/94, HK/G9, and SH/F98) and various genotypes (A-W, G1-G81) [15-17]. This lineage is distributed in Asian countries, such as China, Vietnam, Cambodia, Indonesia, and Myanmar. In Vietnam, which borders China, the Y280 lineage of H9N2 AIV has circulated mainly in poultry since 2012 [4,18]. Recently, it was reported in Japan and eastern Russia, which does not border China [19,20]. In addition, the Y280 lineage of H9N2 AIV was first isolated from LBMs in South Korea in June 2020 and has spread rapidly nationwide [9,10].

Wild birds are the natural host of AIVs, but H9N2 AIV began to be reported in poultry, such as chicken, quail, guinea fowl and partridge in Asia [4], in the mid-1990s and has become endemic in poultry beyond the species barrier without pre-adaptation. The endemicity of Asian H9N2 AI in poultry has promoted the emergence of various novel AIVs and the evolution of H9 AIVs [2]. Infection of H9N2 AIV is an important issue for animal diseases and public health [21]. Previous studies have shown that H9N2 AIVs donated internal gene sets to other human infecting viruses, including H5N1, H5N6, H7N9, and H10N8 [22-27].

In South Korea, the Y439 lineage of H9N2 AIV, which occurred for a long period, has not been reported since 2018, but the Y280 lineage of H9N2 AI was newly introduced in 2020. Considering the history of the endemicity of the H9N2 AI in many countries, including South Korea, once H9N2 AI is introduced, it is more difficult to eradicate than high pathogenicity avian influenza (HPAI). This study reviewed the history of Korean H9N2 AI, the genetic and pathogenic characteristics of H9N2 AIVs, and the control strategies, including vaccination in South Korea.

HISTORY AND CURRENT SITUATION OF Y280 LINEAGE OF H9N2 AIV IN ASIA

Since the mid-1990s, the Y280 lineage of H9N2 AIV has become the dominant strain and circulated in chickens in China [2,28,29]. From 1995 to June 2022, 8,968 cases of the Y280 lineage of H9N2 AIVs were isolated worldwide (**Supplementary Fig. 1**, excluding mammal infections, based on the Global Initiative for Sharing All Influenza Data [https://www.gisaid.org/]). Of these, 8,311 cases, approximately 92.7%, were reported in China, where the outbreaks have increased dramatically since 2009. Although vaccination programs for chickens have been in place for a long time in China [17,27,29], the Y280 lineage of H9N2 AIV has been endemic to poultry and has increased the genetic diversity of the virus due to the high proportion of traditional small-scale mixed breeding and the preference for fresh poultry trading through the LBMs [30,31]. According to Gu et al. [27], at least 23 genotypes of Y280 lineage of H9N2 AIV isolated in China from 1994 to 2014 were identified, of which three types were suggested to be major genotypes: A, H, and S. In particular, genotype S is a reassortant of the PB2 and M genes of the G1 lineage of H9N2 AIV based on the gene

constellation of the Y280 lineage of H9N2 AIV and has become dominant in China since 2010 [32]. The Y280 lineage of H9N2 AIV, which was almost restricted to China before 2010, has spread to other Asian countries, including Vietnam, Cambodia, Indonesia, and recently South Korea (**Fig. 1**) [4,9,18,33,34].

In Hong Kong, the Y280 lineage of H9N2 AIV was first isolated in 1997, and some cases subsequently occurred in poultry and humans. Since the early 2000s, cases of H9N2 AI infection have been reported sporadically until recently [1,35,36]. Interestingly, in Japan, the Y280 lineage of H9N2 AIV was first isolated in imported chicken meat products collected in 1997, 2001, and 2002. In addition, in 2015–2016, H9N2 AIVs were isolated in illegally imported poultry products by flight passengers from China and Taiwan into Japan during the quarantine process [19,37].

In Vietnam and Cambodia, Y280 H9N2 AI was reported in 2009 and 2013. Since then, the Y280 lineage of H9N2 AIV has become dominant in poultry, mainly in LBMs [38-40]. The Y280 lineage of H9N2 AIV of the two countries was genetically closely related to the strain in China. These viruses may have flowed into adjacent countries locally through active poultry trading [19,33,40].

Since the mid-2010s, the Y280 lineage of H9N2 AI has been spreading further in Southeast Asia, and the virus has also been identified in Myanmar, Indonesia, and Laos (**Fig. 1**, **Supplementary Fig. 1**). These viruses are genetically closely related to the Y280 lineage of

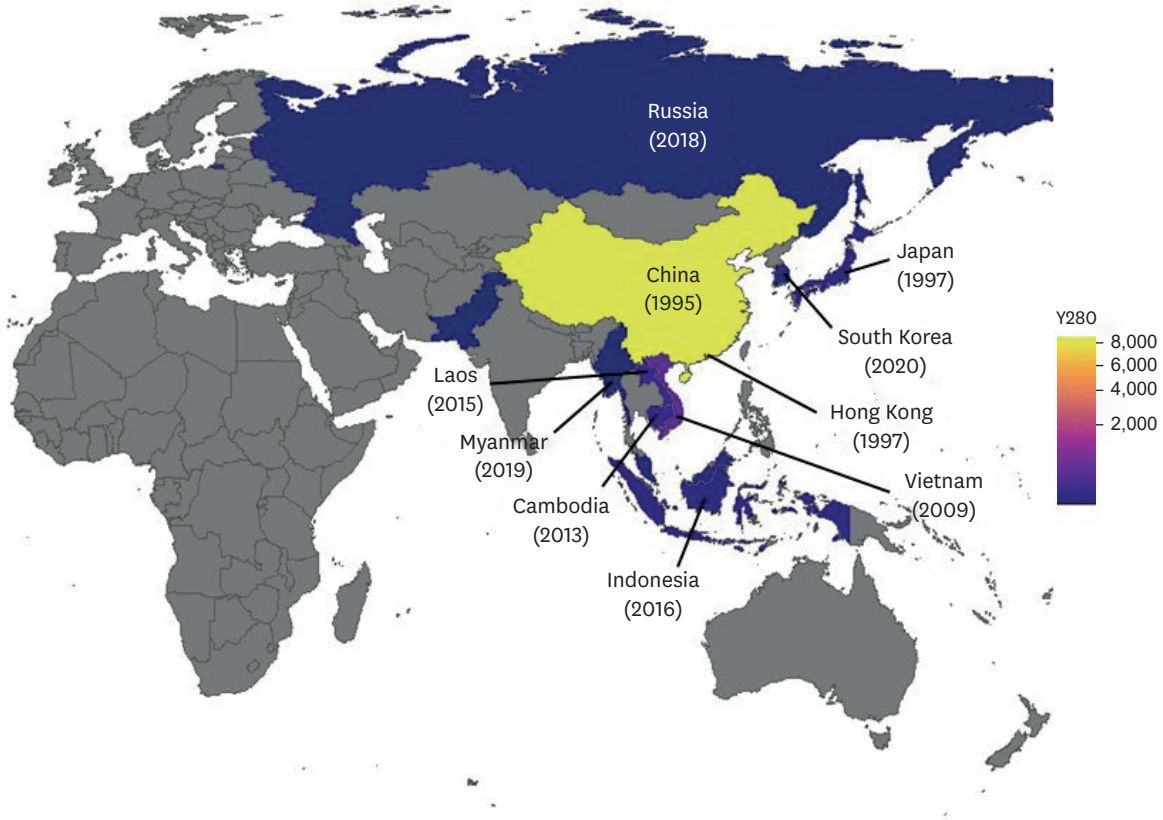

Fig. 1. As of July 2022, the global geographical distribution Y280 lineage of H9N2 avian influenza, including the first reporting year by major Asian countries (square) and the number of genetic information of Y280 H9N2 (yellow to deep blue), uploaded to the Global Initiative for Sharing All Influenza Data database (the hemagglutinin gene sequence collected from January 1995 to June 2022, http://gisaid.org/).

H9N2 AIV in China [41-43]. In addition, The H9N2 AIV was first identified in Russia in 2012 but was not defined genetically. Later, in 2018, the Y280 lineage of H9N2 AIV was isolated at a poultry farm in Primorsky Krai, Far East region of Russia, and was found to be genetically related to that isolated in Tajikistan, Central Asia [20].

Long-distance migrating wild birds, as shown in the high pathogenicity H5 AIV, are one of the factors of AIV transmission and spread [44-47]. In China, there have been several sporadic reports of H9N2 AIV detection in wild birds since 2010 [48-52]. Most H9N2 AIVs in wild birds have been identified as the Eurasian aquatic origin, but some cases were North American and poultry-derived G1 or Y280 lineages. Thus far, there is no direct evidence that the Y280 lineage of H9N2 AIV has been transmitted between countries or continents by wild birds, despite the surveillance programs conducted in several countries [9,53-55]. Although the detection of poultry-derived H9N2 AIV in wild birds was limited, the virus can be disseminated by wild migratory birds if this virus acquires more adaptability to wild waterfowl.

Considering the spread of the H9N2 AIV in neighboring countries of China and the detection of H9N2 AIV through the quarantine process in Japan, the Y280 lineage of H9N2 AIV could be transmitted by the movement of contaminated poultry products, people, or goods [56,57]. Another transmission factor, the LBM, is a central point in generating and spreading novel viruses to other species due to the high prevalence and genetic diversity of H9N2 AIV and should be considered a hotspot for surveillance programs [18,30,58].

THE OUTBREAK AND GENOTYPE OF H9N2 AI IN SOUTH KOREA

Since the first outbreak of H9N2 in South Korea in 1996, the Y439 lineage of the H9N2 virus has been endemic since the 2000s (**Fig. 2**). Nationwide outbreaks of H9N2 AI, which have caused considerable economic losses, have led to the use of vaccination programs since 2007 [7]. Since then, the outbreaks of H9N2 AI in poultry farms, such as layers and breeders, have decreased gradually, but the virus was not completely eradicated and was circulated continuously, mainly in Korean native chickens in LBM, until 2018 (data not shown). The Y439 lineage of H9N2 AIV, which has circulated in South Korea for a long time, has continuously evolved by antigenic drift and reassortment with other AIVs from wild birds and domestic ducks in LBMs [5,59,60]. The Y439 lineage of H9N2 AIV in South Korea is divided broadly into two genotypes according to their gene constellation (**Fig. 3**). The first is the MS96-like genotype, represented by A/chicken/Korea/MS96/1996 (H9N2) and its reassortant viruses with the genes from domestic ducks and wild bird origin, which was distributed in poultry until 2008 (designated as K1, K2, and K3 genotype in Youk et al. [59]). Second, the A146/09-like genotype, represented by A/chicken/Korea/A146/2009 (H9N2), is a reassortant of the hemagglutinin (HA) and nucleoprotein genes of the MS96-like virus with six internal genes originating from wild aquatic birds; this strain has become the dominant strain (designated as K4 genotype in Youk et al. [59]). In South Korea, the national active surveillance program was established for HPAI control, and various measures have been applied, including movement restriction, disinfection, and the culling of infected animals since 2008 (**Fig. 4**). These preventive measures may play a role in reducing the low pathogenicity avian influenza (LPAI) virus and HPAI virus, particularly in domestic ducks and LBM. Consequently, the emergency of reassortant viruses has been reduced, and finally, the Y439 lineage of H9N2 AIV has disappeared in South Korea since 2018.

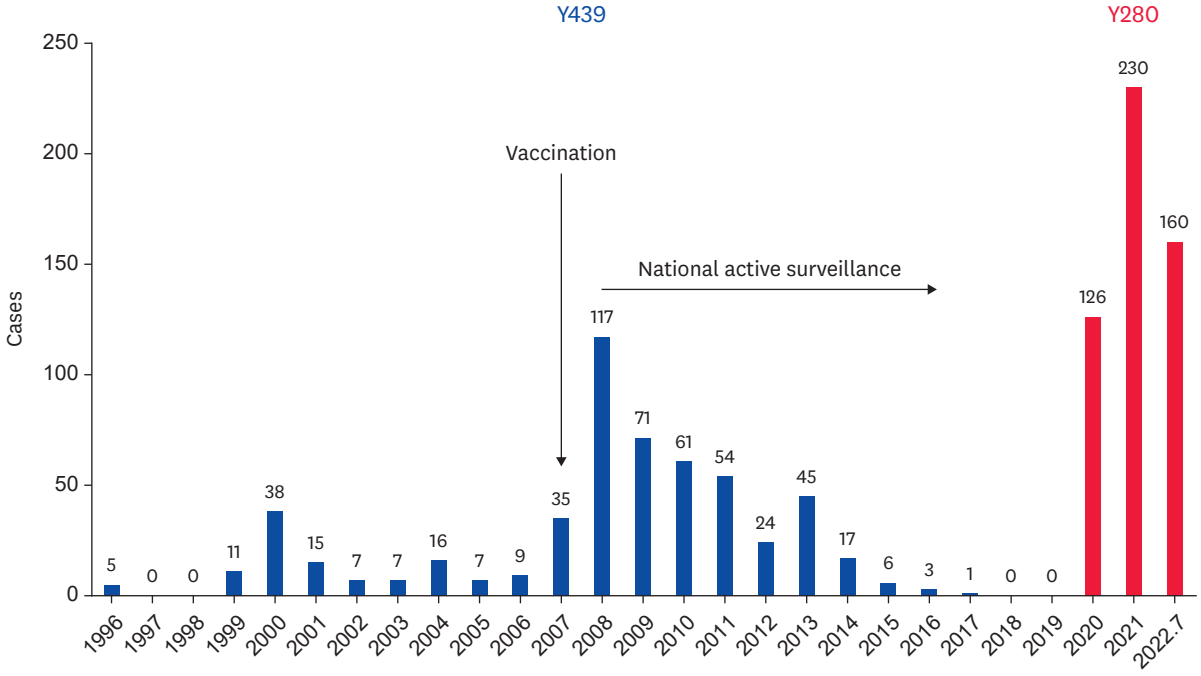

Fig. 2. Number of H9N2 avian influenza (Y439 and Y280 lineage) outbreak cases in South Korea from 1996 to July 2022. Before 2007, data was collected from avian disease pathological diagnosis reports in Animal and Plant Quarantine Agency were used, and from 2008, updated from National Animal Disease Statistics in Korea Animal Health Integrated System.

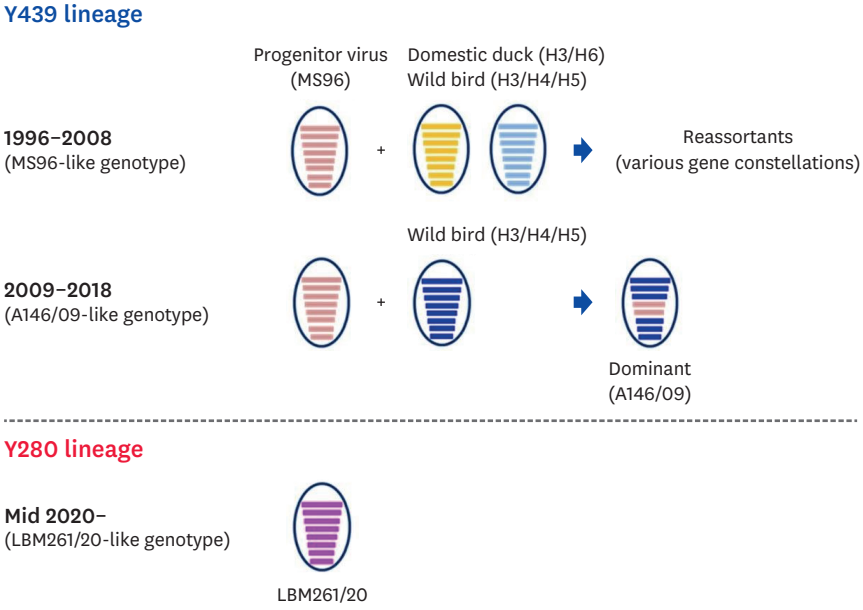

Fig. 3. Illustrative scheme of the gene constellation from Korean representative H9N2 viruses (Y439 and Y280 lineage) depending on their epidemic year. The eight horizontal bars in circle (from top to bottom) represent PB2, PB1, PA, HA, NP, NA, M, and NS genes, respectively.
LBM, live bird market.

In June 2020, the Y280 lineage of H9N2 AIV was first isolated from Korean native chickens in LBM by active surveillance programs, and has since spread nationwide (**Fig. 2**). A/chicken/Korea/LBM261/2020 (H9N2), which was the virus of the index case in South Korea, was

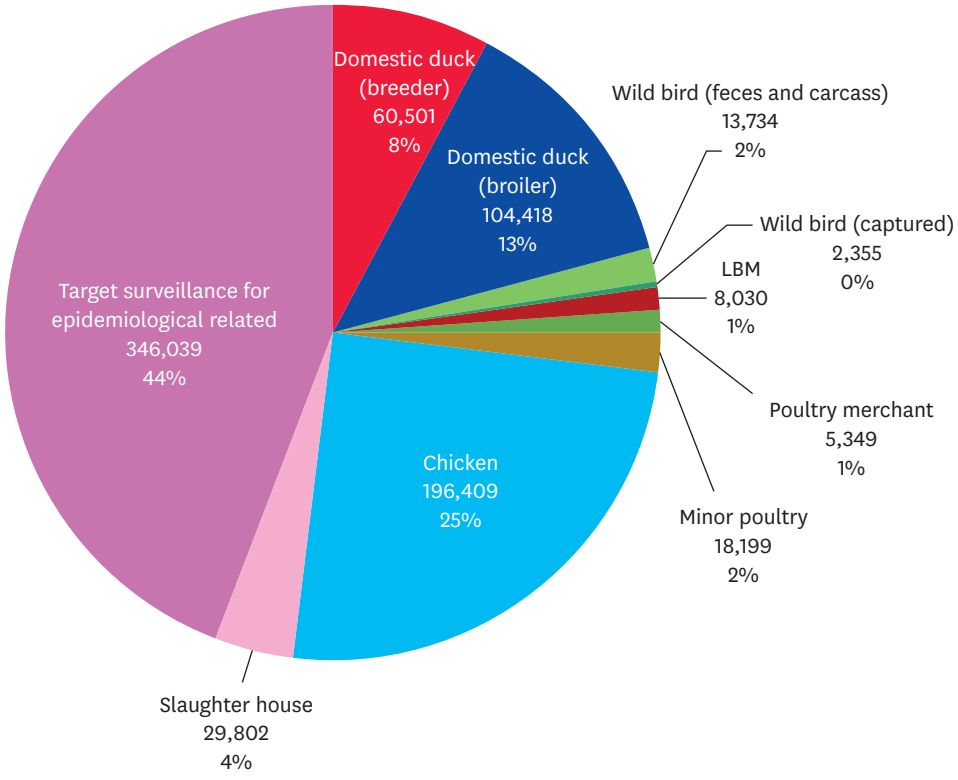

Fig. 4. AI national active surveillance and monitoring results of South Korea in 2021. A total of 784,836 laboratory diagnostic tests were conducted annually in 10 categories, including domestic chickens, ducks, and LBMs (Annual Report on Avian Influenza Surveillance Results for 2021, Animal and Plant Quarantine Agency).
LBM, live bird market.

closely related to the A/chicken/Shandong/1844/2019 (H9N2) virus of China. The Korean Y280 lineage of H9N2 AIV is designated as the LBM261/20-like genotype, which belongs to a subgroup of genotype S in China (**Fig. 3**) [9]. Five hundred sixteen cases of the Korean Y280 lineage of H9N2 AI have been detected nationwide in various breeds, such as Korean native chickens, layer and broiler chickens by active surveillance of domestic poultry from June 2020 to July 2022 (**Fig. 2**). Although the route of introduction of the novel H9N2 AIV into South Korea remains unclear, the likelihood of introduction by wild migratory birds is considered low. This is because the poultry-derived Y280 lineage of H9N2 AIV in wild birds is rarely reported even in China [48-52], and there is no virus isolation in wild birds, including feces, captive birds, carcass through intensive active surveillance in South Korea. Therefore, the virus is likely to be introduced through contaminated poultry products or human activities, as shown in the periodical AIV detection cases in the quarantine process in Japan [9].

PATHOGENIC CHARACTERISTICS OF H9N2 AIV IN CHICKENS AND DUCKS

Although H9N2 AIV is classified as a low pathogenicity virus in poultry, it is causing economic damage to the poultry industry by the decrease in spawning and some mortality rates in commercial chickens. Most chickens infected with H9N2 AIV at farms showed typical signs of influenza, such as respiratory symptoms, egg drop, and mortality (0% to 40%) (summarized in **Table 1**) [2,5,61,62]. On the other hand, experimental infections in specific-

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Table 1. Summary of clinical signs of H9N2 viruses from the Y439 and Y280 lineage in farms and animal experiments, respectively

Cases	Species	Y439 lineage		Y280 lineage	
		Clinical signs	Reference	Clinical signs	Reference
Field (farm)	Chicken (commercial)	**Egg drop**, respiratory sign, depression, diarrhea, weight loss, decreased feed intake, **mortality (0%–30%)**	[2,5,61,63]	**Egg drop, respiratory signs (coughing, sneezing, gasping), mortality (10%–40%)**	[1,2,62]
Animal experiment	Chicken (SPF)	**No mortality**, depression **Viral shedding: higher titer via CL route**	[5,63,65,66]	**No mortality**, depression, diarrhea, decreased feed intake **Viral shedding: higher titer via OP route**	[16,17,19,64,66, 71,89,97,102]
	Mice	**Mostly no clinical signs and mortality**, weight loss, inappetence	[5,92,107]	**Inappetence**, huddling, **ruffled fur**, labored breathing, hunched posture, respiratory distress, **weight loss**, **mortality (0%–30%)**	[2,36,52,82,83, 89,91,92,93]

Bold: observed major clinical symptoms.
SPF, specific-pathogen-free; OP, oropharyngeal; CL, cloacal.

pathogen-free chickens showed no mortality and only mild symptoms, such as depression and decreased feed intake [5,19,63-66]. This disparity between laboratory and field infections with H9N2 AIV suggested that the pathogenicity of H9N2 AIV can vary depending on ages, breeds, the level of immunity, and another secondary opportunistic pathogen infection [5,64,67-69].

Previous studies have shown that similar clinical signs were observed in infection between the Y439 and Y280 lineage of H9N2 AIVs (**Table 1**). In the viral shedding, however, there was a significant difference in the preferential replication between the two viruses. The Y280 lineage of H9N2 AIV was replicated more efficiently in the respiratory tract, while the Y439 lineage of H9N2 AIV was replicated more efficiently in the intestinal tract [5,19,65,66]. Thus, the Y280 lineage of H9N2 AIV can be transmitted airborne more efficiently via the oral-to-oral pathway than the Y439 lineage of H9N2 AIV. This feature can cause a more efficiently spread virus between poultry in the same space. It can be a risk factor that increases the chance of viral infection even between species in contact with infected poultry [2,70,71].

Domestic ducks are intermediate species between poultry and wild waterfowl and have susceptibility and resistance to AIVs [72-74]. Experiments with H9N2 AIV infections in domestic ducks are limited, but the results show that most infected ducks were asymptomatic [2,66,75,76]. In addition, viral replication was not detected in most infected ducks and was identified as low titers in oropharyngeal (OP) and cloacal (CL) swabs from a few infected ducks. According to Wang et al. [76], it was confirmed that the genotype S of the Y280 lineage of H9N2 AIV could replicate with relatively high titers in the respiratory tract of the Muscovy duck. Despite the limited cases, some experimental results have shown viral replication of H9N2 AIV in ducks. If the chicken-adapted H9N2 AIV replicates more efficiently in ducks, it can be a potential risk factor in AIV transmission by domestic ducks and wild migratory ducks.

HUMAN INFECTION BY H9N2 AIV

Human infection by the H9N2 AIV was first reported in Hong Kong in 1998 [4]. Since then, sporadic cases have been reported continuously in various countries, mainly China. As of June 2022, 112 cases have been identified in eight countries, including China, Egypt, Bangladesh, and Cambodia. Cases of infection have been reported mainly in people in close contact with infected poultry and meats or exposed to contaminated environments [77,78]. Children under the age of 10 were most infected with H9N2 AIV but developed mild

symptoms [79]. On the other hand, the H9N2 AIV is closely involved in other fatal human infections as well as direct infections. The high pathogenicity H5N1 AIV in Hong Kong in 1997 was found to have reassorted from six internal genes of the G1 lineage of H9N2 AIV, excluding HA and neuraminidase [22,80]. In addition, the internal genes of H7N9 AIV, which has 1,568 human infections, including 616 fatal cases (case fatality rate, 39%) in China since 2013, originated from the Y280 lineage of H9N2 AI [23].

Poultry-adapted AIVs exhibit asymptomatic or weak signs and can evolve as potential infection sources in mammals through circulation in poultry [81-83]. The HA protein of AIV is determined in the host range by binding with sialic acid on the surface of the host cell. In general, AIV has the highest binding affinity with the α2,3-linked sialic acid of birds, but mutations on the receptor binding sites for high affinity with α2,6-linked sialic acid have been found to increase infectivity in mammals [84,85]. Previous studies reported that leucine (L) in position 226 of the HA proteins plays an important role in the binding affinity to sialic acid as a representative mammalian affinity marker [86,87]. Thus far, the human infection cases by H9N2 AIV were only reported in Y280 and G1 lineages, most of which have a Q226L substitution on the HA protein. In addition, the genotype S of the Y280 lineage, which has been dominant in poultry in China since 2010, has acquired various mammalian affinity markers: H183N, T190V, and Q226L in the HA protein; A588V in the PB2 protein; K356R and S409N in the PA protein; V15I in the M1 protein; I28V and L55F in M2 protein [4,65,88-92]. Newly introduced H9N2 AIV into South Korea in 2020 belonged to genotype S of the Y280 lineage of H9N2 AIV, which has similar genetic characteristics [9].

Although the Y439 lineage of H9N2 AIV had circulated for a long period (1996–2018), there have been no human infection cases in South Korea (**Fig. 2**). The Korean Y439 lineage of H9N2 AIV had retained poultry affinity markers rather than mammals [9,86]. In mouse experiments, the Y280 lineage of H9N2 AIV replicated well in the respiratory tract of infected mice without adaptation and showed various clinical signs, body weight loss, and mortality, whereas the Y439 lineage of H9N2 AIV showed mostly no clinical signs or mild symptoms, such as inappetence and weight loss (**Table 1**) [5,89-93]. These results show that the Y439 lineage of H9N2 AIV is at least less lethal in mammalian infections than the Y280 lineage of H9N2 AIV.

CONTROL STRATEGIES OF H9N2 AI IN SOUTH KOREA

National active surveillance for AI has been conducted since 2008 to monitor HPAI in South Korea. Although there is a slight difference annually, 784,836 laboratory diagnostic tests were conducted in 2021 (**Fig. 4**). The main targets of active surveillance were domestic chickens (approximately 25%), domestic ducks (approximately 21%), wild birds (approximately 2%), LBMs and poultry traders (approximately 2%), and the epidemiological-related places with HPAI outbreaks (approximately 44%). Surveillance has been applied to wild birds for an early warning of HPAI introduction, including fecal samples, captive wild birds, and carcasses. High pathogenicity H5Nx AIVs have been detected in wild birds at the early time of migration before poultry outbreaks [94,95]. Domestic ducks are considered an important target of active surveillance because they can be a potential viral transmission factor, despite not showing clinical symptoms when infected with HPAIV [72]. LBM, which has a high risk of viral transmission by live bird trading, is one of the main targets of surveillance [18,30,58]. For effective control of AIV, surveillance has also been conducted in the place

of poultry merchants and farms related to LBMs. Through the surveillance of LBM, the introduction of the Y280 lineage of H9N2 AIV into South Korea was also found [9]. Overall, intensive national active surveillance and followed control measures, such as disinfection, restriction of movement, ban of poultry trading, and stamping out of HPAI-infected birds, have gradually reduced LPAI as well as HPAI in South Korea. Therefore, active surveillance programs are essential to monitor the emergence of new viruses and to control the spread of the viruses in the early stages after detection.

As a preventive measure, vaccination has been used to control H9N2 AI in many countries, particularly in endemic regions. China has implemented vaccination programs for H9N2 AI on chicken farms since 1998 [65,96]. On the other hand, the H9N2 AI still has a high prevalence in China (**Supplementary Fig. 1**). Moreover, the long-term circulation of the H9N2 AIV in a vaccinated population has caused many virus mutations [17,97-102]. This is considered to have been compositely caused by factors, such as inefficient application of vaccines, low doses, low vaccination coverage, and limited updates of vaccine strains [98,100,103]. At least 20 commercial vaccines have been used in China to cope with various viruses, which need to be updated regularly [97,98,101].

The H9N2 AIV has been prevalent nationwide in South Korea since 2000 but officially reported outbreaks were limited (**Fig. 2**) [7]. Therefore, since 2007, Korean animal health authorities have permitted the use of H9N2 vaccines, which use a single vaccine strain (A/chicken/Korea/01310/2001) of the Y439 lineage of H9N2 AI in layer and breeder chicken to prevent damage to the poultry industry [104,105]. Although outbreaks of the Y439 lineage of H9N2 AI have decreased since the vaccine program, it took more than a decade to disappear from the field (**Fig. 2**). The H9N2 AIV had remained especially in LBMs and small-scale Korean native chicken farms for a long time. This fact suggests a limit to controlling the H9N2 AI with vaccination alone.

Another factor to consider in vaccination strategy is the possibility of virus mutations and the need to update the vaccine strain. Immune pressure by long-term vaccination may cause genetic and antigenic changes, as shown in China and South Korea [8,28,59,65,101,106,107]. This leads to a gradual decrease in the suitability of vaccine strain and vaccine efficacy in the field. Although the vaccine strain for the Y439 lineage of H9N2 AIV has never been updated in South Korea, but depending on the situation in which the Y439 lineage of H9N2 AI is circulated in poultry again, it will be necessary to update the vaccine strain by the genetic and antigenic characteristics of the field virus.

Unfortunately, as the Y280 lineage of H9N2 AIV was newly introduced into South Korea in 2020, previously authorized vaccines against the Y439 lineage of H9N2 AIV may not be an appropriate option to control the current Y280 lineage of H9N2 AIV because of the difference in the genetic and antigenic features (81.8% nucleotide similarity) [108]. In animal experiments, the Y439 lineage of the vaccine showed only limited efficacy to heterogeneous Y280 lineage of H9N2 AIV (Y439 lineage of the vaccine reduced the replication of the Y280 lineage of H9N2 AIV in the cecal tonsils by 37.5%, and also partially inhibits viral shedding in respiratory and intestinal tracts) (**Fig. 5**). By contrast, the rgHS314 virus (derived from A/chicken/Korea/H314/2020), which was newly developed as an autogenous vaccine for the current epizootic Y280 lineage of H9N2 AIV, can reduce viral replication significantly with 100% inhibition of virus recovery in the cecal tonsil and no viral shedding in OP and CL swabs (**Fig. 5**) [108]. New commercial vaccines using the Y280 lineage of the H9N2 vaccine

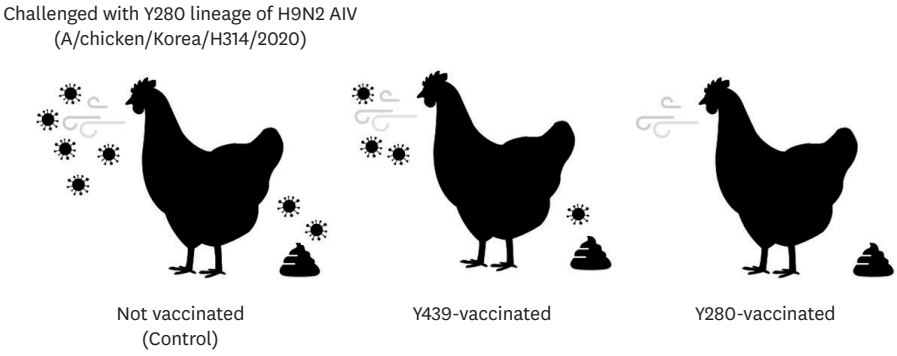

Fig. 5. Assessment of the protective efficacy of the commercial Y439 vaccine and newly developed Y280 vaccine (used homologous strain, A/chicken/Korea/H314/2020) when challenged with the Y280 H9N2 virus. In an animal experiment, the commercial Y439 vaccine has been found only partially to inhibit viral replication and shedding and has been shown to provide incomplete protection against the Y280 H9N2 virus [108].
AIV, avian influenza virus.

seed strain may be available in the field in the first half of 2023. However, active surveillance and enhanced biosecurity levels must be combined with vaccination to control the H9N2 AI effectively [86,103].

CONCLUSION

This report provides an overview of the history of outbreaks and the control strategies for H9N2 AI in South Korea. Unlike many endemic countries, including China, where new variants of H9N2 AIV are emerging by genetic mutations, in South Korea, the Y439 lineage of H9N2 AI has disappeared by effective control measures, such as continued large-scale national surveillance, improved levels of biosecurity, appropriate vaccination, and culling of poultry in the case of HPAI. Therefore, in order to control the new Korean Y280 lineage of H9N2 AI, measures such as updating vaccine strain, organizing surveillance based on the potential risks of H9N2 AI (breeds and prevalence rate, etc.) and strengthening follow-up monitoring of LBM's supply farms and distribution networks are urgently needed. These intensive measures and strategies will help control the Y280 lineage of H9N2 AI as soon as possible. This review paper is expected to assist in establishing control strategies and provide insight for low pathogenicity H9N2 AI in endemic countries.

SUPPLEMENTARY MATERIAL

Supplementary Fig. 1

Y280 lineage H9 subtype virus detection graph by country and year (total 8,967 cases based on the GISAID database).

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REVIEW

Alarming situation of emerging H5 and H7 avian influenza and effective control strategies

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ABSTRACT

Avian influenza viruses continue to present challenges to animal and human health. Viruses bearing the hemagglutinin (HA) gene of the H5 subtype and H7 subtype have caused 2634 human cases around the world, including more than 1000 deaths. These viruses have caused numerous disease outbreaks in wild birds and domestic poultry, and are responsible for the loss of at least 422 million domestic birds since 2005. The H5 influenza viruses are spread by migratory wild birds and have caused three waves of influenza outbreaks across multiple continents, and the third wave that started in 2020 is ongoing. Many countries in Europe and North America control highly pathogenic avian influenza by culling alone, whereas some countries, including China, have adopted a "cull plus vaccination" strategy. As the largest poultry-producing country in the world, China lost relatively few poultry during the three waves of global H5 avian influenza outbreaks, and nearly eliminated the pervasive H7N9 viruses that emerged in 2013. In this review, we briefly summarize the damages the H5 and H7 influenza viruses have caused to the global poultry industry and public health, analyze the origin, evolution, and spread of the H5 viruses that caused the waves, and discuss how and why the vaccination strategy in China has been a success. Given that the H5N1 viruses are widely circulating in wild birds and causing problems in domestic poultry around the world, we recommend that any unnecessary obstacles to vaccination strategies should be removed immediately and forever.

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Influenza A viruses are important pathogens that continually challenge both human and animal health. The genome of influenza A virus comprises eight gene segments: basic polymerase 2 (PB2), basic polymerase 1 (PB1), acidic polymerase (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and nonstructural protein (NS). Each of these segments encodes one to three proteins. On the basis of the antigenicity of the HA and NA proteins, influenza viruses are divided into different subtypes. Currently, 16 HA subtypes and nine NA subtypes have been detected in avian species. H1N1, H2N2, and H3N2 viruses have caused four influenza pandemics since 1918, and H1N1 and H3N2 viruses continue to co-circulate in humans globally. Viruses of several other subtypes that circulate in animals have also jumped to humans on multiple occasions [1–8], and some of them have shown pandemic potential [9–12].

The avian influenza viruses are maintained and circulate in wild birds. Although different subtypes of viruses have been detected in domestic poultry, especially waterfowl that come into close contact with wild birds, only three HA subtypes—H5, H7, and H9—have spread and been detected in domestic poultry across wide geographic areas. Some strains bearing the HA gene of the H5 or H7 subtypes are highly pathogenic for poultry and have caused severe problems for the global poultry industry. In this review, we briefly summarize the H5 and H7 influenza outbreaks and the damage they have caused to the global poultry industry and public health, analyze the evolution and spread of H5 viruses, and discuss the effectiveness of the poultry vaccination strategy for highly pathogenic avian influenza control.

Avian influenza outbreaks caused by H5 viruses

In the last century, avian influenza outbreaks caused by different H5 viruses have occurred in eight countries or regions. The first recorded highly pathogenic avian influenza outbreak was caused by H5N1 virus in chickens in Scotland in 1959 [13]; in 1966, an avian influenza outbreak in turkeys in Canada was caused by H5N9 virus [14]; an H5N2 virus caused

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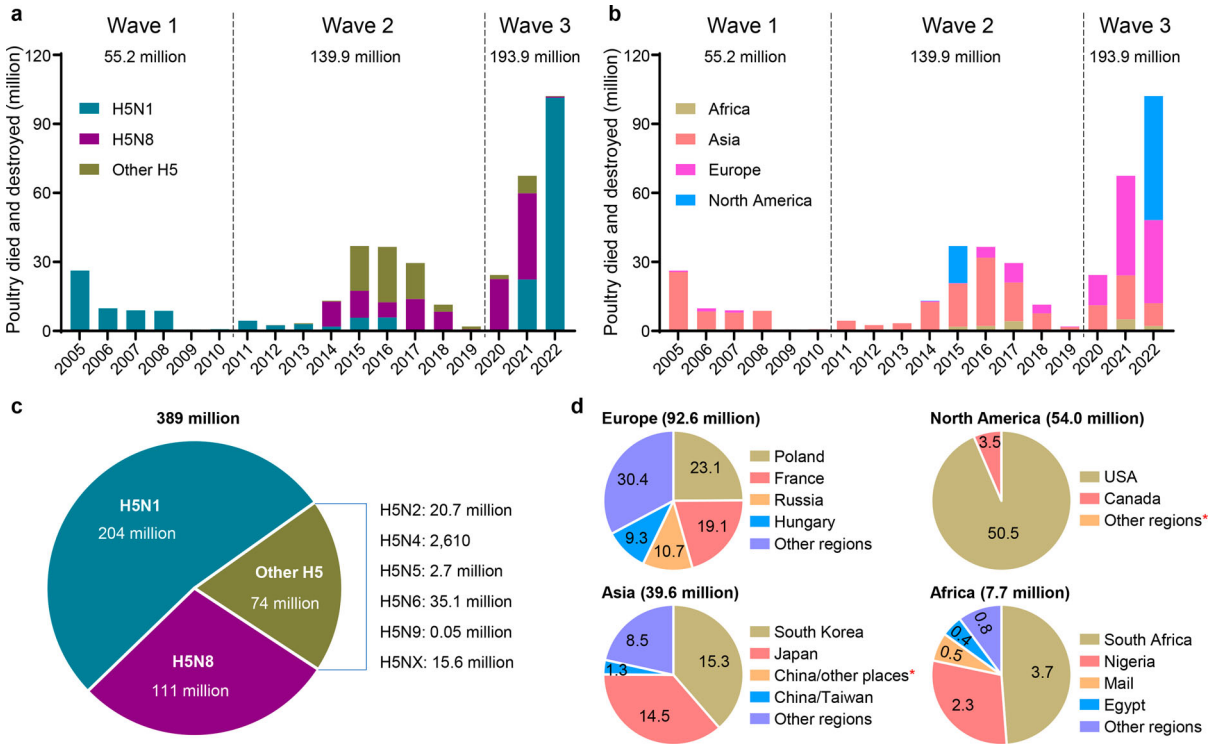

Figure 1. Damage caused to the global poultry industry since 2005 by different H5 avian influenza viruses based on information reported in the OIE-World Animal Health Information System. The number of poultry that died or were destroyed during outbreaks caused by different subtypes of H5 influenza viruses (a, c) in different continents (b), and (d) the number of poultry that died or were destroyed in different countries or regions since 2020. *, fewer than 10,000 birds died or were destroyed in the indicated country or regions.

multiple outbreaks in chickens and turkeys in the US from 1983 to 1985 [15]; in 1983, an H5N8 virus caused disease outbreaks in turkeys, chickens, and ducks in Ireland [16]; in 1991, an H5N1 virus caused a disease outbreak in turkeys in England [17]; an H5N2 virus caused multiple outbreaks in chickens and turkeys in Mexico from 1994 to 1995 [18]; and in 1997, an H5N1 virus and an H5N2 virus caused outbreaks in chickens in Hong Kong and Italy, respectively [19,20].

In this century, the first H5 avian influenza outbreak occurred in Hong Kong in 2002, caused by an H5N1 virus [20]. In 2003 and 2004, avian influenza outbreaks caused by H5N1 viruses were reported in several Asian countries, including Vietnam, Thailand, Indonesia, China, Japan, South Korea, Cambodia, and Lao [21]. The number of poultry lost in the outbreaks that occurred before 2004 is not available; however, between January 2005 and November 2022, H5 highly pathogenic avian influenza viruses have caused 8534 outbreaks and the loss of 389 million poultry around the world (Figure 1(a)), according to the information reported in the OIE-World Animal Health Information System (OIE-WAHIS, https://wahis.woah.org). The viruses caused three waves of outbreaks in multiple countries in Asia, Africa, Europe, and North America. The first wave, which occurred from 2005–2010, was caused by H5N1 viruses and 55.2 million poultry died or were destroyed. The outbreaks that occurred during this period were mainly reported in Asian countries, although some African and European countries were also affected (Figure 1(b)). The second wave, which occurred from 2011 to 2019, was caused by multiple subtypes of H5 viruses and 139.9 million poultry died or were destroyed (Figure 1(a)). The outbreaks in this period were reported in Asia, Europe, Africa, and North America (Figure 1(b)). The third wave started in 2020 and was mainly caused by H5N8 and H5N1 viruses; 193.9 million poultry died or were destroyed as of the end of November 2022 (Figure 1(a)). The outbreaks in this period were mainly reported in Europe and North America, although some were also reported in Asian and African countries (Figure 1(b)).

Of the 389 million poultry that died or were destroyed, H5N1 viruses were responsible for 204 million, H5N8 viruses were responsible for 111 million, and the other 74 million poultry losses were caused by other H5 viruses (Figure 1(c)). Of note, 92.6 million, 54 million, 39.6 million, and 7.7 million poultry died or were destroyed in Europe, North America, Asia, and Africa, respectively, since 2020 (Fig 1(d)). The large number of birds that died or were destroyed in the third wave in a relatively short period of time suggests that the ongoing third wave will be much more serious than previous ones, if control measures taken in Europe and North America do not change.

Table 1. Outbreaks caused by H7 highly pathogenic avian influenza viruses between January 2005 and November 2022 around the world.

Year	Continent	Country	Subtype	Outbreaks	Number of poultry dead/destroyed
2005	Asia	Democratic People's Republic of Korea	H7N7	3	218,788
2007	North America	Canada	H7N3	1	49,100
2008	Europe	United Kingdom	H7N7	1	25,000
2009	Europe	Spain	H7N7	1	308,640
2012–2022	North America	Mexico	H7N3	75	29,813,496
2012	Oceania	Australia	H7Nx*	1	50,000
2013	Europe	Italy	H7N7	2	1,178,861
2013	Oceania	Australia	H7N2	1	490,000
2015	Europe	Germany	H7N7	1	10,104
2015	Europe	United Kingdom	H7N7	1	179,865
2016	Europe	Italy	H7N7	1	66,972
2016	North America	United States	H7N8	1	43,500
2017–2018	Asia	China	H7N9	12	745,665
2017	North America	United States	H7N9	1	74,000
2020	North America	United States	H7N3	1	34,160
2020	Oceania	Australia	H7N7	3	435,378
Total				106	33,723,529

*The NA subtype was not reported.

Avian influenza outbreaks caused by H7 viruses

In the last century, avian influenza outbreaks caused by different H7 viruses have occurred in five countries. The first outbreak was caused by H7N3 virus in turkeys in England in 1963 [22]. Five outbreaks occurred in domestic poultry in Australia in 1976, 1985, 1992, 1994, and 1997, respectively, and were caused by H7N7 virus (1975 and 1985), H7N3 virus (1992 and 1994), and H7N4 virus (1997) [23–25]. In 1979, H7N7 virus caused outbreaks in domestic poultry in Germany and England [26]; in 1995, an H7N3 virus caused outbreaks in chickens in Pakistan [27]; and in 1999–2000, H7N1 virus caused outbreaks in multiple species of domestic poultry in Italy [28].

In 2002, outbreaks in chickens caused by H7N3 virus occurred in Chile [29]; in 2003, outbreaks in different domestic poultry caused by H7N7 virus occurred in The Netherlands, Belgium, and Germany [30]; and in 2004, an H7N3 virus caused outbreaks in chickens in Canada [31]. The number of birds killed or destroyed in the outbreaks that occurred before 2004 is not available.

Between January 2005 and November 2022, different H7 highly pathogenic avian influenza viruses caused 106 outbreaks and the loss of over 33 million poultry around the world, according to data reported in the OIE-WAHIS (Table 1). These outbreaks occurred in 10 countries across Asia, Europe, North America, and Oceania, including Australia, Canada, Mexico, the US, the Democratic People's Republic of

Table 2. Human infections caused by H5 viruses around the world from January 2003 to April 2022*.

	Case information				
Country	Total Number	Year	Virus subtype	Number infected	Number of fatalities
Azerbaijan	8	2006	H5N1	8	5
Bangladesh	8	2008, 2011–2013, 2015	H5N1	8	1
Cambodia	56	2005–2014	H5N1	56	37
Canada	1	2013	H5N1	1	1
China	127	2003, 2005–2015	H5N1	53	31
		2014–2022	H5N6	74**	32
Djibouti	1	2006	H5N1	1	0
Egypt	359	2006–2017	H5N1	359	120
India	1	2021	H5N1	1	1
Indonesia	200	2005–2015, 2017	H5N1	200	168
Iraq	3	2006	H5N1	3	2
Lao	4	2007, 2020	H5N1	3	2
		2021	H5N6	1	0
Myanmar	1	2007	H5N1	1	0
Nepal	1	2019	H5N1	1	1
Nigeria	1	2007	H5N1	1	1
Pakistan	3	2007	H5N1	3	1
Russia	7	2020	H5N8	7	0
Thailand	25	2004–2006	H5N1	25	17
Turkey	12	2006	H5N1	12	4
UK	1	2021	H5N1	1	0
US	1	2022	H5N1	1	0
Viet Nam	127	2003–2005, 2007-2010, 2012–2014	H5N1	127	64
Total	947	/	/	947	488

*Data obtained from the WHO website.
**Forty-nine of the 75 human cases infected with H5N6 virus have occurred since January 2021.

Table 3. Human infections caused by H7 viruses around the world since 1959.

	Case information					
Country	Total Number	Time period	Virus subtype	Pathotype	Number infected	Number of fatalities
Australia	1	1977	H7N7	Highly pathogenic (HP)	1	0
Canada	2	2004	H7N3	HP	2	0
China	1569	Feb. 2013–Sept, 2017	H7N9	Low pathogenic (LP)/HP	1564	615
		Oct. 2017–Sept, 2018	H7N9	HP	3	1
		2018	H7N4	LP	1	0
		2019	H7N9	HP	1	0
Italy	10	2002–2003	H7N3	LP	7[#]	0
		2013	H7N7	HP	3	0
Mexico	2	2012	H7N3	HP	2	0
The Netherlands	89	2003	H7N7	HP	89	1
UK	6	1996	H7N7	LP	1	0
		2006	H7N3	LP	1	0
		2007	H7N2	LP	4	0
US	8	1959	H7N7	HP	1	0
		1979	H7N7	LP	4	0
		2002–2003, 2016	H7N2	LP	3	0
Total	1687	/	/	/	1687	617

[#]Serologic evidence only.

Korea, China, the UK, Spain, Italy, and Germany. Of note, 77 outbreaks in countries in North America were caused by H7N3 viruses, resulting in the loss of more than 29 million birds. The H7N7 viruses caused 10 outbreaks in European countries and the Democratic People's Republic of Korea, and H7N9 viruses caused outbreaks in the US and China. At least three different subtypes of H7 viruses were responsible for the outbreaks in Australia. These facts indicate that the H7 viruses are actively circulating in nature and continue to pose a threat to the global poultry industry.

Human infections caused by H5 and H7 viruses

In 1997, H5N1 avian influenza viruses transmitted from birds to humans in Hong Kong causing the deaths of 6 of 18 infected persons [3]; this was the first report of human infection with lethal H5N1 virus and attracted wide attention. Since 2003, 865 human cases of H5N1 virus infection have been reported in more than 20 countries across Asia, Africa, Europe, and North America (Table 2). Seventy-five human cases of H5N6 virus infection have been reported in China and Lao, whereas seven human cases of H5N8 virus infection were reported in Russia (Table 2). Among the 947 human cases involving different viruses reported from 2003 to April 2022, 488 were fatal (Table 2). Studies have identified several key amino acids in the HA of H5N1 viruses that increase the affinity of these viruses for human-type receptors [10,11,32], and several research groups have demonstrated that H5N1 virus can become transmissible via respiratory droplets in ferrets or guinea pigs after obtaining certain mutations or reassorting with human influenza viruses [10–12].

As described above, different subtypes of H7 highly pathogenic influenza viruses have caused disease outbreaks in poultry around the world. Historically, both low and highly pathogenic H7 influenza viruses caused human infections, and a total of 1687 human cases were documented in eight countries between 1959 and 2019 (Table 3). The cases reported in Australia, Canada, Italy, Mexico, the UK, and the US ranged from one to 10, and all of the infected individuals survived the infection (Table 3) [5,7,8,33–43]. Eighty-nine human cases infected with highly pathogenic H7N7 virus were reported in The Netherlands in 2003, and one veterinarian died from the infection [6]. One human case infected with H7N4 virus and 1568 human cases infected with H7N9 viruses were reported in China [4]; 616 of the H7N9 virus infections were fatal (Table 3).

The thousands of human cases of infection with H5 or H7 viruses indicate that humans are highly susceptible to these viruses. Epidemiology studies have shown that humans become infected mainly through exposure to virus-infected poultry or a contaminated environment [44]; human-to-human transmission has been very limited. Therefore, before the H5 and H7 viruses acquire the ability to transmit from human to human, control of these viruses in animals is essential and effective to prevent them from infecting humans.

Evolution and spread of H5 viruses by migratory wild birds

Influenza viruses evolve mainly through the accumulation of mutations in their genomes and reassortment between different strains. The HA genes of H5 viruses detected since 2003 can be roughly divided into nine different clades, and some clades have been further divided into different subclades [45]. Viruses bearing the HA gene of the same clade may have different NA and internal genes, and may therefore belong to different genotypes. Most H5 viruses have been

detected only in certain countries or regions, and only strains that infect long-distance migratory birds have spread over different continents and caused disastrous consequences. Of the four large-scale intercontinental transmissions of H5 viruses in the past 20 years, two originated in Asia (H5N1 in 2005 and H5N8 in 2014) and two originated in Europe (H5N8 in 2020 and H5N1 in 2021).

The first H5 virus that was widely spread by migratory wild birds was the so-called Qinghai Lake-like H5N1 virus. In May 2005, migrating bar-headed geese carrying at least three different genotypes of H5N1 virus bearing the clade 2.2 HA gene flew over the Himalayas to the egg island in Qinghai Lake in western China, a major breeding ground for migratory birds [21,46]. The viruses spread to several other species on the island, including great black-headed gulls, brown-headed gulls, great cormorants, ruddy shelducks, and whooper swans, and caused the death of over 6,000 wild birds at the lake from 4 May to 29 June 2005 [21]. The viruses were subsequently spread by whooper swans to Mongolia and Russia in August 2005, and were then widely detected in wild birds and domestic poultry in European and African countries in 2006 [47]. These viruses were eradicated in China and many other European countries in a relatively short time, but they circulated in poultry for many years and caused severe disease in poultry and humans in Egypt [48].

The second H5 virus that was widely spread by migratory birds was the H5N8 virus bearing the subclade 2.3.4.4 HA gene. In early 2014, a novel H5N8 virus bearing the subclade 2.3.4.4 HA gene caused multiple outbreaks in migratory birds and domestic ducks in South Korea [49], and was subsequently spread to Europe, North America, and East Asia by migratory birds [50]. The H5N8 viruses continued to evolve and spread and caused numerous outbreaks in wild birds and domestic poultry in countries in Asia, Europe, and Africa [51]. Although similar H5N8 viruses were also detected in swans and grey-legged geese in China at the end of 2016 and in earlier 2017 [52], they did not infect and spread among domestic poultry in China, probably because the vaccine used in poultry in China was effective against these H5N8 viruses [53].

The third H5 virus that was spread widely by migratory birds was the H5N8 virus bearing the subclade 2.3.4.4b HA gene. In January 2020, a novel H5N8 virus bearing the clade 2.3.4.4b HA caused outbreaks in chickens in Poland and then started a new wave of outbreaks in poultry and wild birds in countries in Europe, Africa, and Asia [54]. By the end of March 2022, the H5N8 viruses were reported in more than 42 countries, and nearly 60 million domestic poultry had died or were destroyed (http://empres-i.fao.org/eipws3 g/). The HA genes of these H5N8 viruses formed two different branches that probably separated in early 2018 [55]. The viruses with the branch I HA circulated in domestic poultry and wild birds in Poland, Hungary, Germany, and Czech Republic in the spring and summer of 2020, and were then detected in domestic poultry and wild birds in Japan and Korea in the winter of that year. In January 2021, a virus bearing the branch I HA was detected in a whopper swan in Shandong Province, China. The virus bearing the branch II HA was first detected in chickens in Iraq in May 2020, then caused multiple disease outbreaks in domestic poultry in July and August 2020 in Russia, and was responsible for subsequently widespread disease outbreaks in wild birds and domestic poultry in Russia and many countries in the Middle East, Europe, Africa, and Asia. The virus bearing the branch II HA began to be detected in swans and other wild birds in China from October 2020, and was also detected in ducks and geese in 2021. Of note, H5N8 viruses bearing the branch II HA gene were also detected in humans in Russia and in seals and a fox in the UK [56–58].

Emergence, evolution, and global dissemination of the recent H5N1 influenza viruses bearing the clade 2.3.4.4b HA

During their circulation in nature, the H5N8 viruses reassorted with other influenza viruses and generated several other subtypes of H5 viruses that bear the clade 2.3.4.4b HA gene. H5N2 viruses were detected in wild birds in Serbia and domestic poultry in Taiwan, China and Bulgaria [54,59]; H5N3 viruses were detected in wild birds in Denmark, France, Germany, Ireland, and The Netherlands [54]; H5N4 viruses were detected in wild birds in Germany, The Netherlands, and Sweden [54]; H5N5 viruses were detected in wild birds and domestic poultry in Iran and many countries in Europe [54]; and H5N6 viruses were detected in ducks in China and have caused multiple cases of human infection [2,60]. The most important descendant of the H5N8 virus is the novel H5N1 virus that was first detected in The Netherlands [61]. Unlike the H5N2, H5N3, H5N4, H5N5, and H5N6 viruses, each of which has only been detected in countries on one or two continents, the H5N1 virus bearing the clade 2.3.4.4b HA gene took over from H5N8 virus and started the fourth large-scale, intercontinental spread. This novel H5N1 virus has caused 4,284 disease outbreaks, as of the end of March 2022, in many countries in Europe, Africa, Asia, and the Americas since it emerged in The Netherlands in October 2020 [54].

Genetic analysis revealed that the novel index H5N1 virus A/Eurasian wigeon/Netherlands/1/2020 (H5N1) is a reassortant of five different viruses: an

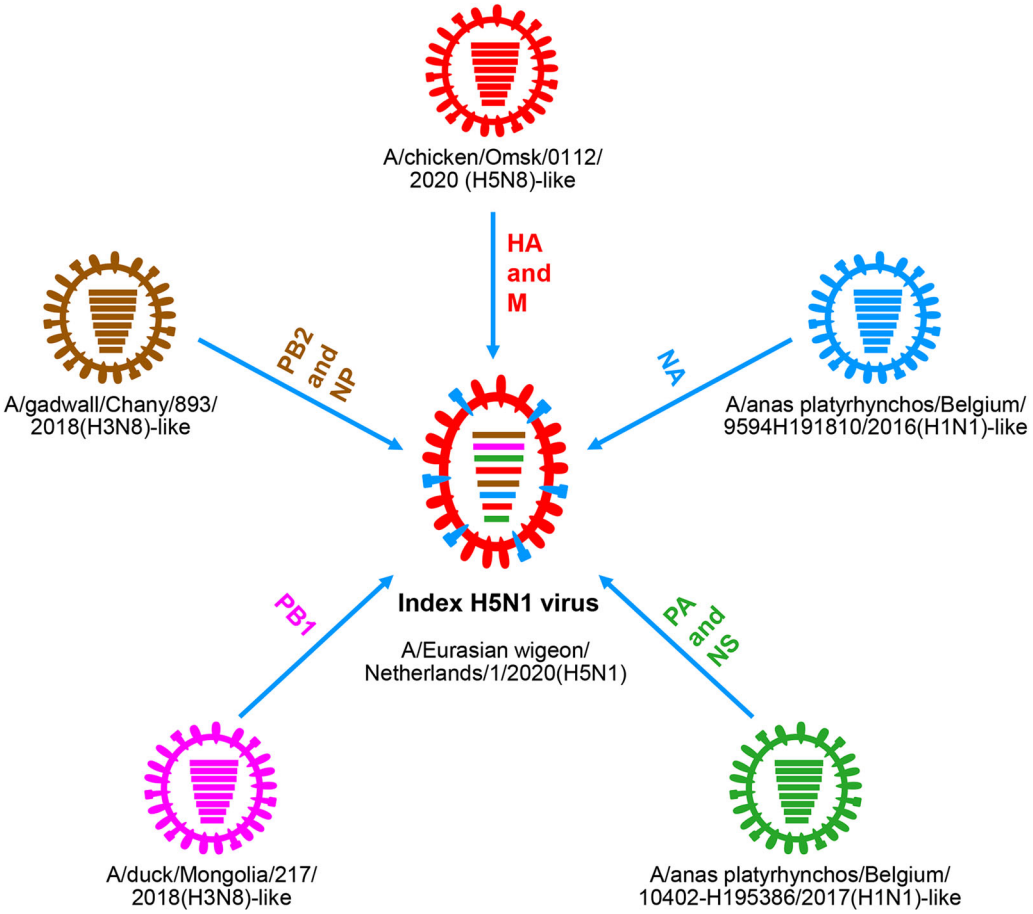

Figure 2. Formation of the index H5N1 virus bearing the 2.3.4.4b HA gene in 2020. The eight bars represent the eight gene segments (from top to bottom: PB2, PB1, PA, HA, NP, NA, M, and NS), and the colour of the bar indicates the closest donor strain of the gene segment.

H5N8 virus provided the HA and M genes, an A/gadwall/Chany/893/2018(H3N8)-like virus provided the PB2 and NP genes, an A/duck/Mongolia/217/2018(H3N8)-like virus provided the PB1 gene, an A/anas platyrhynchos/Belgium/10402-H195386/2017 (H1N1)-like virus provided the PA and NS genes, and an A/anas platyrhynchos/Belgium/9594H191810/2016 (H1N1)-like virus provided the NA gene (Figure 2) [62].

Cui et al. analyzed 233 representative H5N1 viruses bearing the clade 2.3.4.4b HA gene detected in Europe, Africa, Asia, and North America that were reported from October 2020 to March 2022 and revealed their spatiotemporal spread. They found that these viruses formed 16 different genotypes (G1-G16) (Figure 3(a)), and viruses of nine genotypes were only detected in one country or one region (Europe was analyzed as four regions—Northern Europe, Southern Europe, Central and Eastern Europe, and Western Europe – in Figure 3 of this review): G2, G11, G15, and G16 were only detected in Western Europe, G13 was only detected in Central and Eastern Europe, G3 and G6 were only detected in Russia, and G9 and G14 were detected in China and Bangladesh, respectively. Viruses of the other seven genotypes spread between countries, regions, or continents (Figure 3(b–e)). Between October 2020 and August 2021, the G1 virus circulated in multiple countries in Europe and Africa (Figure 3(b)); between August 2021 and November 2021, viruses of the G4, G5, G8, and G12 genotypes spread among European countries, and the G1 virus spread from southern Europe to Russia and from northern Europe to China (Figure 3(c)). In the following month, the G1 virus spread from Western Europe to the US, and the G7 virus was generated in Japan/Korea and spread to China (Figure 3(d)). The G10 virus was generated and detected first in Russia in October 2021 and then spread to and was detected in China in March 2022 (Figure 3(e)). Of note, H5N1 viruses are still circulating in multiple countries and causing disease outbreaks in wild birds and poultry [54], and continued reassortment and spread of the viruses are inevitable.

Control of H5 influenza by vaccination: the China experience

Different countries have adopted different strategies to control highly pathogenic avian influenza. Many countries in Europe and North America control highly

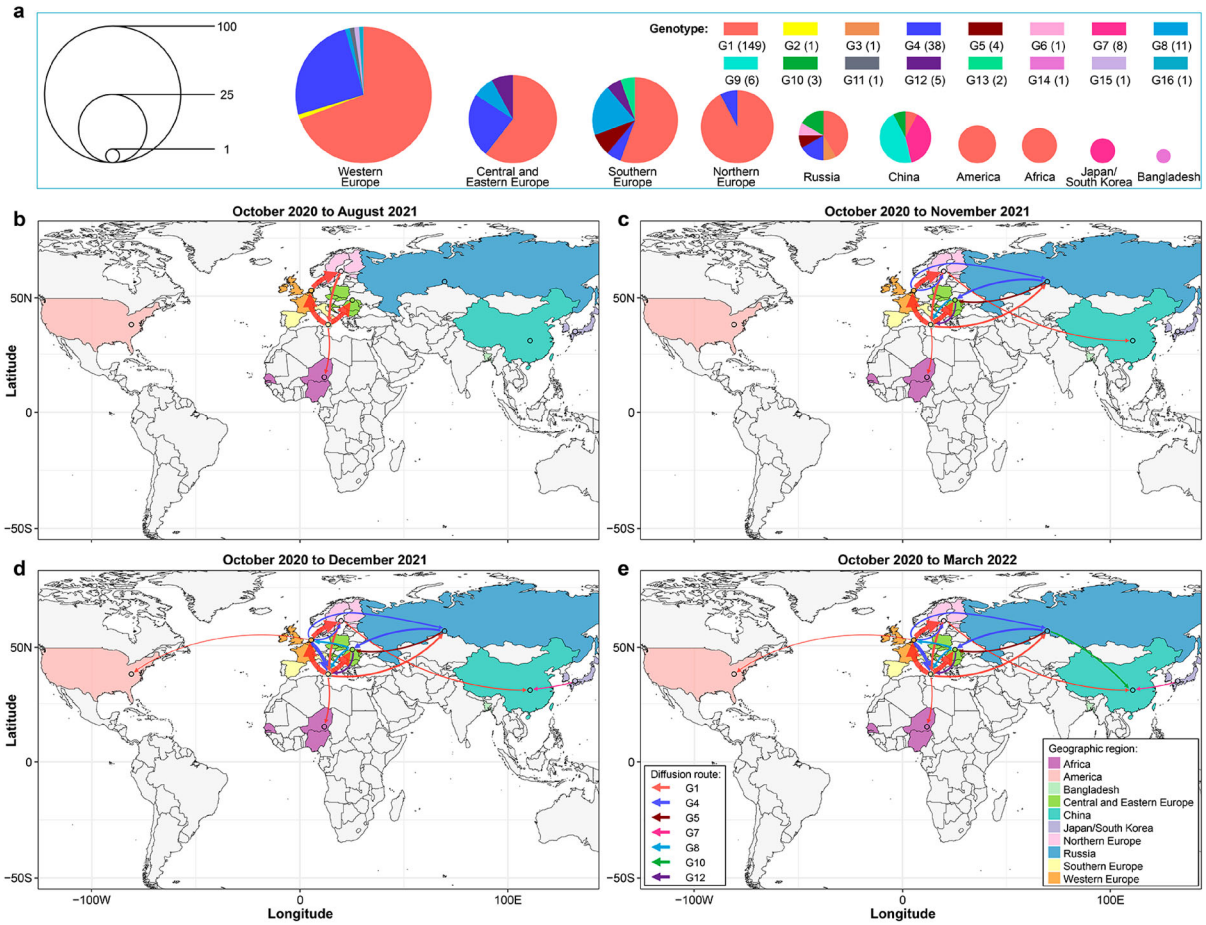

Figure 3. Spatiotemporal spread of H5N1 viruses bearing the clade 2.3.4.4b HA gene. (a) Genotype and distribution of 233 H5N1 viruses isolated from 28 countries between October 2020 and March 2022. (b–d). Emergence and spread of the indicated seven genotypes that were detected in more than one country/region/continent.

pathogenic influenza by culling infected and suspected birds (also called the stamping-out strategy), whereas some countries, including China, have adopted a "cull plus vaccination" strategy.

Over 17 billion poultry, including 4 billion ducks, are reared annually in China. Many birds, especially ducks and geese, are often reared in open fields with no biosecurity measures. We started to develop an H5 vaccine as soon as the first highly pathogenic H5N1 virus was detected in Guangdong in 1996 [63]. In addition to the inactivated vaccine described below, a novel Newcastle virus (NDV)-vectored H5 avian influenza bivalent live vaccine has been used in chickens in China since 2006 [64], and the first H5 DNA vaccine was approved in 2018 [65], but has not been used yet due to vaccine updates. A duck enteritis virus (DEV)-vectored bivalent live vaccine has been constructed and found to provide fast and complete protection in ducks against H5N1 avian influenza virus and highly lethal duck enteritis virus [66]. Most importantly, the DEV-vectored vaccine provided good cross-protection against challenge with different clades of viruses [67]. The DEV-vectored vaccine is not yet officially used to control avian influenza in ducks, as its licence is still pending.

An inactivated vaccine produced with the naturally isolated H5N2 low pathogenic virus A/turkey/England/N28/73(H5N2) was used in China from 2004 to 2006. However, influenza virus mutates easily, and mutation of the HA gene often causes antigenic variation. The biggest challenge for the vaccination strategy is ensuring that the vaccine matches the circulating virus. To address this challenge, a platform for generating vaccine seed viruses by using reverse genetics was established, and an ideal vaccine seed virus containing the modified HA gene and native NA gene of a prevalent H5 virus and the internal genes of the high-growth A/Puerto Rico/8/1934 (H1N1) (PR8) virus can be generated within a week.

Similar to the introduction of H5N8 and H5N1 viruses into China over the past two years, viruses carrying different clades or subclades of HA genes have been introduced into China over the past two decades [52,55,62,68–70]. In response, since 2004, ten different H5 seed viruses generated by reverse genetics have been used for inactivated vaccine production to control and eliminate these viruses (Table 4). Unlike the NDV-vectored vaccines, which are used only in chickens, the reverse genetics inactivated vaccines have

Table 4. Inactivated vaccine seed viruses generated by reverse genetics for the control of highly pathogenic avian influenza in China since 2004[a].

Seed virus (subtype)	HA donor virus (clade)[b]	Application period[c]	Effective against influenza virus of a different subtype (clade)	Reference
H5-Re1 (H5N1)	GS/GD/1/1996(H5N1) (0)	03/2004–03/2008	H5 (0, 1, 2.2, 2.3.4)	[68,72,73]
H5-Re4 (H5N1)	CK/SX/2/2006(H5N1) (7.2)	07/2006–04/2014	H5 (7.2)	[76]
H5-Re5 (H5N1)	DK/AH/1/2006(H5N1) (2.3.4)	03/2008–06/2012	H5 (2.3.4)	[76]
H5-Re6 (H5N1)	DK/GD/S1322/2010(H5N1) (2.3.2)	06/2012–09/2017	H5 (2.3.2)	[75]
H5-Re7 (H5N1)	CK/LN/S4092/2011(H5N1) (7.2)	04/2014–09/2017	H5 (7.2)	[74]
H5-Re8 (H5N1)	CK/GZ/4/2013(H5N1) (2.3.4.4g)	12/2015–12/2018	H5 (2.3.4.4g)	[53]
H5-Re11 (H5N1)	DK/GZ/S4184/2017(H5N6) (2.3.4.4h)	12/2018–12/2021	H5 (2.3.4.4h)	[76]
H5-Re12 (H5N1)	CK/LN/SD007/2017(H5N1) (2.3.2.1f)	12/2018–12/2021	H5 (2.3.2.1f)	[76]
H5-Re13 (H5N6)	DK/FJ/S1424/2020(H5N6) (2.3.4.4h)	01/2022–	H5 (2.3.4.4h)	[77]
H5-Re14 (H5N8)	WS/SX/4-1/2020(H5N8) (2.3.4.4b)	01/2022–	H5 (2.3.4.4b)	[77]
H7-Re1 (H7N9)	PG/SH/S1069/2013(H7N9)	09/2017–12/2018	H7N9	[33,34]
H7-Re2 (H7N9)	CK/GX/SD098/2017(H7N9)	12/2018–07/2020	H7N9	[76,82]
H7-Re3 (H7N9)	CK/IM/SD010/2019(H7N9)	07/2020–12/2021	H7N9	[77]
H7-Re4 (H7N9)	CK/YN/SD024/2021(H7N9)	01/2022–	H7N9	[77]

[a]Only vaccine seed viruses prepared by the Harbin Veterinary Research Institute are listed in this table.
[b]Abbreviations: GS, goose; CK, chicken; DK, duck; WS, whooper swan; PG, pigeon; GD, Guangdong; SX, Shanxi; AH, Anhui; LN, Liaoning; GZ, Guizhou; FJ, Fujian; SH, Shanghai; GX, Guangxi; IM, Inner Mongolia; YN, Yunnan.
[c]When two or three seed viruses are used at the same time, it means that these seed viruses are used for bivalent or trivalent inactivated vaccine production.

been used in chickens and waterfowl, and their effectiveness in these species is well documented [64,71]. The H5-Re1 vaccine seed virus, which derives its HA and NA genes from A/goose/Guangdong/1/1996 (H5N1), started to be used in 2004 and provided solid protection against viruses bearing the clade 0 HA, clade 1 HA, clade 2.2 HA, or 2.3.4 HA gene [68,72,73]. In March 2008, the H5-Re1 seed virus was replaced by the H5-Re5 seed virus, which derived its HA and NA genes from A/duck/Anhui/1/2006 (H5N1). The H5N1 viruses bearing the clade 2.3.4 HA gene were eliminated by using the H5-Re5 vaccine and use of the vaccine was suspended in June 2012 (Table 4). The H5-Re4 and H5-Re7 vaccine seed viruses were developed in 2006 and 2014, respectively, to control viruses bearing the clade 7.2 HA gene, which were completely eliminated in China in 2017 (Table 4) [74]. The H5-Re6 and H5-Re12 vaccine seed viruses were developed and used in 2012 and 2018, respectively, to control viruses bearing the clade 2.3.2 HA gene and viruses bearing the clade 2.3.2.1f HA gene, respectively; the use of these vaccines was stopped in 2017 and 2021, respectively, when the viruses were eliminated in China (Table 4) [75,76]. The H5-Re8, H5-Re11, H5-Re13, and H5-Re14 vaccine seed viruses were developed to control H5 viruses bearing different subclades of 2.3.4.4 HA that have been introduced into China in recent years, and currently only the H5-Re13 and H5-Re14 vaccines are used to control the local H5 virus bearing clade 2.3.4.4h HA and the globally circulating H5 viruses bearing clade 2.3.4.4b HA (Table 4) [53,76,77]. Of note, the vaccine used in China is updated when a clear antigenic difference between the vaccine and the newly detected virus is observed, even though sometimes the vaccine could still provide complete protection against the emerging virus.

Emergence, evolution, and effective control of H7N9 influenza virus in China

In February 2013, the H7N9 virus emerged in the live poultry markets in China. Genetically the H7N9 virus is a reassortant of three different viruses: A/duck/Zhejiang/12/2011(H7N3)-like virus provided the HA gene, A/wild bird/Korea/A14/2011(H7N9)-like virus provided the NA gene, and the local H9N2 viruses provided the six internal genes (Figure 4(a)) [78]. Animal studies indicated that the early H7N9 viruses were low pathogenic in chickens and hardly infected ducks [79]; however, after four years of circulation in nature, the viruses obtained certain amino acids in their HA cleavage site and became highly pathogenic in chickens in Guangdong in 2017 (Figure 4(a)) [9]. The increased replicative ability of highly pathogenic H7N9 viruses enabled them to reassort with other duck viruses and generate novel lethal H7 viruses in ducks [33].

The H7N9 viruses have high potential to cause a human influenza pandemic. The H7N9 viruses bind to human-type receptors with high affinity and to avian-type receptor with very low affinity [9,79], which allows the virus to infect humans very easily, as evidenced by the fact that the virus caused over 1560 human infections in five waves from February 2013 to 30 September 2017, with a mortality rate of nearly 40%. Between 1 October 2016 and 30 September 2017, there were 766 human cases (48.9% of the total) reported [33,34], which raised concerns that an even large number of human infections may occur in the subsequent wave. Sequence analysis indicated that after replication in humans, over 78% of the H7N9 strains acquired the 627K mutation in their PB2 gene [9], and Liang and colleagues found that the low polymerase activity attributed to the viral PA protein

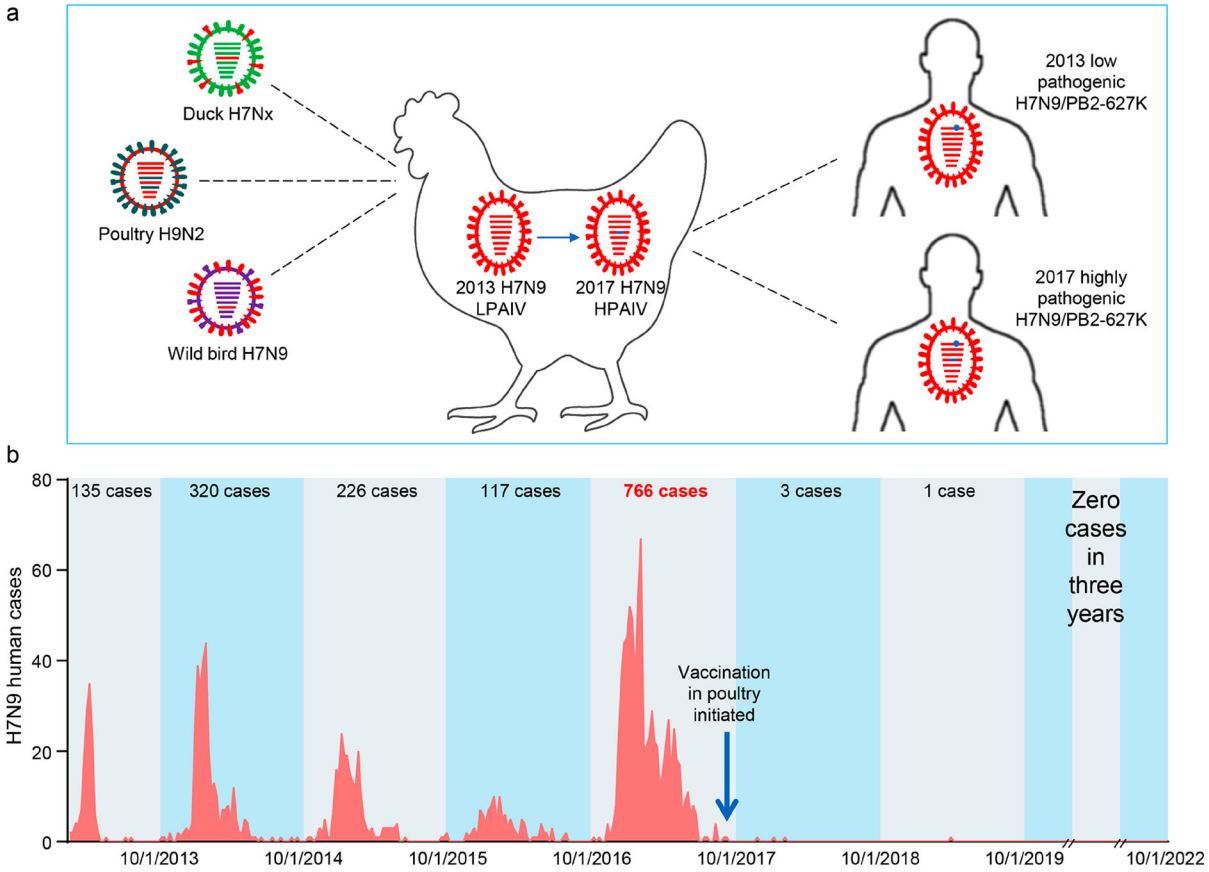

Figure 4. H7N9 viruses detected in China and the human infections they have caused since 2013. (a) Diagram of the emergence and evolution of H7N9 viruses in China. LPAIV, low pathogenic avian influenza virus; HPAIV, highly pathogenic avian influenza virus. (b) Human infections with H7N9 viruses in China.

is the intrinsic driving force behind the emergence of PB2 627K during H7N9 virus replication in mammals [80]. This PB2 627K mutation dramatically increases the replication and virulence of H7N9 virus in mammals, and promotes the respiratory droplet transmission of the H7N9 viruses in mammalian animal models [9]. A recent study indicated that efficient replication of H7N9-PB2/627K virus in the lungs of mice activates gasdermin E-mediated pyroptosis in alveolar epithelial cells and triggers a lethal cytokine storm in mice, thereby revealing the underlying mechanism behind the lethality of H7N9 virus infection in humans [81].

The H7N9 viruses not only caused severe public health problems and concerns, but also caused considerable damage to the poultry industry in China. During each human H7N9 infection wave, in addition to culling poultry from poultry markets that were positive for the virus, tons of uninfected poultry and poultry products were destroyed because people were afraid to consume them. The highly pathogenic H7N9 virus that emerged in early 2017 caused several disease outbreaks on chicken farms in many provinces [33]. Given the damage the H7N9 lethal virus has and will cause to poultry and the high risk it poses to human health, control and eradication of both the low and highly pathogenic H7N9 viruses became the highest priority for animal disease control authorities in China in 2017. Five waves of human infections combined with the emergence of the highly pathogenic H7N9 virus suggested that stamping-out was not a successful measure for H7N9 control; therefore, a vaccination strategy was developed.

To save labour and increase the efficiency of the poultry vaccine strategy, an H5/H7 bivalent inactivated vaccine was developed by using the H7N9-Re1 and H5-Re8 viruses as seed viruses (Table 4) [33,34]. The vaccine was extensively evaluated for safety and efficacy in the laboratory setting with different H5 and H7 viruses. The vaccine provided solid protection against the H7N9 low pathogenic virus and different H7N9 highly pathogenic viruses in chickens [33,34], and its application in poultry was initiated in September 2017 in China. The prevalence of H7N9 virus in poultry was largely prevented, as evidenced by the fact that the isolation rate of H7N9 virus in poultry was reduced by 93.3% after birds were inoculated with the H5/H7 vaccine [33]. More importantly, the vaccination of poultry successfully eliminated human infections with H7N9 virus: only three human cases and one human case were reported during the sixth and seventh waves, respectively, and no human case has been detected since April 2019 (Figure 4(b)).

The H7N9 viruses has still occasionally been detected in chickens, and the vaccine seed virus has been updated three times (Table 4) [76,77,82]. One study indicated that the recent H7N9 viruses have lost the ability to bind to human-type receptors [82], suggesting that the risk to public health posed by the recent viruses may be reduced compared with the earlier ones.

In summary, H5 and H7 subtype avian influenza viruses have caused severe problems to the global poultry industry with more than 389 million domestic birds dying or being destroyed since 2005, including 193.9 million birds lost between January 2020 and November 2022. These viruses also pose severe threats to public health and have caused 2634 human cases with over 1000 fatalities. Vaccines have been used in poultry to successfully prevent highly pathogenic influenza virus infection in China; even though the globally circulating H5 viruses have been detected in many species of wild birds and occasionally in ducks or geese in recent years, they have never caused problems on routinely vaccinated poultry farms in China, and the pervasive H7N9 viruses have been nearly eliminated in China. H5N1 viruses bearing the clade 2.3.4.4b HA gene are widely circulating in wild birds and causing problems in domestic poultry in numerous countries around the world. To improve animal welfare, reduce economic damage, and reduce human infections, vaccination should be immediately and seriously considered as a control strategy not only in underdeveloped countries, but also in developed countries. Any unnecessary obstacles to vaccination strategies should be removed immediately and forever.

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Disclosure statement

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Author contributions

J.S., X.Z., P.C., and C.Y. analyzed the data; J.S. and H.C. wrote the manuscript.

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