EPIDEMIOLOGY OF AVIAN INFLUENZA VIRUSES

EDITED BY: Irene Iglesias, Timothée Vergne, Mathilde C. Paul, Paolo Mulatti and Thanawat Tiensin

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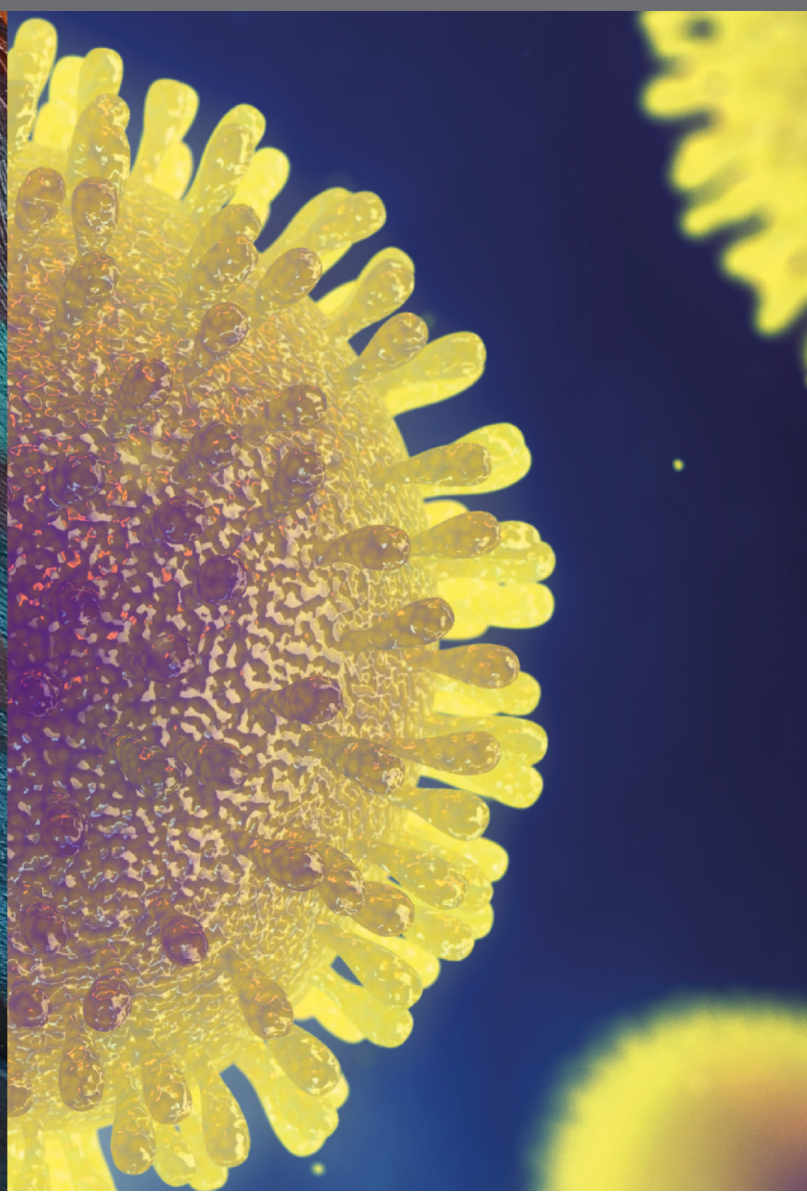

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EPIDEMIOLOGY OF AVIAN INFLUENZA VIRUSES

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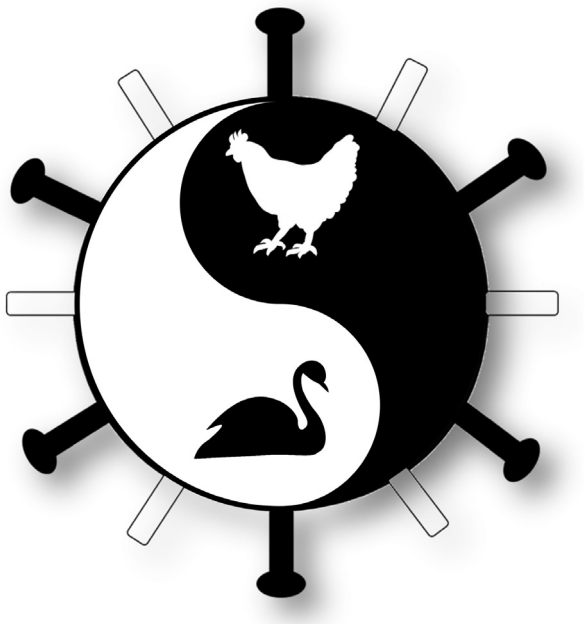

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Avian influenza is a highly contagious viral disease, characterized by intense circulation in the wild waterbird reservoirs, with periodical introductions into the domestic poultry sector. AI viruses have been the source of devastating economic losses in the poultry industry over the last three decades, and have become a major veterinary and public health concern due to their zoonotic potential. The most emblematic illustration of this impact has been the emergence of the HPAI H5N1 virus in southern China in the mid-1990s, followed by its continental spread across East and Southeast Asia, and the unprecedented epidemics recorded in 2003–2004. More recently (from 2014 to 2017), several subtypes of HPAI (including H5N1, H5N6, H5N8) emerged in East Asia and spread intercontinentally, stressing the crucial role of this geographical hotspot as a source of new HPAI subtypes. The international dimension and the difficulty to effectively control those epidemics highlight the need for a global approach to

HPAI surveillance and a comprehensive knowledge on epidemiology and patterns of the disease. This Research Topic aims at contributing to fill this gap. It includes ten papers which supplement the knowledge of the epidemiology of AI and offer new approaches on control strategies in various regions of the world.

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EDITORIAL
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Editorial: Epidemiology of Avian Influenza Viruses

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Keywords: avian influenza, epidemiology, surveillance, animal health, control strategies, domestic poultry, wild birds

Editorial on the Research Topic

Epidemiology of Avian Influenza Viruses

Avian influenza (AI) is a highly contagious viral disease, characterized by an intense circulation in many wild waterbird reservoir populations, with periodical introduction into the domestic poultry sector. AI viruses have been the source of devastating economic losses in the poultry industry over the last three decades and have become a major veterinary and public health concern due to their zoonotic potential (1, 2). Outbreaks caused by highly pathogenic avian influenza (HPAI) viruses have caused serious animal health crises worldwide, such as the high case fatality rates in poultry, the control measures that are applied (massive pre-emptive culling or vaccination) and the consequences of virus detection on the international poultry produce trade.

The most emblematic illustration of this impact was the emergence of the HPAI H5N1 virus in southern China in the mid-1990s, followed by its continental spread across East and Southeast Asia, and the unprecedented epidemics recorded in 2003–2004. More recently (from 2014 to 2017), several subtypes of HPAI (including H5N1, H5N6, H5N8) have emerged in East Asia and spread intercontinentally, stressing the crucial role of this geographical hotspot as a source of new HPAI subtypes (3, 4). The international dimension and the difficulties in effectively controlling these epidemics, highlight the need for more scientific information in relation to the epidemiology and patterns of the disease in affected countries, especially in East Asia, as well as the need for effective policies against HPAI. This Research Topic aims at contributing to fill this gap. It includes 10 papers which supplement the knowledge of the epidemiology of AI and offer new approaches and insights for surveillance and control strategies in various regions of the world (including France, Germany, the USA, Vietnam, Australia, and Indonesia).

Undoubtedly, the rapid and continuous evolution of AI viruses make their surveillance and control particularly challenging. Dhingra et al. collated all emergence events of H5 and H7 HPAI subtypes, reported since 1959, and used spatial and phylogeographical analyses to shed new light on the emergence processes of highly pathogenic strains. An increase in viral reassortment rates and an antigenic diversity found in China and Vietnam emphasizes the need for further research in these HPAI emergence hotspots. Furthermore, the identification of differences in the spatio-temporal patterns and risk factors for HPAI subtypes in Vietnam, as developed by Mellor et al. highlights the challenge of tailoring surveillance and intervention strategies to the epidemiological contexts and subtypes of interest. Control can be particularly challenging in endemic areas, such as Indonesia, where multiple HPAI virus subtypes and clades may circulate, as described by Durr et al. The authors illustrate the importance of the seed strain used in vaccine developments and of the antigens used to assess sero-protection of vaccinated flocks, under field conditions.

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Another great challenge of HPAI control is the intense circulation of AI viruses in waterfowl populations, which act as a natural reservoir with periodic spill-over to domestic poultry. Globig et al. conducted a detailed examination of the distribution of the HPAI H5N8 cases that were reported in wild or captive birds in Germany (2016–2017). They emphasize the lessons learnt during the epidemic in terms of prevention and control, highlighting substantial gaps in farm biosecurity.

AI management is further complicated by the fact that viruses are able to spread through a large number of transmission routes. Identifying these pathways is key in the development of appropriate prevention and control strategies. Walz et al. described the types of potentially infectious or contaminated materials that are disposed of in different US poultry sectors and suggested that poultry farm garbage management and disposal practices may well-contribute to the spread of HPAI viruses between farms. The potential of airborne transmission was questioned by Scoizec et al. who detected the presence of the AIV genome in some air samples collected up to 110 m outside of infected premises during the French HPAI H5N8 epidemic (2016–2017). Based on these results, the authors stress the challenge of implementing the depopulation of infected farms, without contributing to the airborne diffusion of the virus.

Veterinary epidemiology has an eminently applied nature, generating valuable tools of risk analysis that assist decision-making in the animal health sector. The two studies presented by Scott et al. illustrate the relevance of such approaches in the context of early warning systems in disease-free areas. Scenario tree modeling approaches made it possible to assess the pathways of LPAI exposure, as well as to quantify the risk of LPAI and HPAI spread within and between Australian commercial chicken farms.

Timely information is required to optimize the emergency response during outbreaks. In this regard, the questionnaire developed by Umber et al. based on the experience and lessons learnt during an HPAI outbreak in the USA, provides an essential tool in establishing poultry premises status, and tailoring future outbreak management measures. The heterogeneity of actors and organizations involved in poultry production chains is another challenge that needs to be addressed in the design of appropriate measures for AI. Indrawan et al. used a value chain analysis to establish a theoretical framework that makes it possible to examine biosecurity and HPAI control in Western Java, Indonesia, where the disease remains endemic despite extensive efforts. Their results highlight that a proper understanding of the chain governance structure is vital to improve the effectiveness of HPAI control measures, target the incentives, and design fit-for-purpose interventions.

The papers gathered in this Research Topic provide a broad overview of the challenges posed by the surveillance and control of AI viruses (both low and highly pathogenic) in a wide diversity of epidemiological contexts (from disease-free to endemic situations) in different countries. This Research Topic contributes to generating new insights into the epidemiology of avian influenza, which could be used to inform prevention, surveillance and intervention strategies in domestic poultry.

AUTHOR CONTRIBUTIONS

The main text has been redacted by MP, TV, PM, and II. All authors revised and approved the editorial.

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ORIGINAL RESEARCH
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Highly Pathogenic Avian Influenza H5N8 Clade 2.3.4.4b in Germany in 2016/2017

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Here, we report on the occurrence of highly pathogenic avian influenza (HPAI) H5Nx clade 2.3.4.4b in Germany. Between November 8, 2016, and September 30, 2017, more than 1,150 cases of HPAI H5Nx clade 2.3.4.4b in wild birds and 107 outbreaks in birds kept in captivity (92 poultry holdings and 15 zoos/animal parks) were reported in Germany. This HPAI epidemic is the most severe recorded in Germany so far. The viruses were apparently introduced by migratory birds, sparking an epidemic among wild birds across Germany with occasional incursions into poultry holdings, zoos and animal parks, which were usually rapidly detected and controlled by stamping out. HPAI viruses (mainly subtype H5N8, in a few cases also H5N5) were found in dead wild birds of at least 53 species. The affected wild birds were water birds (including gulls, storks, herons, and cormorants) and scavenging birds (birds of prey, owls, and crows). In a number of cases, substantial gaps in farm biosecurity may have eased virus entry into the holdings. In a second wave of the epidemic starting from February 2017, there was epidemiological and molecular evidence for virus transmission of the infections between commercial turkey holdings in an area of high poultry density, which caused approximately 25% of the total number of outbreaks in poultry. Biosecurity measures in poultry holdings should be adapted. This includes, *inter alia*, wearing of stable-specific protective clothing and footwear, cleaning, and disinfection of equipment that has been in contact with birds and prevention of contacts between poultry and wild water birds.

Keywords: highly pathogenic avian influenza, H5N8, clade 2.3.4.4b, Germany, wild water birds, outbreak investigations, primary incursion, farm-to-farm spread

INTRODUCTION

Avian Influenza is an infectious disease of poultry caused by influenza A viruses, which are enveloped viruses of the family *Orthomyxoviridae* with a segmented single-stranded RNA genome. These viruses occur in two pathogenicity variants (low/highly pathogenic) and a multitude of different subtypes. Wild water birds (*Anseriformes*) as well as gulls, terns, and wader birds (*Charadriiformes*) are regarded as the natural reservoir for all low pathogenic avian influenza viruses (LPAIVs), i.e., viruses of the subtypes H1–H16 and N1–N9. While LPAIV of the subtypes H5 and H7 may cause almost no or only mild disease in domestic poultry, these subtypes have the capacity to evolve spontaneously into highly pathogenic forms [highly pathogenic avian influenza viruses (HPAIVs)].

The underlying mutational steps seem to be associated with adaptation to domestic poultry after transmission of the low pathogenic progenitors from wild birds (1). The highly pathogenic form clinically manifests itself in poultry as fowl plague, which causes drastic losses especially in turkeys and chickens. In ducks and geese, however, the clinical signs of an HPAIV infection may be mild, and mortality can be considerably lower than in turkeys and chickens. Therefore, HPAIV may circulate in waterfowl undetected, whereas mortality is always very high in *Galliformes* [75–100% (2)].

Upon exposure to a high infectious dose, usually by direct contact to infected birds, some avian influenza viruses (AIVs) (e.g., HPAIV H5N1 and H5N6, LPAIV H7N9 in China, of which a HPAI variant has recently been detected) can be transmitted to humans and may cause fatal disease. Due to the segmented genome of influenza A viruses, new viruses can evolve, when simultaneous infections of a single host with different influenza A viruses allow mixing (reassortment) of the genome segments. Therefore, there is a permanent risk for the generation of novel influenza viruses with pandemic potential if different zoonotic influenza A virus strains cocirculate (3).

In 1996, a HPAIV of subtype H5N1 originating from geese (goose/Guangdong/96, gs/GD) in southern China caused outbreaks in chickens and disease in 18 humans with six fatalities. This virus evolved steadily during the following two decades into various phylogenetic clades, subtypes, and genotypes within the so-called gs/GD lineage. A combination of blanket vaccination of poultry against HPAI H5, trading at live bird markets and the traditional way of keeping waterfowl, for example, in rice fields, in contact to wild or feral water birds is a perfect source for the genesis, emergence, and evolution of new HPAIVs in large parts of Asia, especially in South East Asia. Migratory water birds mixing with poultry may contribute to the development of new viruses by reassortment and eventually give rise to intra- and intercontinental spread. Many of the gs/GD H5-descendants caused serious outbreaks of fowl plague in poultry in South East Asia and some were detected in Europe as well: in 2005/2006 (H5N1 clade 2.2), in 2010 (H5N1 clade 2.3.2.1c), and in 2014 (H5N8 clade 2.3.4.4a). This led to a massive increase of HPAI outbreaks worldwide since 1996 (4–6). Some, but not all of these HPAI H5 strains can also cause severe infections in humans. The generation of a potentially pandemic virus from this lineage that is able to spread within the human population is of worldwide concern and under careful observation. Genetic analysis and animal experiments showed that there was no indication of a zoonotic potential of the clade 2.3.4.4 H5N8a and b viruses (7) and no human infections with this virus have been reported so far. However, 2.3.4.4c H5N6 viruses, which have hitherto only been detected in South East Asia, bear a zoonotic potential (8).

In September 2016, the FAO released a risk alert about the potential westward spread of a novel HPAIV H5N8 of clade 2.3.4.4b, which was detected through surveillance of wild migratory birds in the Tyva Republic, Russian Federation, in June 2016 (9). Only one month later, Hungary and then Poland notified the first cases of HPAIV H5N8 clade 2.3.4.4b detection in dead wild birds (a swan in Hungary and ducks as well as gulls in Poland).

Here, we provide a brief account of the course of the HPAI epidemic that took place in wild and kept birds in Germany in 2016–2017.

MATERIALS AND METHODS

Case and Outbreak Data

Records of cases of HPAIV infections in wild birds and HPAI outbreaks in kept birds in Germany, i.e., commercial and backyard poultry holdings as well as zoos, were obtained from the German National Animal Disease Data Base (10). In brief, all cases of HPAIV detection in wild and captive birds were submitted to the database by the competent veterinary authorities at the district level.

Records on HPAI cases in wild birds in Germany were retrieved from the "Wildvogelmonitoring-Datenbank", the National Avian Influenza Data Base run by the Friedrich-Loeffler-Institut (11). Data on the type of surveillance (active or passive), the sampled wild bird species and the laboratory result were entered by the veterinary investigation centers of the respective federal states.

Data on outbreaks in poultry and cases in wild birds in Europe were obtained from the European Animal Disease Notification System[1] and EMPRES Global Animal Disease Information System (FAO[2]). Data were analyzed in Excel spreadsheets (Microsoft Excel, 2016). Maps were created using ArcGIS software (ESRI, Redlands, CA, USA).

Epidemiological Outbreak Investigations

Epidemiological outbreak investigations were conducted in affected poultry holdings and zoos according to Council Directive 2005/94/EC as previously described (12). In brief, data were obtained by on-site visits to the holdings and by structured interviews with farm or zoo managers, employees, and veterinarians who had visited the farm or zoo. Additional data were extracted from invoices, trade documents (purchase of poultry and feed), and stable records of the affected holdings if available. Touring records of the veterinarians and of vehicles (feed transports, rendering lorries, etc.) were checked for their potential role in virus introduction into the affected holdings.

RESULTS

HPAI H5N8 Clade 2.3.4.4b Outbreaks in Europe and Germany

On November 7, 2016, shortly after the first detection of HPAIV H5N8 clade 2.3.4.4b in Hungary and Poland, an increased mortality of uncertain cause was first reported in tufted ducks (*Aythya fuligula*) at Lake Constance in Baden-Württemberg, in the southwest of Germany. One day later, on November 8, 2016, HPAIV H5N8 was identified in wild birds (mostly tufted ducks) at Lake Constance as well as in tufted ducks found dead at Lake

[1] https://ec.europa.eu/food/animals/animal-diseases/not-system_en
[2] http://empres-i.fao.org/eipws3g/

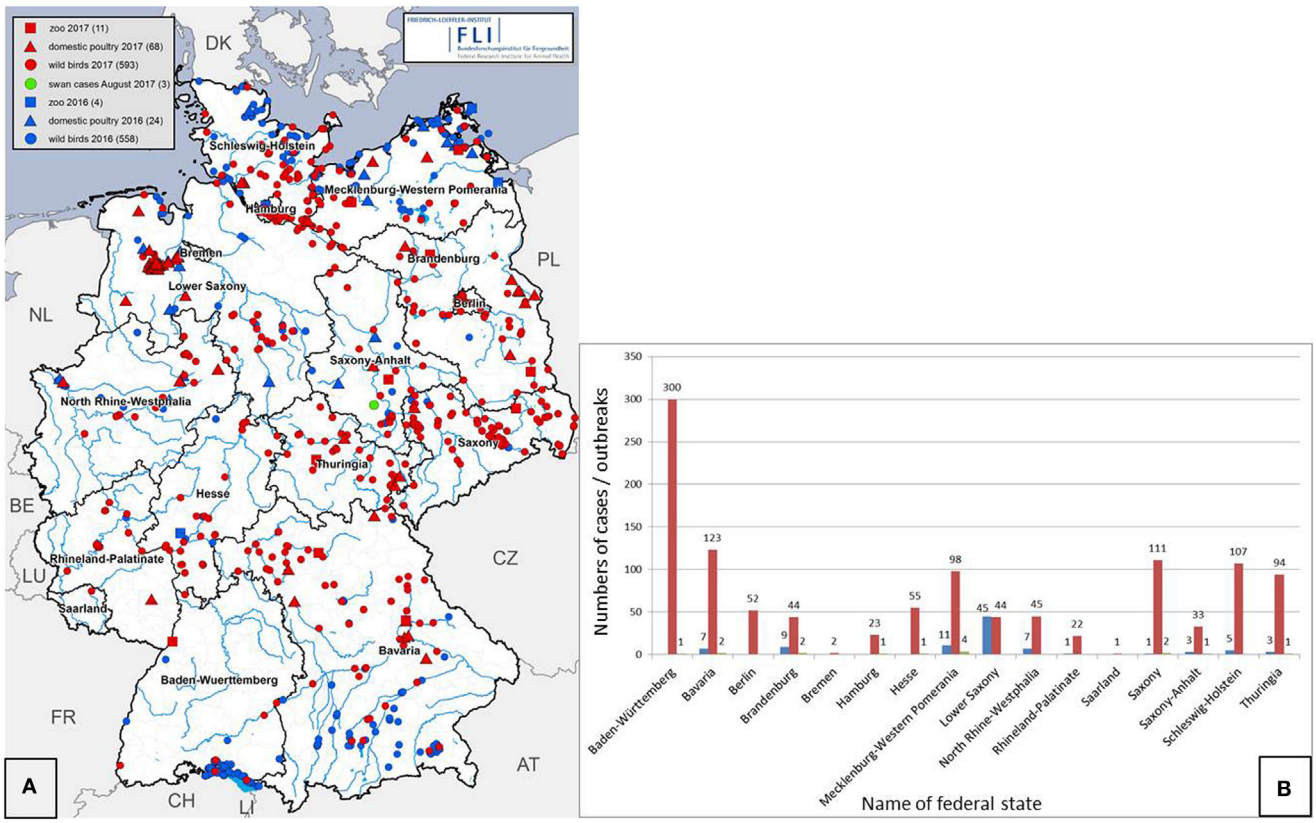

FIGURE 1 | Reported highly pathogenic avian influenza (HPAI) clade 2.3.4.4b H5Nx cases in wild birds (points) and outbreaks in poultry holdings (triangles) and zoos (squares) in the German federal states in 2016 (blue) and 2017 (red). Green points refer to mute swans found HPAIV H5N8 infected in August 2017 **(A)**. Number of cases in wild birds (red) and outbreaks in poultry holdings (blue) and zoos (green) in each German federal state **(B)**.

Plön in Schleswig-Holstein, northern Germany. Simultaneously, an increased number of wild water birds and sea gulls were found dead at the eastern coast of Schleswig-Holstein, around Lake Constance in Switzerland, Austria, and Germany (Bavaria and Baden-Württemberg) as well as at the Baltic Sea Coast in Mecklenburg-Western Pomerania, northeastern Germany (**Figure 1A**, blue points).

Soon, the HPAI H5N8 infections widened to an epidemic across Germany (**Figure 1A**, red points) affecting mainly wild water birds of the orders *Anseriformes, Podicipediformes, Charadriiformes, Phalacrocoraciformes, Ardeiformes*, and *Ciconiiformes* overwintering at lakes and rivers or along the coast, and scavenging birds of the orders *Accipitriformes, Falconiformes*, and *Strigiformes* as well as in few cases also crows that had apparently fed on infected carcasses. The virus was isolated from at least 53 wild or feral bird species (**Table 1**). Almost all other European countries were affected by the epidemic as well (**Figure 2**).

Between November 8, 2016, and September 30, 2017, more than 1,150 cases of HPAI H5N8 in wild birds and 107 outbreaks in birds kept in captivity (92 poultry holdings and 15 zoos or animal parks) were reported in Germany (**Figures 1**, **3** and **4**). The vast majority of cases in wild birds were detected in the context of passive surveillance (sick and dead birds). The last outbreak in poultry so far was reported on May 9, 2017. Thus, the HPAI epidemic seemed to be waning in Germany since April 2017 (**Figure 4**). Rise in ambient temperature and increasing UV radiation as well as lower densities of overwintering waterfowl on lakes and rivers may have influenced the decrease of observed cases since the tenacity of AIV is in general regarded as low (13, 14). However, in August 2017 feral mute swans in central Germany were found dead and tested positive for HPAIV H5N8.

Generally, the temporal course of the epidemic in wild birds was characterized by at least two waves, with maxima in mid-November 2016 and mid-February 2017, respectively (**Figure 4**). A few days after the detection of HPAIV H5N8 in wild birds, the first outbreaks were reported in non-commercial poultry (backyard) and a small animal park close to the coast of the Baltic Sea. Subsequently, large commercial poultry farms were also affected. By the end of February 2017, all federal states of Germany had reported HPAIV H5N8 infections in wild birds or poultry (**Figures 1**, **3** and **4**). During the second wave of the epidemic, further HPAIV H5 reassortants were found in wild birds and domestic poultry (turkeys) in Schleswig-Holstein. These strains could be clearly distinguished from the first reported strains as they belonged to different genotypes involving several gene segments including another NA subtype (N5).

TABLE 1 | Species of wild or feral (marked with *) birds infected with HPAIV clade 2.3.4.4b H5N8/N5.

Species	Latin name
Order *Anseriformes*	
Diving ducks	***Aythya***
Tufted duck	*Aythya fuligula*
Common pochard	*Aythya ferina*
Common goldeneye	*Bucephala clangula*
Red crested pochard	*Netta Rufina*
Greater scaup	*Aythya marila*
Common eider	*Somateria mollissima*
Common scoter	*Melanitta nigra*
Dabbling ducks	***Anas***
Mallard	*Anas platyrhynchos*
Northern pintail	*Anas acuta*
Gadwall	*Mareca strepera*
Eurasian wigeon	*Anas penelope*
Perching ducks	***Anatini***
Wood duck	*Aix sponsa*
Ruddy ducks	***Oxyura***
Ruddy duck*	*Oxyura jamaicensis*
Shelducks	***Tadorninae***
Common shelduck	*Tadorna tadorna*
	Podiceps
Great crested grebe	*Podiceps cristatus*
Red-necked grebe	*Podiceps grisegena*
Little grebe	*Tachybaptus ruficollis*
Merganser	***Mergus***
Merganser	*mergus*
Common merganser	*Mergus merganser*
Goose	
Greylag goose	*Anser anser*
Bean goose	*Anser fabalis*
Canada goose	*Branta canadensis*
White-fronted goose	*Anser albifrons*
Pink-footed goose	*Anser brachyrhynchus*
Barnacle goose	*Branta leucopsis*
Dark-bellied brant	*Branta bernicla*
Red-breasted goose*	*Branta ruficollis*
Lesser white-fronted goose	*Anser erythropus*
Swans	***Cygnus***
Mute swan	*Cygnus olor*
Black swan*	*Cygnus atratus*
Whooper swan	*Cygnus cygnus*
Order *Charadriiformes*	
Gulls	***Laridae***
Black-headed gull	*Chroicocephalus ridibundus*
European herring gull	*Larus argentatus*
Great black-backed gull	*Larus marinus*
Mew gull	*Larus canus*
Little gull	*Hydrocoloeus minutus*
Lesser black-backed gull	*Larus fuscus*
Sandpipers	***Scolopacidae***
Red shank	*Tringa totanus*
Order *Gruiformes*	
Rail	***Rallidae***
Common coot	*Fulica atra*
Order *Ardeiformes*	
Grey heron	*Ardea cinerea*
Western great egret	*Ardea alba*
Order *Accipitriformes*	
	Accipitridae
Common buzzard	*Buteo buteo*
Rough-legged buzzard	*Buteo lagopus*
White-tailed eagle	*Haliaeetus albicilla*
Northern goshawk	*Accipiter gentilis*
Eurasian sparrowhawk	*Accipiter nisus*
Order *Phalacrocoraciformes*	
Great cormorant	*Phalacrocorax carbo*
Order *Passeriformes*	
Crows	***Corvidae***
Carrion crow	*Corvus corone*
Magpie	*Pica pica*
Order *Ciconiiformes*	
Storks	***Ciconiidae***
White stork	*Ciconia ciconia*
Order *Falconiformes*	
Falcons	***Falco***
Peregrine falcon	*Falco peregrinus*
Order *Strigiformes*	
Owls	***Strigidae***
Long-eared Owl	*Asio otus*
Tawny owl	*Strix aluco*

Phylogenetic analyses indicated that multiple independent incursions of HPAIV into Germany had occurred more or less at the same time (15).

Epidemiological Outbreak Investigations

A total of 68 commercial poultry holdings were affected by the epidemic, including 52 turkey, 5 laying hen, 9 duck, and 2 geese holdings (**Figure 3**). Moreover, 24 small scale, non-commercial poultry holdings were also infected by HPAIV H5N8. They were distributed almost all over Germany. Similar to the outbreaks in captive birds in zoos, they were most likely caused by primary virus incursions into the holdings/zoos *via* direct contact to infected wild birds (where captive birds were kept outdoors and with access to ponds also visited by wild birds) or *via* indirect contact (feces or material contaminated by infected carcasses). No evidence for the transmission of HPAIVs through trade of live animals, feed, or products of animal origin was detected in the course of the epidemiological outbreak investigations.

The majority of outbreaks in large commercial poultry holdings were apparently caused by single incursion events, often affecting only one out of several stables of the respective holding. In a number of cases, substantial gaps in farm biosecurity may have eased virus entry. This refers to outdoor storage of bedding material, lack of personal hygiene when entering the stables (no changing of footwear and protective clothing, lack of appropriate disinfection), regrouping of poultry flocks (mainly turkeys) during fattening, attraction of wild water birds close to the stables either by ponds or by storing silage on the premise as supply for a biogas plant. Only in the late phase of the epidemic,

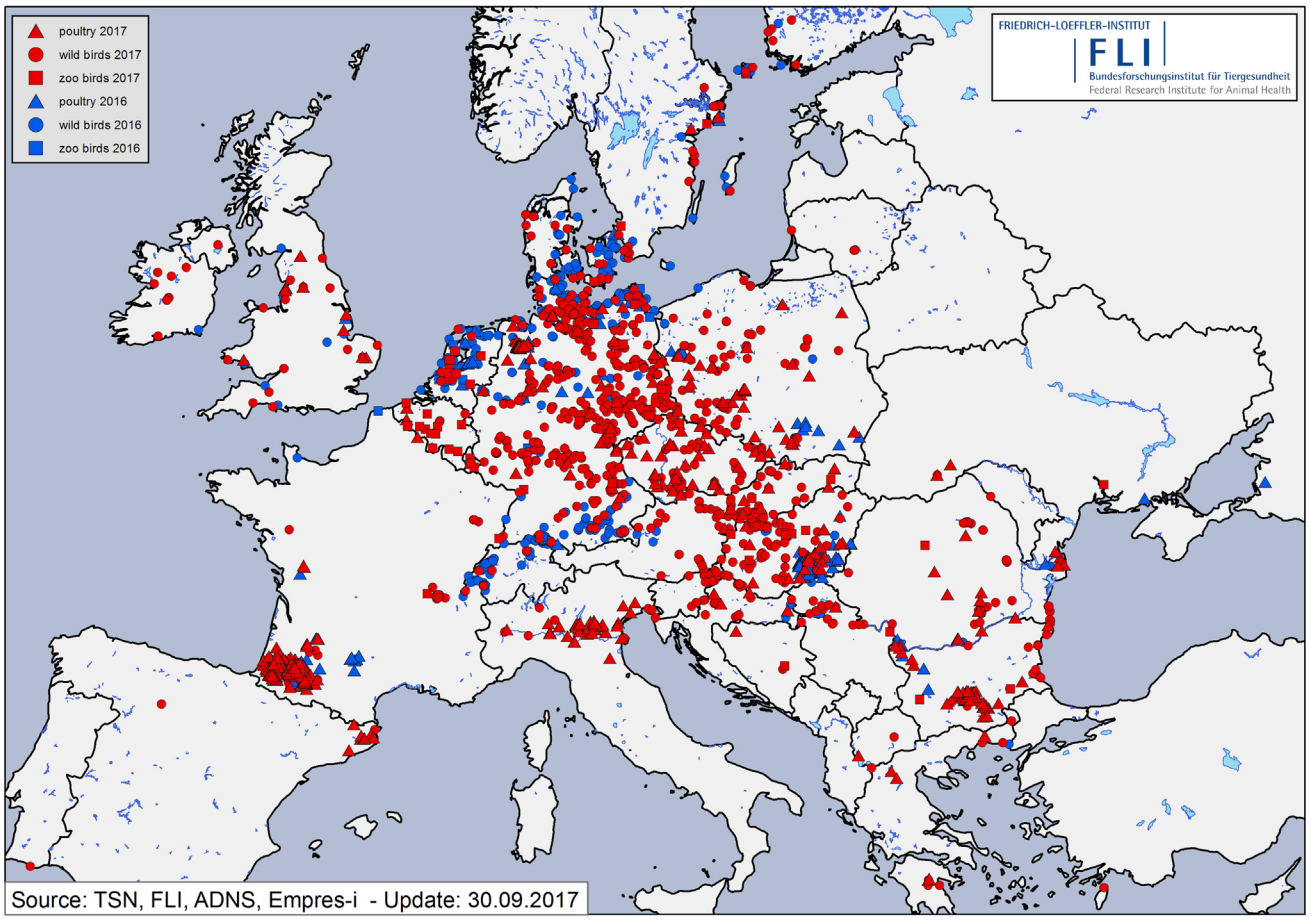

FIGURE 2 | Distribution of reported highly pathogenic avian influenza clade 2.3.4.4b H5Nx cases in wild birds (points) and outbreaks in poultry holdings (triangles) and captive birds in zoos (squares) in 2016 (blue) and 2017 (red) in Europe.

there was epidemiological and molecular evidence for direct farm-to-farm transmission affecting mainly turkey holdings in the area with the highest poultry densitiy, which caused approximately 25% of the total number of outbreaks (**Figure 3**, within red circles). The mode of farm-to-farm spread remained elusive, but was in a few cases found to be potentially related to sharing a single carcass bin by some holdings and possible vehicle contacts between farms.

Approximately 1.2 million birds died or had to be killed, and the economic losses (direct costs) were estimated as in excess of 17 million Euros.

DISCUSSION

Continuous cocirculation of HPAIVs and LPAIVs in poultry with frequent spill-over transmissions into migratory wild birds has been observed in several parts of Asia over more than two decades. Chances to eradicate these viruses at their source in poultry in Asia are estimated to be low. Similarly, in Egypt and West-Africa HPAIV H5N1 2.3.2.1c and HPAIV H5N8 2.3.4.4b are continuously circulating. Therefore, the poultry industry, risk managers and poultry associations must anticipate future incursions and improve their preparations for prevention and control. Fortunately, the recent HPAIV H5N8 clade 2.3.4.4a and b had no zoonotic potential, but this is prone to change as new viruses within this clade (2.3.4.4c and d) that may lead to fatal infections in mammals have already evolved in Asia (8). Efficient measures to prevent the spread of notifiable AIV include prompt detection of infection, closing affected holdings already in the case of suspected infections, immediate depopulation and cleansing/disinfection, as well as a temporary ban on restocking (7). Moreover, potential contact to wild birds, mode and frequency of farm visits, biosecurity practices, and the density of poultry holdings in a specific region are relevant risk factors for the introduction and the spread of HPAIVs (16).

Historically, HPAI outbreaks were usually geographically limited and mainly restricted to poultry, i.e., the viruses causing the outbreaks did not circulate in wild birds. This situation has fundamentally changed since the expansion of Gs/GD HPAIVs H5 to other continents, including Europe, which has led to a panzootic (5, 6). Although the epidemic of HPAIV H5N8 clade 2.3.4.4b in poultry came to a hold in late spring 2017, sporadic cases in wild water birds have continuously been reported from European countries during the summer of this year. As demonstrated by

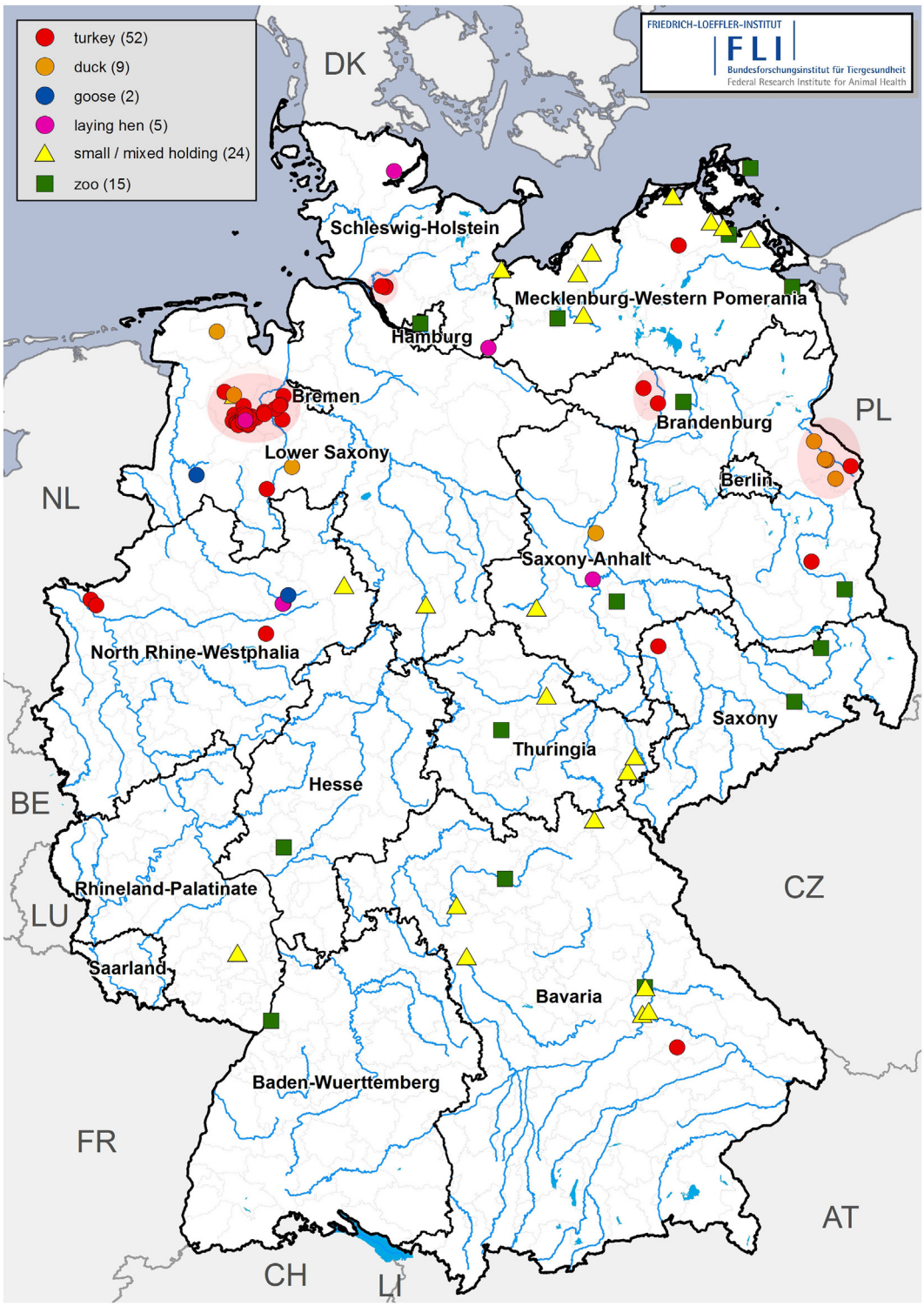

FIGURE 3 | Highly pathogenic avian influenza in holdings of captive birds in Germany since November 2016. Red points: turkeys (52), orange points: ducks (9), blue points: geese (2), pink points: laying hens (5), yellow triangles: small scale, mixed holdings (24), and green squares: zoos (15). Red circles indicate outbreaks where farm-to-farm spread most likely occurred.

the cases detected in mute swans in central Germany in August 2017 and by several outbreaks in poultry and wild birds in Italy, Belgium, and the UK during summer 2017, continuing low level circulation among kept birds or repeated introduction into wild bird populations and vice versa cannot be excluded as long as there is the chance for direct or indirect contact to infected wild

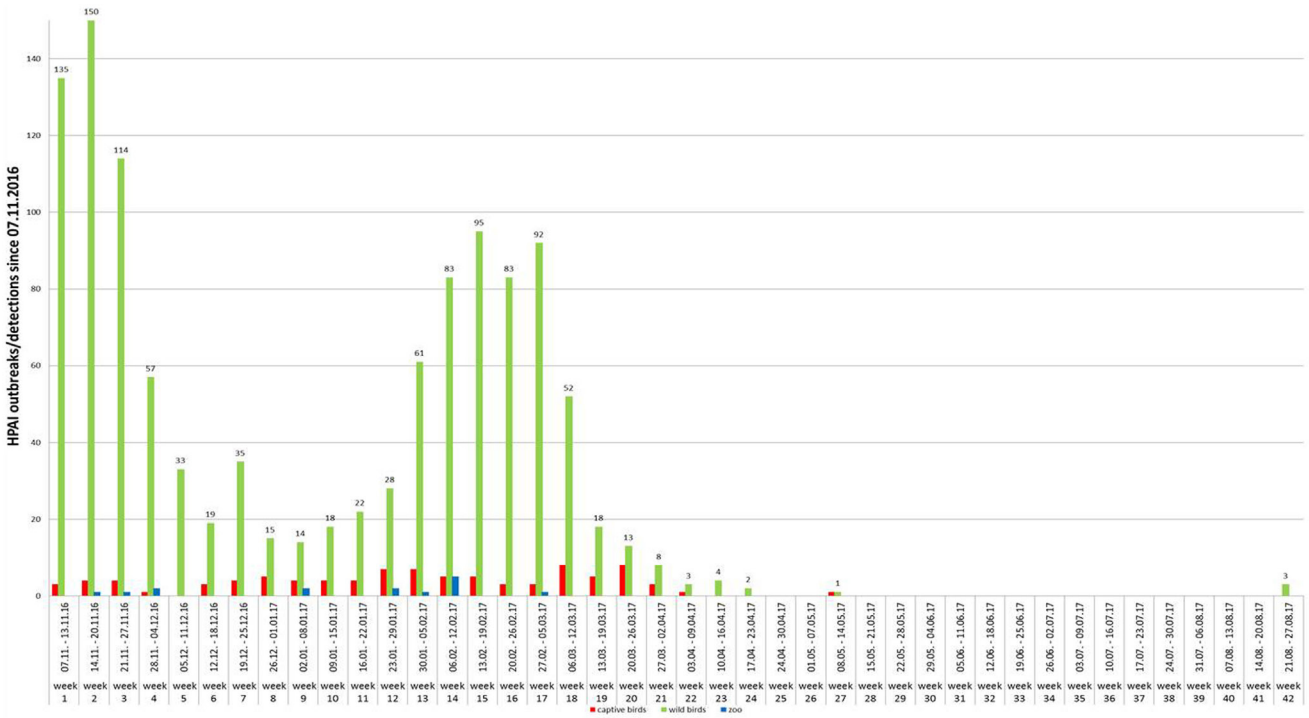

FIGURE 4 | Weekly number of outbreaks of highly pathogenic avian influenza in poultry (red columns), zoos (blue columns) and cases in wild birds (green columns) in Germany (November 2016–August 2017).

birds. This applies in particular to zoos or animal parks where birds are kept on ponds that are also frequented by wild water birds.

Adequate farm biosecurity is essential to decrease the risk of introduction and spread in poultry farms, which is particularly relevant in areas with high poultry density, particularly during epidemics. In high-risk periods and locations, losses should be compensated according to the level of biosecurity established and enforced on the affected holdings.

The most important lesson learned during the epidemic was the finding of substantial gaps in farm biosecurity and the impact of HPAI in an area of high poultry density, i.e., substantial farm-to-farm spread.

In general, protection of domestic poultry holdings from infection with HPAIV H5N8 has highest priority. Emphasis is put on the creation of a physical and functional barrier between wild bird habitats and domestic poultry holdings. Among other biosafety measures, mandatory indoor housing of poultry or the use of protected shelters (fenced and covered with fabric) minimize the risk of direct and indirect contact with infected wild birds. In particular, indirect introduction routes, e.g., through feed contaminated by wild birds, contaminated water, litter, and objects (shoes, wheelbarrows, vehicles, etc.) must be interrupted and adequate disinfection measures applied. Revision, optimization and strict implementation of biosafety measures are of utmost importance.

The HPAI H5N8 epidemic has taught the German veterinary authorities some limitations, but also the use of possible exceptions from culling as laid down in the national legislation, e.g., minimizing culling of birds kept in zoos. Based on the experience made, the national legislation is currently under revision. Furthermore, the German legislation on biosafety in poultry holdings has been amended. Not only commercial poultry farms but also small holders must now follow rules and principles that aim at reducing the risk of introduction of HPAIV into poultry farms. An online tool for an assessment of the quality of farm biosecurity by the farmers themselves is under development.

AUTHOR CONTRIBUTIONS

AG, CS, and FC designed the study and analyzed the data. AG and FC drafted the manuscript. CS-L, KD, TH-B, CP, JG, KD, CG, TH, ES, AP, DH, MB, and TM collected or analyzed data and edited the manuscript.

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Garbage Management: An Important Risk Factor for HPAI-Virus Infection in Commercial Poultry Flocks

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Garbage management represents a potential pathway of HPAI-virus infection for commercial poultry operations as multiple poultry premises may share a common trash collection service provider, trash collection site (e.g., shared dumpster for multiple premises) or disposal site (e.g., landfill). The types of potentially infectious or contaminated material disposed of in the garbage has not been previously described but is suspected to vary by poultry industry sector. A survey of representatives from the broiler, turkey, and layer sectors in the United States revealed that many potentially contaminated or infectious items are routinely disposed of in the trash on commercial poultry premises. On-farm garbage management practices, along with trash hauling and disposal practices are thus key components that must be considered to evaluate the risk of commercial poultry becoming infected with HPAI virus.

Keywords: United States, poultry, farms, chickens, turkeys, risk, waste disposal facilities, highly pathogenic avian influenza

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INTRODUCTION

In past avian influenza (AI) outbreaks in US poultry, evidence of lateral disease spread has been documented *via* transfer of people, vehicles, and shared equipment or visitors between farms (1). Before 2015, however, epidemiological trace-back questionnaires in AI outbreaks on commercial poultry farms in the United States did not specifically investigate garbage management services as a risk factor for disease spread.

Many and likely most commercial poultry operations in the United States use third-party companies to collect and transport trash to off-site disposal locations. Garbage management poses a risk for potential HPAI-virus infection of a commercial poultry flock through a number of pathways. These include: multiple poultry premises (commercial and backyard operations) sharing a common trash collection service provider, sharing a trash collection site (i.e., common dumpster for multiple premises) or disposal site (i.e. landfill). HPAI virus may be carried onto a poultry premises *via* contaminated garbage transport vehicles or drivers, and it is hypothesized that garbage contents within the truck may contain virus-laden trash items. Garbage trucks coming near the barns (within 15 ft) were identified as a significant risk factor in a case–control study in the 2015 United States HPAI H5N2 outbreak. It was shown that egg layer flocks in Nebraska and Iowa that had garbage trucks coming near the barns were 14.7 times more likely to be infected (at the farm level) than flocks that did not have garbage trucks come near the barns ($p < 0.001$) (2). Of note, the frequency with which garbage trucks visited the farms in this study is not known.

To date there are no known studies describing disposal practices used by commercial poultry operations in the United States To more fully evaluate the risk of HPAI infection to commercial poultry *via*

garbage management, we initiated a survey of the poultry industry to refine the risk and establish mitigation measures.

METHODS

A convenience sample of veterinarians and other managers in the poultry industry was surveyed between June and August 2016 on standard practices for garbage management on farms that they manage or supervise. A URL link to the survey was distributed to members of the Secure Egg, Turkey, and Broiler Supply working groups *via* email; these groups consisted of industry veterinarians and production managers within major United States poultry producing companies (Appendix S1 in Supplementary Material). The survey was administered using an online polling service.[1] Participants were surveyed anonymously, minimal opt-in demographic questions (such as company name or job position within the organization) were also included. Some minor differences in the survey wording were used to match common terminology for the commodity (broiler, turkey, or layer) to which it was distributed. In addition, participants were given the option to decline to answer any question within the survey. Respondents were stratified by industry sector (broiler chicken, layer chicken, or turkey) and descriptive statistics were calculated for each. The study was submitted to the University of Minnesota Institutional Review Board and determined to be exempt from review.

RESULTS

A total of 63 surveys were completed. Respondents represented the turkey ($n = 15$), broiler ($n = 8$), and layer ($n = 40$) commodities. The types of potentially infectious or contaminated material disposed of in the garbage varied by sector of the poultry industry, and many potentially contaminated or infectious materials were reported as routinely disposed of in the trash as listed in **Table 1**. One or more items classified as a risk (e.g., poultry or wild bird carcasses and items that contacted birds or bird feces) were reported to be disposed of in trash on premises managed by 79.4% of all respondents (layers 75% $n = 30$; broilers 75% $n = 6$; and turkeys 93.3% $n = 14$).

Approximately half of broiler and turkey sector respondents reported that the garbage truck may collect waste from multiple poultry premises before depositing the load at a landfill (43 and 53% respectively), while an additional 48% ($n = 23$) of respondents from all three sectors reported they did not know if the garbage truck route included other poultry premises.

The dumpster or garbage collection area may be located at various locations on a premises (reported proximity to the nearest barn of <100 ft (30.48 m) to >250 ft (76.2 m); **Figure 1**), however only a minority of respondents ($n = 2$; 3.3%) reported sharing a trash collection location between multiple premises. Representatives of all three industry sectors suggest it is common practice for the dumpster or trash collection point to be located at the entrance or perimeter of the farm. This exact distance to the nearest poultry barn may vary; however, this appears to represent a distance of at least 100 ft (30.48 m) to the nearest barn for a majority of respondents.

DISCUSSION

In our study, respondents identified potential HPAI contaminated or infectious material (i.e., dead wildlife, poultry carcasses, egg shells, and materials that have contacted poultry) that are regularly disposed of in the garbage on their poultry premises. Estimates of HPAI-virus concentrations in chicken and turkey secretions,

[1]Qualtrics© 2015 Provo, UT, USA. http://www.qualtrics.com.

TABLE 1 | Survey results of material disposed of in the garbage on premises in the broiler, turkey, and layer industries.[a]

Item	Broiler sector ($n = 8$ respondents)	Turkey sector ($n = 15$ respondents)	Layer sector ($n = 39$ respondents)
Dead wildlife/wild birds	Yes (1/8)	Yes (5/15)	Yes (1/39)
Rodents	Yes (3/8)	Yes (5/15)	Yes (10/39)
Dead poultry or poultry carcasses	No (0/8)	Yes (1/15)	Yes (9/39)
Eggs or egg products[b]	Yes (1/8)	Yes (1/15)	Yes (8/39)
Manure	No (0/8)	No (0/15)	Yes (1/39)
Spilled feed	Yes (2/8)	Yes (8/15)	Yes (7/39)
Disposable chick transport boxes[b]	Yes (4/8)	Yes (4/15)	Yes (24/39)
Used needles/syringes/diagnostic supplies that have contacted birds[b]	Yes (1/8)	Yes (5/15)	Yes (14/39)
Personal protective equipment (boot covers, gloves, coveralls, etc.)	Yes (8/8)	Yes (14/15)	Yes (36/39)
Feathers	No (0/8)	Yes (2/15)	Yes (4/39)
Offal	No (0/8)	No (0/15)	No (0/39)
Equipment or supplies from inside barns[c]	Yes	Yes	Yes (22/39)
Household garbage from farm manager or any other residence[c]	–	Yes	Yes (20/39)
Trash associated with waterfowl hunting[c]	–	–	No (0/39)
Garbage from processing operation[c]	–	–	Yes (23/39)
Lunch room and restroom garbage[c]	–	–	Yes (37/39)

[a]*Yes indicates materials disposed of in the garbage by one or more survey respondents within each industry. In parenthesis, numerator indicates number of survey respondents reporting disposal of item and denominator indicates total number of respondents.*

[b]*Language of selection choice modified in survey distributed to representatives of layer industry.*

[c]*Item only explicitly asked in survey distributed to representatives of layer industry. Yes in the broiler and turkey industries for these items represent at least one write-in response indicating disposal of that item.*

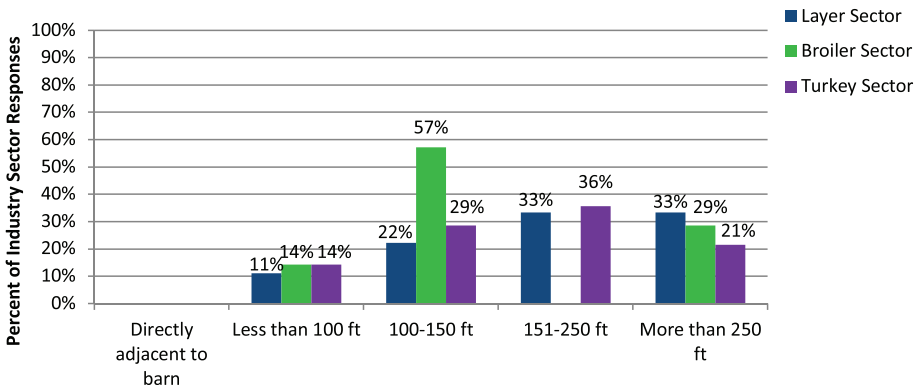

FIGURE 1 | Histogram of frequencies of responses from poultry industry representatives regarding the distance of the dumpster or trash collection point from the nearest poultry barn (layer sector: $n = 36$; broiler sector: $n = 7$; turkey sector: $n = 14$). In the survey of layer industry representatives, it was specified that the nearest poultry barn may be on the same premises or neighboring premises.

feces, feathers, and other tissues generally range between 10^3 and 10^7 EID_{50} per gram of solid or per milliliter of liquid (3–10), and virus persistence is generally longer at cooler temperatures and in more humid conditions. Virus survival on materials that may be disposed of in the garbage, such as poultry carcasses, feathers, egg shells, egg trays, wood, steel, glass, and personal protective equipment, has been reviewed elsewhere (11–15). Viruses may survive days to weeks or longer depending on environmental conditions. Thus, we suggest the potential for HPAI virus to be present in the garbage and survive in that environment is sufficient to infect a bird should the bird become exposed to that material.

Study participants reported that garbage management contractors used by some turkey and broiler premises visit multiple poultry premises on one route before depositing a load at the landfill; thus, the pathway by which HPAI virus-contaminated garbage from infected premises may be present on the truck when it arrives at the next poultry farm appears to be viable. The types of potentially contaminated trash from non-commercial poultry operations and related industries (e.g., backyard poultry, processing facilities, and live bird markets) are not known, but are likely to include materials similar to those reported in garbage from commercial poultry operations. Poultry carcasses have been reported in the trash of backyard chicken keepers during an exotic Newcastle disease outbreak in California in 2002 (A. Jones, personal communication, September 2017). In the Netherlands, poor waste management practices pertaining to liquid waste (e.g., waste water) and solid waste have been identified as potentially increasing the risk of AI transmission in the neighborhood of infected farms (A. Ssematimba, personal communication, August 2016) (16). A shared dumpster or common trash collection point for multiple poultry premises, while not a common practice in the United States poultry industry, represents an additional site of potential cross-contamination between commercial poultry operations related to garbage management.

Garbage trucks and drivers typically do not contact live poultry while completing contracted duties on poultry premises. Biosecurity recommendations and site-specific biosecurity plans may not stipulate specific biosecurity measures for garbage truck drivers; however, it is recommended in recent updates to the National Poultry Improvement Plan guidance that all visitors and vehicles remain as far from poultry barns as possible (e.g., outside the "Perimeter Buffer Area" or PBA), and for those vehicles which must come near poultry barns, all must be cleaned and disinfected (17). If garbage management activities and pickups occur outside of the PBA, there may be a decreased likelihood of contaminated garbage vehicles, personnel, or virus-laden garbage on the truck contacting farm personnel or equipment which may access the poultry house and expose birds to HPAI virus.

An overwhelming majority of respondents in our survey indicated that they hire a contractor for some or all of their garbage transport needs. Similar to activities of other third-party contractors, cleaning and disinfection of garbage transport vehicles, pickup routing, and landfill practices may be difficult to control and may not be easily influenced by the poultry grower or integrator if using a contractor to haul garbage.

The use of hauling routes that include multiple farms and the use of communal landfills increase the likelihood of contact with infectious garbage. It appears reasonable that garbage within a truck upon arrival to a commercial poultry farm could originate from both commercial and non-commercial (live poultry markets and backyard) poultry operations. In previous outbreaks of HPAI in non-commercial poultry operations, disposal of dead poultry in garbage was noted as a practice which correlated with risk for AI infection. In an evaluation of risk factors for live bird markets in New York, New Jersey, Pennsylvania, and New England, markets that disposed of dead birds and offal in the trash were 2.4 times more likely to have a repeated presence of LPAI H5 and H7 viruses (OR: 2.4; 95% CI, 1.8–3.4) (18). In an analysis of risk factors associated with H5N1 in backyard poultry in Egypt from 2010 to 2012, disposing of dead birds and poultry feces in garbage piles outside was highly correlated with infection in the regression model ($F = 15.7$; $p < 0.0001$) (19). Whether disposing birds in the garbage represented a risk for infection on one's own premises, or rather is indicative of likelihood for other high-risk practices in these non-commercial operations is not clear. The final destination of the garbage and garbage vehicles, such as to a landfill, also can contribute to the risk of HPAI-virus contamination. Landfills may serve as a potential

site for cross-contamination as contracted garbage management services for poultry premises may transport garbage to the same landfill; it has been noted that upon arrival at landfills, garbage hauling vehicles may drive over previously deposited garbage (D. Halvorson, personal communication, June 2016). This risk of vehicle contamination likely increases if landfills are used as an off-site disposal method for infected depopulated flocks, which has been reported in previous LPAI outbreaks (20, 21). Landfills also attract wild birds, including scavenger species such as gulls which are susceptible to HPAI viruses and are a known reservoir of AIVs (22, 23).

This survey used a purposive sampling method focused on recruiting participants with significant experience in the poultry industry and was subsequently limited by small sample size. Members of the surveyed working groups were encouraged to share the survey with others within their companies who might have first-hand knowledge of garbage management practices on poultry farms. Therefore, it is not possible to calculate a reliable response rate for this survey and results may not be generalizable to the entire United States commercial poultry industry. Still, the data are informative for the purpose of risk assessment and serve to illustrate the variations in industry practices and potential differences between poultry sectors that may operate in the same geographic area. As such, we suggest the absence of an affirmative response to a high-risk activity does not definitively indicate it is not occurring, and that further evaluation of the prevalence of such practices on an industry-wide scale may be warranted based on this exploratory survey.

CONCLUSION

This exploratory survey identified items in garbage that may contain infectious HPAI virus, some of which may carry high titers of infectious virus. Given that there is potential for HPAI virus to be associated with trash contents and garbage management practices, and taking into account the ease with which virus could be introduced into the poultry house, the potential for a commercial poultry flock becoming infected with HPAI virus due to garbage management during an outbreak should be considered. Further research is needed to determine prevalence of garbage management practices in different production systems and across geographic regions in the United States and producers should develop appropriate mitigation measures in the event of a HPAI outbreak in commercial poultry.

ETHICS STATEMENT

The study was submitted to the University of Minnesota Institutional Review Board and determined to be exempt from review.

AUTHOR CONTRIBUTIONS

Survey design and distribution: EW, FC, JU, and EL. Manuscript writing: EW, EL, DH, CC, and MC. Manuscript editing: DH, EL, JU, CC, MC, and FC.

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SUPPLEMENTARY MATERIAL

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CASE REPORT
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Airborne Detection of H5N8 Highly Pathogenic Avian Influenza Virus Genome in Poultry Farms, France

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In southwestern France, during the winter of 2016–2017, the rapid spread of highly pathogenic avian influenza H5N8 outbreaks despite the implementation of routine control measures, raised the question about the potential role of airborne transmission in viral spread. As a first step to investigate the plausibility of that transmission, air samples were collected inside, outside and downwind from infected duck and chicken facilities. H5 avian influenza virus RNA was detected in all samples collected inside poultry houses, at external exhaust fans and at 5 m distance from poultry houses. For three of the five flocks studied, in the sample collected at 50–110 m distance, viral genomic RNA was detected. The measured viral air concentrations ranged between 4.3 and 6.4 $\log_{10}$ RNA copies per m^3, and their geometric mean decreased from external exhaust fans to the downwind measurement point. These findings are in accordance with the possibility of airborne transmission and question the procedures for outbreak depopulation.

Keywords: avian influenza, highly pathogenic avian influenza, H5N8, clade 2.3.4.4, airborne, transmission, ducks, chickens

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INTRODUCTION

A H5N8 clade 2.3.4.4 strain of highly pathogenic avian influenza (HPAI) virus (HPAIV) was first detected in France in November 2016. Until the 3rd of March 2017, 348 cases of HPAI H5N8 and 136 cases of HPAI H5Nx strain closely related to HPAIV H5N8 were detected in poultry, with 80% of cases occurring in waterfowl farms (mainly duck farms) (1). In the area affected by the outbreak (zones from 0 to 5 km distance from a poultry case), the mean proportion of poultry farms affected was around 15 and 24% where the poultry farm density was greater than $1/km^2$. In the southwestern region of France, the virus spread rapidly especially in high poultry farm density zones, despite the implementation of routine control measure. This rapid regional spread and the proportion of farms affected in some areas, drove us to question the potential role of airborne transmission in HPAI H5N8 viral spread.

The capacity of poultry to transmit influenza virus *via* the airborne route, was evidenced by experimental studies in chickens infected with the H5N1 HPAIV strain (2, 3) and was further supported by field studies as the ones detailed below. Thus, the detection and isolation of strains of AIV in air samples, with particles sizes partly compatible with respiratory contamination, in wet poultry markets could explain human infections reported after a visit of a wet poultry market without any direct contact with live poultry or poultry stalls (4–6). Detection of different AIV strains, with or without quantification, have been performed on air samples collected outside, inside and downwind

from infected poultry premises, up to 59 m for low pathogenic strains and up to 1,000 m for highly pathogenic ones (7–9) and occurred partly on particles respirable fraction. Isolation of HPAIV H5N2 clade 2.3.4.4 has been performed on air samples collected inside, 5 m outside and even 70–150 m outside from poultry barns (8, 9).

The capacity of poultry flock to be infected through the airborne route is strongly suggested by epidemiological studies. For example, pig farm proximity to turkey premises has been associated with turkey seropositivity to swine-origin influenza A virus (IAV) and the detection and quantification of swine IAV in air samples collected inside and outside swine barns (10), support the hypothesis of airborne transmission (11). Modeling studies on the outbreak of HPAI H7N7 in the Netherlands in 2003, estimated the contribution of a possible wind-mediated mechanism to the total amount of spread to be around 18% (12) and showed that the wind-borne route could contribute substantially to the spread over short distance ranges, explaining, for example, 24% of the transmission over a distance up to 25 km (13).

The first observations of the French H5 clade 2.3.4.4 epizootic short distance diffusion (<10 km) (14) are compatible with a contribution of wind-born transmission to the spread when compared with the Dutch H7N7 2003 outbreak. Thus the objective of this study was to determine whether AIV could be detected in air samples collected inside, outside, and downwind from poultry barns infected by H5N8 HPAIV under field conditions. This study was designed and performed as part of a rapid outbreak response.

MATERIALS AND METHODS

Flock Selection/Description

The study was conducted in January and March 2017. The selection of flocks was carried out in collaboration with departmental animal health authorities regarding the confirmed infected status, the not-yet depopulation of flocks and the agreement of the farmer, at the time of the field team availability. Three duck flocks (A, B, and C) and two chicken flocks (D and E), located in Landes and Pyrénées Atlantiques departments were selected. All selected flocks had an officially confirmed diagnosis of HPAI H5N8 at the time of sampling, according to the European diagnostic manual for avian influenza (15). Sampling was performed 2–7 days after confirmation date. At the sampling event, three of the five selected flocks were confined totally in-house (C, D, and E). Loading for culling occurred during the sampling process for one flock (E). A part of the ducks for the flocks A and B, had still an access to the open free range at the sampling event. Characteristics of flocks are summarized in **Table 1** and their location within the affected region presented in the **Figure 1**.

Air Sampling Procedures and Sampling Scheme

To detect AIV genome in aerosols, air samples were collected using a cyclone-based bioareosol sampler, Coriolis®μ microbial air sampler (Bertin Technologies, St-Quentin en Yvelines, France): 300 L/min, 10 min/sample, in 10–12 mL of 0.005% Triton X-100 (Sigma Aldrich) solution prepared in demineralized water and placed into a sterile sampling cone. The collected sample was poured directly after collection from the sampling cone into a sterile 50 mL tube.

After each sample collection, the air sampler was cleaned and disinfected, the cone removed and the sample stored at 0–4°C. The disinfection was performed by spraying Aniospray Surf 29 (Laboratoires Anios, France) on external surface and inside and outside the air intake and the aspiration tube. The samples were transported to a nearby laboratory (from accredited laboratories national network) within 12 h where they were stored at −80°C until testing.

For each flock, air samples were collected in the following order: downwind from the barn at 50–110 m distance, at 5 m distance, at external exhaust fans and finally inside the barn. For one flock (E), the loading of the flock for culling started during the sampling process, the air samples were collected downwind at 110 m distance, inside the barn and at 1 m distance from the animal transport truck. One control sample was collected at 5 km distance from any poultry farm. The sampler was placed directly against the exhaust fans and on the ground for the other sampling locations.

Detection and Quantification of AIV RNA Genome

Collected air samples were concentrated using a Amicon® Ultra-15 30K centrifugal filter device (Merck Millipore Ltd., Ireland). After centrifugation (for 30 min at 5,000 *g*), RNA was purified from 200 μL eluate using the RNeasy Mini Kit© (Qiagen GmbH, Hiden, Germany), and 2 μL RNA extract from the 50 μL obtained from purification was tested by real-time reverse-transcription polymerase chain reaction (rRT-PCR) targeting the matrix gene (M gene) of avian influenza type A viruses, as previously described by Ref. (16, 17). Samples with a detection of M gene signal were tested by subtype specific H5 rRT-PCR (16, 18). We will refer to samples with a detection of viral genome signal by rRT-PCR as positive in the text that follows. For the positive samples, the number of M gene copies in the volume of analyzed sample is estimated from the cycle threshold (Ct) value obtained in RT-PCR, according to a calibration curve relating decimal dilution series of a synthetic RNA transcript of known concentration (determined by fluorimetric quantitation) to Ct values: each dilution point of the RNA transcript was tested twice.

For each sample, the number of AIV M gene copies per m^3 air was calculated according to the formula:

$$\text{M gene copies} / m^3 = \text{M gene copies PCR} \times (\text{Vextract} \div \text{Vpcr}) \div (U \times t),$$

where Vextract is the sample final reduced volume obtained after centrifugation and RNA extraction, Vpcr is the volume analyzed by RT-PCR, U is the air flow rate (m^3 per min), and t is the sampling duration (min).

Ethic Statement

Air sampling was performed with the permission of the farmers and the departmental animal health authorities.

TABLE 1 | Attributes of flocks studied and environmental conditions at sampling events.

Farm ID	French depart.[a]	Specie/type	House	Flock initial size	House poultry density[b]	Positive confirmation date[c] (dd/mm/yyyy)	Proportion of positive pools[d]	Clinical signs	Air sampling date (dd/mm/yyyy)	Sampling location/distance (m)	Ambient temperature (°C)	Wind velocity (km/h)
A	40	Ducks/PAG[e]	Tunnel[f]	2,500	7/m^2	29/01/2017	2/2	Mortality/ symptoms	31/01/2017	Inside External exhaust fans Outside 5 m Downwind 50 m	NR NR NR 20	<5
B	64	Ducks/PAG	Tunnel	3,000	0.5/m^2	09/03/2017	5/24	None	16/03/2017	Inside External exhaust fans Outside 5 m Downwind 80 m	NR NR NR 24	10
4	64	Ducks/FF[G]	Barn	800	2.5/m^2	11/03/2017	12/12	None	16/03/2017	Inside External exhaust fans Outside 5 m Downwind 60 m	NR NR NR 17	<5
D	40	Chickens/grow	Barn	4,000	1/m^2	14/03/2017	2/2	Mortality/ symptoms	21/03/2017	Inside External exhaust fans Outside 5 m Downwind 50 m	NR NR NR 12	<5
E	64	Chickens/grow	Barn	4,400	8/m^2	18/03/2017	8/8	Mortality/ symptoms	22/03/2017	Inside Loading for culling Downwind 110 m	NR NR 2	≈0

[a]Department is an administrative division unit in France (the median land area of French metropolitan departments is 5,960 km^2).

[b]At sampling event.

[c]Date of the official sampling that permitted to confirm the avian influenza H5 infection of the flock.

[d]Proportion of pools of five swabs (cloacal or oropharyngeal) positive to rRT-PCR targeting the matrix gene at the official sampling.

[e]PAG, growing ducks for "foie gras" production.

[f]Tunnel: open sided tunnel.

[g]FF ("foie gras" production).

NR, not recorded; FF, force feeding period; PAG, prêts à gaver.

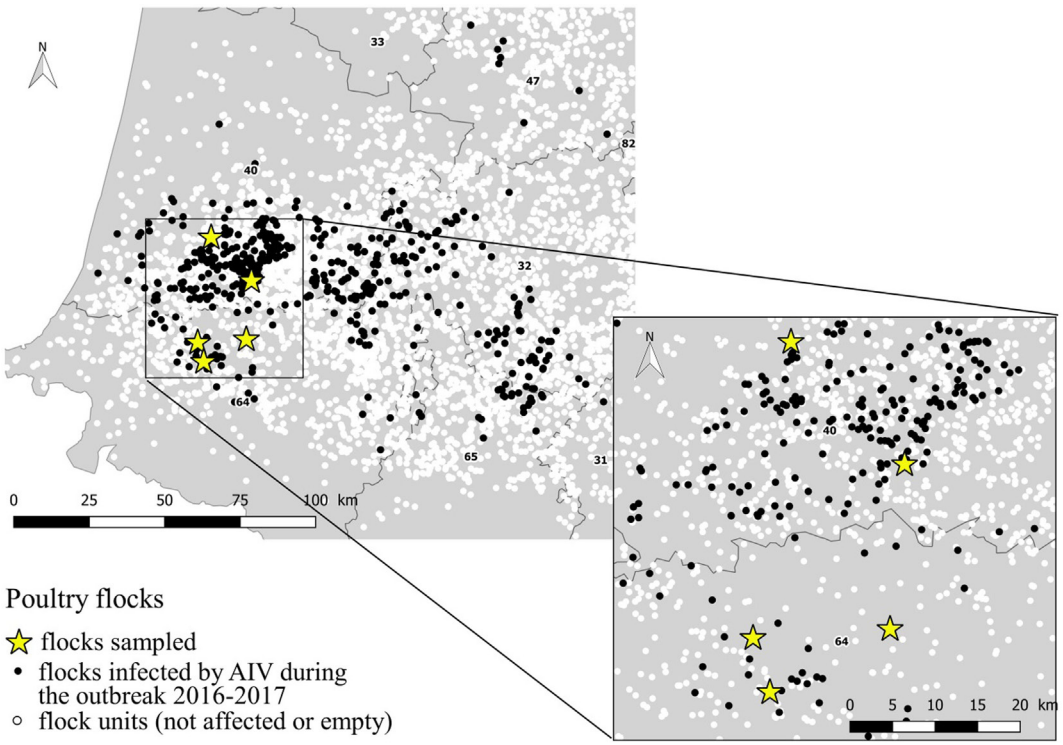

FIGURE 1 | Location of the sampled flocks within the area affected by the AIV 2016–2017 outbreak.

TABLE 2 | Detection of influenza virus genome in air samples by rRT-PCR inside and outside poultry barns infected by HPAI subtype H5N8 clade 2.3.4.4.

Farm ID	Specie/type	M gene rRT-PCR Ct value/H5 gene rRT-PCR Ct value				
		Inside	External exhaust fans	Outside 5 m	Downwind (distance in m)	Loading for culling
A	Ducks/PAG[a]	32.4/34.9	32.7/35.8	32.3/36.1	33.6/35.4 (50)	NT
B	Ducks/PAG	35.6/39.7	31.2/34.8	33.9/35.8	Not detected (80)	NT
C	Ducks/FF[b]	29.8/30	31/30.7	30.5/31.1	Not detected (60)	NT
D	Chickens/grow	34.9/35.3	33.1/34.4	33.1/36.2	34.2/38.8 (50)	NT
E	Chickens/grow	31.5/32	NT	NT	34.2/37.5 (110)	28.7/29.3

[a]*PAG, growing ducks for "foie gras" production.*
[b]*FF ("foie gras" production).*
Ct, cycle threshold; PAG, prêts à gaver; FF, force feeding period; NT, not tested.

RESULTS

Detection of HPAI Viral Genome in Air Samples

In the control sample, no viral genome signal was detected by M gene rRT-PCR. All positive air samples detected in this study, were both positive by M gene and H5 subtype rRT-PCR. All air samples collected inside (5/5), at external exhaust fans (4/4), 5 m outside the barn (4/4) were positive. Three of the five samples collected downwind from the barn were also positive (**Table 2**). Regarding samples collected downwind, the positive samples correspond to the flocks with clinical signs (mortality) and to an ambient temperature at sampling event of 2, 12, and 20°C and the negative samples to the asymptomatic flocks and to an ambient temperature of 17 and 24°C. The two flocks (B and C) with no detection of viral genome in air sample collected downwind also had low housing poultry densities. The sample collected during the animal loading was positive. In the five flocks studied, all air samples collected inside and at least one sample collected outside at 5–110 m distance from the barn were positive.

Quantification of HPAI Viral Genome in Air Samples

The quantity of virus (expressed in $\log_{10}$ RNA copies per m^3) estimated in positive air samples, ranged from 4.33 to 6.09 and from 4.54 to 6.43, in duck and chicken flocks, respectively (**Figure 2**; **Table 3**). The maximum air viral RNA concentration

was found at the animal loading point. For two of four flocks (one duck, one chicken, flocks B and D), the concentration found at the external fans was higher than inside the barn. There was a higher concentration variability between flocks (**Figure 2**; **Table 3**) for the samples collected inside barns than at the other sampling locations. The two lowest air concentrations measured inside barns corresponded to the lowest house poultry densities flocks (B and D) at sampling event. Furthermore the lowest of these two air concentrations mentioned above also corresponded to the flock (B) with the lowest proportion of pools of five swabs positive by rRT-PCR targeting the matrix gene (**Table 1**). The highest concentrations measured inside and at the short distance outside (external exhaust fans and 5 m distance) corresponded to the flock (C) of ducks at the force feeding period. Outside of the barns, there was a decrease of the geometric mean of positive air sample RNA concentrations measured against an increasing distance from the barns (**Table 3**).

DISCUSSION

The rapid spread of H5N8 or H5Nx HPAI clade 2.3.4.4 virus during the winter of 2016–2017 in South West France raised questions about the possibility of airborne transmission contribution to the global spread. As a first step in the investigation of airborne transmission hypothesis, we detected H5 gene viral RNA from air samples collected inside, outside and downwind of H5N8 HPAI infected poultry facilities and this detection occurred inside and outside poultry facilities in all of the five flocks studied.

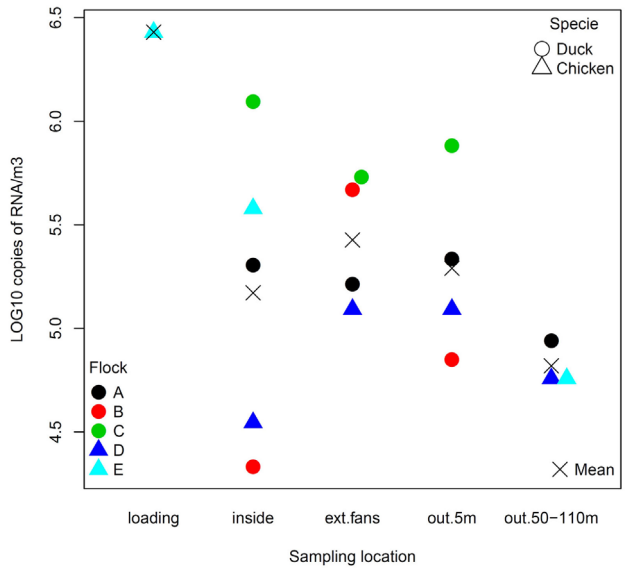

FIGURE 2 | Viral RNA concentration (log_{10} copies of RNA per m^3) of positive air samples by sampling location.

The percentage of actively infected birds, the poultry density and environmental conditions inside and outside barns at the time of sampling, were expected to influence the detection and the concentration of viral genome in air samples. This seems to be particularly the case for the measurement inside the barns. The decrease of positive air sample proportion, as well as the decrease of viral genome concentration in air samples between the samples collected inside or at short distance outside poultry facilities and the ones collected at 50–110 m distance, likely reflect decreasing virus concentration by dilution as a function of distance from the source. The time of sampling, which took place late morning (10 a.m.) for the flocks D and E and early afternoon (2 p.m.) for the other flocks (A, B, and C), could have also influenced the results due to the ambient temperature. Indeed, it could have contributed to the no detection of viral genome at 50–110 m distance for two of the three flocks collected early afternoon.

The levels of viral detection (proportion of positive samples and positive air sample viral RNA concentrations) were comparable to the ones found around H5N2 clade 2.3.4.4 HPAI poultry facilities during the 2015 spring outbreaks in the United States (8) and higher than the results around LPAI poultry facilities in the Netherlands (7) and at live poultry markets in China (4, 6). The virus viability in the air samples collected could not be investigated in this study, due to the sample processing (nature of the solution used). However, based on previous studies with different strains of AIV (6) or the same clade of AIV (8), we hypothesize that viable virus was likely captured in our sampling given the high levels of viral RNA concentrations.

For the airborne transmission of HPAIV to potentially occur, it would require not only the transport of viable virus on aerosolized particles, but also the capacity of viral contaminated particles to infect birds. The fact that experimentally, H5N1 HPAI airborne transmission has been performed with chickens (2, 3, 19) with air viral genome concentrations (all air fractions included) comparable to Ref. (3) our findings, is in favor of the hypothesis of infective capacity of the contaminated aerosolized particles present in the positive air samples collected. Even considering that the infectivity of AIV, considering the infectious

TABLE 3 | Quantity (RNA copies/m^3 of air) of H5N8 HPAIV in positive sampling events collected inside and outside duck and chicken premises.

Sampling location	Duck				Chicken			
	n	GM	GSD	max	*n*	GM	GSD	max
Inside	3	1.76E + 05	7.6	1.25E + 06	2	1.15E + 05	5.4	3.79E + 05
External exhaust fans	3	3.46E + 05	1.9	5.38E + 05	1	1.24E + 05	–	–
Outside 5 m	3	2.27E + 05	3.3	7.64E + 05	1	1.24E + 05	–	–
Downwind (50–110 m)	1	8.72E + 04	–	–	2	5.73E + 04	0	–
Loading for culling					1	2.69E + 06	–	–

GM, geometric mean; GSD, geometric standard deviation.

dose, is both host-dependent and virus strain-dependent (20–23), the fact that a low mean bird infectious dose (<2–3 $\log_{10}$ EID_{50}) by intranasal route has been determined with H5 HPAIV clade 2.3.4.4 (H5N8 and H5N2) United States index viruses in Pekin ducks and Chinese geese (24) and that the infectivity of AIV can be much higher (30 times) by aerosol route as compared with intranasal route, as established for eight strains of subtype H5N1 HPAIV in chickens (19), suggests that the airborne transmission through infected aerosols could require a very low dose of AIV with domestic ducks for such strains.

Infectious particles with aerodynamic diameter smaller than 10 µm are more susceptible to cause infection as they are inhaled into the lower respiratory tract. In future studies, the infectious particle size distribution should be investigated to confirm the infective potential of the exhausted air from H5N8 HPAI infected poultry facilities in case of new outbreaks, as was performed around H5N2 clade 2.3.4.4 HPAI infected poultry facilities with results indicating that viral RNA can be associated with fine particles (8, 9).

Despite the limitations of the study, our results suggest that exhaust air from H5N8 HPAI infected poultry facilities could be an important source of environmental contamination by deposition of infected dust on surfaces surrounding the infected premises, generating fomites. This phenomenon would be highly influenced by the environmental conditions such as temperature, relative humidity, UV exposure, etc. The quantity of viruses emitted in the air by an infected flock considering the downwind estimated air viral concentration and the duration of the flock excreting period (estimated, for example, at 7 days at least for the flock B) could be considered as potentially important enough to infect a nearby large poultry flock close. However, this possibility doesn't only depend on environmental conditions but also on factors influencing infected aerosol dispersion such as wind and factors influencing animal receptivity such as species.

Our results also question the management of infected flocks. The confinement inside housing does not seem to be effective enough to prevent viral diffusion into the environment surrounding infected premises and the culling process requiring the loading of the animals into containers located outside the poultry house seems to generate an important emission of potentially infectious dust and/or aerosols into the environment. It would be essential to reduce this diffusion by rapidly implementing the depopulation using a method that reduces the air viral emission. To achieve this goal, new case management methods must require less human resource in terms of time and volume because human resources availability is the main cause of increasing time between the confirmation date and the depopulation. Furthermore, the methods must include a depopulation process minimizing the air viral diffusion to the surrounding environment. Methods such as emergency mass culling of poultry using a foam blanket over birds and in-house carcasses and litter composting could contribute to improve the control of influenza outbreaks (25, 26).

In conclusion, our results sustain the hypothesis of a potential airborne transmission contribution to the spread of the H5N8 HPAIV. However, more investigations would be required to support this hypothesis so as to provide evidence of virus viability in fine particles emitted from poultry outbreaks and epidemiological evidence.

AUTHOR CONTRIBUTIONS

Survey design and field implementation: AXS, RT and SLB. Laboratory analyses: PD. Data analysis: AXS, SLB, EN, AUS. Manuscript writing: AXS. Manuscript editing: SLB, EN, PD, AUS.

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Comparative Epidemiology of Highly Pathogenic Avian Influenza Virus H5N1 and H5N6 in Vietnamese Live Bird Markets: Spatiotemporal Patterns of Distribution and Risk Factors

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Highly pathogenic avian influenza (HPAI) H5N1 virus has been circulating in Vietnam since 2003, whilst outbreaks of HPAI H5N6 virus are more recent, having only been reported since 2014. Although the spatial distribution of H5N1 outbreaks and risk factors for virus occurrence has been extensively studied, there have been no comparative studies for H5N6. Data collected through active surveillance of Vietnamese live bird markets (LBMs) between 2011 and 2015 were used to explore and compare the spatiotemporal distributions of H5N1- and H5N6-positive LBMs. Conditional autoregressive models were developed to quantify spatiotemporal associations between agroecological factors and the two HPAI strains using the same set of predictor variables. Unlike H5N1, which exhibited a strong north–south divide, with repeated occurrence in the extreme south of a cluster of high-risk provinces, H5N6 was homogeneously distributed throughout Vietnam. Similarly, different agroecological factors were associated with each strain. Sample collection in the months of January and February and higher average maximum temperature were associated with higher likelihood of H5N1-positive market-day status. The likelihood of market days being positive for H5N6 increased with decreased river density, and with successive Rounds of data collection. This study highlights marked differences in spatial patterns and risk factors for H5N1 and H5N6 in Vietnam, suggesting the need for tailored surveillance and control approaches.

Keywords: avian influenza, epidemiology, live bird markets, poultry, spatial modelling, Vietnam, emerging infectious disease

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Abbreviations: HPAI, highly pathogenic avian influenza; LBM, live bird market; INLA, integrated nested Laplace approximations; LISA, local indicators of spatial association; DAH, Department of Animal Health; CAR, conditional autoregressive model.

INTRODUCTION

Highly pathogenic avian influenza (HPAI) H5N1 virus (hereafter H5N1) is endemic to multiple Asian countries, including Vietnam, where the first recorded H5N1 outbreak occurred in 2003. Since then, costly control measures have been introduced, including culling of infected birds and vaccination of poultry (1). In addition to the economic impact, H5N1 has a high mortality rate in humans coupled with an ever-present threat of pandemic influenza (2). HPAI H5N6 virus (hereafter H5N6) emerged in Vietnam in April 2014 (3, 4).

Both virus subtypes are highly pathogenic in chickens, may cause asymptomatic infection in ducks, and have been associated with sporadic human infection and deaths in Asia. Numerous studies have explored the epidemiology of H5N1 in Asia, describing its spatial distribution at the regional level and in individual countries. Multiple factors have been associated with H5N1 including increased density of domestic ducks (5, 6) and chickens (7), proximity to high aggregations of human population density, a greater percentage of land used as rice paddy fields, higher rice-cropping intensity and lower average annual precipitation (8, 9). These studies predominantly analysed passive surveillance disease presence data resulting in exposure to temporal and spatial variations in surveillance effectiveness. However, contrast to the extensive literature surrounding H5N1, little has been published on the epidemiology of H5N6 in poultry.

In response to the endemic HPAI status in Vietnam, extensive active surveillance of live bird markets (LBMs) was initiated in 2008, first targeting the H5N1 virus and later expanding to include the H5N6 strain. LBMs were chosen as the foci for surveillance activities, partly because funding constraints precluded active surveillance at the farm level, but also because LBMs act as potential reservoirs for HPAI due to their role as hubs for poultry trade (10–12). Moreover, LBMs in northern Vietnam have been found to be highly connected through contact networks, enabling spread of HPAI not only between markets, but also between regions and even across international borders (12, 13). The key role that LBMs play in endemic spread of the virus was highlighted by the impact of the introduction of various biosecurity measures and infection control policies. Requirements such as the introduction of a day of market closure, cleaning at regular intervals, and for all birds to be sold or slaughtered by the end of trading each day greatly reduced the prevalence of HPAI in Hong Kong LBMs (14).

The aim of this study was to analyse the spatial distribution of H5N1 and H5N6 in Vietnamese LBMs using the same set of predictor variables. The majority of studies investigating HPAI in Asia utilise passive surveillance data, which relies upon detection and testing of clinically affected birds. Whilst HPAI H5N1 has been detected in asymptomatic ducks and poultry in LBM (15, 16), such cases are not detected through passive surveillance. The data collected through active surveillance of Vietnamese LBMs over a 5-year period provide a unique opportunity to explore HPAI epidemiology in Vietnam using virus detection data that are less exposed to reporting bias compared with data from passive surveillance. Specific objectives of this study were to (i) determine the prevalence of H5N1 and H5N6 virus in Vietnamese LBMs between 2011 and 2015; (ii) explore the spatiotemporal distributions of H5N1 and H5N6 virus in Vietnam; and (iii) develop models to quantify the spatiotemporal association between agroecological factors and the two HPAI strains using the same set of predictor variables.

MATERIALS AND METHODS

Surveillance Characteristics

Surveillance Protocol

Sampling was conducted as part of routine governmental active surveillance. All surveillance activities and protocols were approved by the Vietnamese Department of Animal Health (DAH) Epidemiology Division before implementation.

Sampling activities were implemented at specified times and places by the provincial Sub-Department of Animal Health (SDAH). At the LBM level, a sample size of 30 was required for 95% confidence of detection of H5N1 or H5N6, assuming prevalence of 10%, test sensitivity of 90%, and specificity of 99%. Sample testing was conducted in seven Regional Animal Health Office BSL2+ certified laboratories belonging to DAH, using BSL3 biosafety practice.

The surveillance period extended from September 2011 to December 2015 and was divided into six "Rounds" (**Table 1**).

LBM Selection

Selection of sampling locations varied by Round as follows:

1. *Round 1*: samples were collected from the two largest LBMs in each of 30 provinces out of a total of 63 (58 provinces and 5 centrally controlled municipalities (cities) at the same level as provinces). Provinces were selected on the basis of fulfilling one or more of the following criteria: (i) having a previous history of HPAI outbreaks, (ii) presence of an international

TABLE 1 | Graphical illustration of the temporal distribution of the six rounds of active surveillance sampling between September 2011 and December 2015.

	January	February	March	April	May	June	July	August	September	October	November	December
2011									Round 1			
2012	Round 1 (cont)									Round 2		
2013	Round 2 (cont)									Round 3		
2014	Round 3 (cont)				Round 4						Round 5	
2015	Round 6											

border, (iii) having a high density of poultry, or (iv) high human population density.

2. *Rounds 2–5*: samples were collected from provinces distributed throughout Vietnam using the same criteria for province selection as in Round 1. For every round, the DAH selected (i) 40 provinces from which one small-scale LBM was sampled and (ii) 20 provinces from which a large-scale LBM was sampled. The DAH selected 120 districts from the aforementioned 40 provinces (three districts per province) and 20 cities from the aforementioned 20 provinces.

Districts were selected on the basis of (i) having a high duck density and (ii) having a history of H5N1 outbreaks. In each selected district or city, the SDAH of the corresponding province selected one LBM for sampling. LBMs were selected on the basis of (i) size (at least six vendors), (ii) source of birds (within the district for small-scale LBMs, outside the province for large-scale LBMs), and (iii) no inclusion in H7N9 surveillance activities.

Small-scale markets were defined as markets which draw birds from within the same district and/or province. Sampling should therefore capture/represent the local circulation of HPAI. Large-scale markets were defined as markets which draw birds from outside the province and therefore capture/represent the national and/or regional circulation of HPAI.

3. *Round 6*: DAH selected 32 provinces distributed throughout the country; 12 northern provinces that share a border with China or have poultry trading connected to northern border provinces, and 20 central/southern provinces. In the northern provinces, a total of 48 LBM (4 from each province) were sampled. In the central/southern provinces, the largest LBM in each province was sampled.

Data Collection

Sampling

On the day of sampling, the vendors to be sampled were selected randomly from all vendors present at the market selling more than five ducks (or chickens for Round 6). The number and type of samples collected for each market day according to the surveillance design is summarised in **Table 2**.

Oropharyngeal swabs from ducks were collected consistently during each surveillance Round, whilst oropharyngeal swabs from chickens were collected during the last Round only. Environmental sampling started during Round 2 with the collection of faeces, feathers and waste in the resting and slaughter areas at four selected large LBMs. Four samples of each type were collected and tested individually. From Round 5 onwards, environmental sampling was extended to all LBMs regardless of their size and environmental swabs were pooled instead of being tested individually. Pooled samples comprised five merged swab samples (either oropharyngeal or environmental). Depending on the Round, between 93 and 100% of the market days reached the sample size targets (detailed in **Table 2**) for each type of sample.

Case Definition

Samples were tested at Regional Animal Health Offices for the H5 and N1 virus subtypes using real-time reverse transcription polymerase chain reaction. From Round 4 onwards samples were also tested for the N6 subtype. Cycle threshold values of less than 35 were regarded as positive. Samples positive for both the H5 and N1 subtypes were classified as positive for H5N1. Similarly, samples positive for both the H5 and N6 subtypes were classified as positive for H5N6.

The epidemiological unit for this study was a market day at a given LBM on a given date. A market day was classified as positive for H5N1 or H5N6 if one or more samples (individual or pooled) collected from that LBM on that date tested positive.

Agroecological Predictor Data

A review of the published literature served to identify potential predictor variables previously shown to be risk factors for HPAI occurrence and the final set of predictor variables used in this study included the following: density of ducks (heads/km^2) (7, 8, 17, 18), density of chickens (heads/km^2) (7, 8, 18, 19), human population density (heads/km^2) (6–8, 18, 20), travel time (minutes) to the nearest city with a population of ≥50,000, suitability of areas for growing rice (8, 18), river density (km length/km^2) (7, 19, 21, 22), average annual precipitation (mm) (23), average monthly minimum temperature (°C), and average monthly maximum temperature (°C) (23–25). LBM density (number of LBM/10 km^2) was also included.

Digital spatial data layers representing each predictor variable were sourced for Vietnam from the public domain, and all spatial

TABLE 2 | Target number of samples to be collected per market day, according to round and sample type.

Sample type		Numbers refer to pooled samples when not indicated otherwise			
		Round 1	Rounds 2–4	Round 5	Round 6
Oropharyngeal swabs	Ducks	4	6	6	6
	Chicken	0	0	0	6
Environmental swabs from four large live bird markets	Faeces from cage	0	4 individual samples	0	0
	Waste from resting area	0	4 individual samples	0	0
	Feathers	0	4 individual samples	0	0
	Dirt in slaughter area	0	4 individual samples	0	0
Environmental swabs from all sampling sites	Liquid waste	0	0	2	2
	Solid waste	0	0	2	2
	Faeces	0	0	1	1
	Drinking water	0	0	1	1

Pooled samples are the combination of 5 swab samples.

data manipulations and map creation were performed using ArcGIS 10.3.1 (28). Chicken and duck densities were extracted from the *Gridded Livestock of the World* (resolution: 1 km^2),[1] and human population density was obtained from *Gridded Population of the World v4* (resolution: 1 km^2; estimated for 2015).[2] The predicted density of LBMs/10 km^2 was obtained from a model generated by Gilbert et al. (unpublished, model description in Supplementary Material; resolution: 10 km^2), which was resampled to a resolution of 1 km^2. Travel time to the nearest city was obtained from the Global Environment Monitoring Unit in the Joint Research Centre of the European Commission (26). Areas suitable for rice growing were extracted from *Suitability for Rain-fed and Irrigated Rice (High Input)*, a shapefile available from Food and Agricultural Organization's GeoNetwork website (published 2007).[3] The data were converted to raster format (resolution: 1 km^2), and the original eight suitability categories were recategorised as follows: high (very high/high/good), moderate (medium/moderate), low (marginal/very marginal), unsuitable. Open water features were extracted from *VMap0 Perennial Water Courses (Rivers) of the World* (published 1997) (available from the Food and Agricultural Organization's GeoNetwork website; see text footnote 3) and density of rivers per square kilometre calculated using the line density feature in ArcGIS. Average monthly precipitation and minimum and maximum temperature data (based on the time frame 1950–2000) were obtained from the WorldClim website ((27); accessed March 2017). A vector shapefile of Vietnam's provincial boundaries was obtained from the GADM Database of Global Administrative Areas v2.8.[4] All data were processed to ensure that projections and extents matched. Latitude and longitude were available for LBMs, and data for each predictor variable were extracted to the point location of individual LBMs.

Statistical Analysis

Data Management

The Regional Animal Health Offices used Microsoft Excel to compile the sample collection spreadsheet and laboratory results into a single dataset (regional dataset). These regional datasets were submitted to the DAH each month where they were aggregated to provide a single dataset for each surveillance period. However, merged datasets were not available for some periods, and data recording was not harmonised between regional datasets resulting in multiple names identifying the same location. In such instances, markets with different names but the same coordinates were considered to be the same market. Eighty-three LBMs with missing longitude and latitude data were assigned the coordinates of the relevant commune centroid.

Mapping Spatiotemporal Distribution

To preserve LBM anonymity, market days were aggregated by province, and province-level prevalence of H5N1 and H5N6 was calculated for each Round as the number of positive market days in a province divided by the total number of market days sampled in that province.

Choropleth maps of raw rates or standard mortality ratios per area can be misleading; the addition of a small number of cases in an area with a small population at risk can dramatically increase the reported rate of disease for the area. Conversely, the addition of the same number of cases in an area with a large population at risk has little effect on the reported rate of disease for the area. Bayesian approaches allow disease rates to be adjusted through combining the observed rate for an area with rates observed in surrounding areas. When the at risk population of an area of interest is large, and the statistical error of the rate estimate small, higher credibility is given to the observed estimate, and the Bayes adjusted rates are similar to observed rates. However, where the population at risk is small, the rate is adjusted towards the mean rate observed over the wider study area.

Choropleth maps of empirical Bayes-smoothed prevalence were generated for H5N1 and H5N6 using Eqs 1–3 as follows: given that y_i equalled the number of positive market days observed in the ith province, n_i the total number of market days sampled in the ith province, and r_i was the proportion of positive market days for the ith province, then the pooled mean of observed prevalence across all provinces (γ) was calculated as follows:

$$\gamma = \sum \frac{y_i}{n_i}, \tag{1}$$

and the estimate of the population variance of the prevalence based on a weighted sample of the observed prevalences (φ) was calculated as follows:

$$\varphi = \frac{\sum n_i \left(r_i - \gamma\right)^2}{\sum n_i} - \frac{\gamma}{\overline{n}}, \tag{2}$$

then θ, the empirical Bayes-smoothed prevalence for the ith province, was calculated as follows:

$$\theta = \frac{\varphi * \left(r_i - \gamma\right)}{\varphi + \frac{\gamma}{n_i}} + \gamma. \tag{3}$$

Exploring Spatial Autocorrelation and Clustering

Spatial autocorrelation of the smoothed Bayes risk was explored at a global scale using the Moran's I statistic and at a local scale using the Anselin Local Moran's I statistic and Getis-Ord GI* statistic. The global Moran's I statistic was used to assess the presence, strength and direction of spatial autocorrelation over the whole study area, using a queen's contiguity weights matrix and 499 random permutations. A p-value ≤ 0.05 was considered significant. The Local Moran's I and GI* statistics were used to detect clustering of provinces with similar risk of H5N1 or H5N6, and to identify the locations of province-level hot and/or cold spots. The GI* statistic returned a z-score for each province and for statistically significant positive z-scores, the larger the z-score the more intense the clustering of high values (hot spot). For statistically significant negative z-scores, the smaller the z-score the more intense the clustering of low values

[1] https://livestock.geo-wiki.org/home-2/ (Accessed: March, 2017).
[2] http://sedac.ciesin.columbia.edu/data/collection/gpw-v4 (Accessed: March, 2017).
[3] http://www.fao.org/geonetwork/srv/en/main.home (Accessed: March, 2017).
[4] http://www.gadm.org/ (Accessed: March, 2017).

(cold spot). All spatial analyses were conducted using tools provided in ArcGIS 10.3.1 (28).

Modelling Associations Between Agroecological Factors and HPAI

Multivariable logistic regression analyses were used to investigate associations between putative predictor variables and H5N1 or H5N6-positive market days. Univariable analyses for each predictor variable were conducted, with significant variables included in multivariable analysis. All univariable and multivariable statistical analyses were performed in R 3.4.0 (29). Before multivariable analysis, all predictor data were standardised to a mean of 0 and SD of 1, for variables measured at different scales to contribute equally to the analysis. To identify the set of predictors associated with H5N1 and H5N6-positive market days, non-spatial generalised linear models were used, implemented *via* the R *glmulti* package (30), to build every possible non-redundant model for every combination of predictor main effects (interactions were not included due to the number of variables involved). Final best-fit models were chosen using Akaike's information criterion (AIC), which ranks models based on goodness-of-fit and complexity, whilst penalising deviance. The predictors identified in this first step were then included in a mixed-effects logistic regression model with the variable "market" as a random effect to determine whether any predictors were no longer significant after accounting for non-independence of market days. All continuous variables were assessed for linear trend by comparing the model with the continuous version of the variable with a model where the variable was categorised into quartiles. If the likelihood ratio test p-value was <0.05 the categorical version of the variable was included in model.

All identified predictors which remained significant at the 5% level in the mixed-effects logistic regressions were then included in a conditional autoregressive model (CAR) to account for the spatial autocorrelation of observations. Clustering of markets within provinces was accounted for by the inclusion of a spatially varying random effect "Province," using a spatial weights matrix where polygons were classified as neighbouring if they shared a corner or border (queen's contiguity). Clustering of market days within markets was accounted for through the inclusion of the non-spatially varying random effect "Market." The potential temporal effect of sampling heterogeneity was accounted for through inclusion of the variable "Round" in the model.

All CAR models were implemented in R using integrated nested Laplace approximations (INLA) which uses an approximation for inference and avoids the computational demands, convergence issues and mixing problems sometimes encountered by Markov chain Monte Carlo algorithms (31). The model was fitted using R-INLA, with the Besag model for spatial effects specified inside the function. In the Besag model, Gaussian Markov random fields are used as priors to model spatial dependency structures and unobserved effects. In addition, each model was run through INLA whilst excluding the random spatial effect to obtain non-spatial Bayesian estimates and to compare model fit and performance due to the explicit spatial process. Model selection was based on the deviance information criterion (DIC) where a lower DIC indicates a better model fit. In all analyses, an α-level of 0.05 was adopted to indicate statistical significance.

Choropleth maps showing the spatial distribution of the posterior means of the structured random effects obtained from the models were produced in ArcGIS (28).

RESULTS

Sampling Sites and Samples

During the surveillance period 22,185 pooled samples were collected from 459 LBM distributed between 48 provinces (242 districts) (**Table 3**). Each LBM was visited between 1 and 28 times (median 4 visits), providing a total of 3,461 market days for analysis. Sampling intensity was highest in Round 1 and decreased thereafter (**Table 3**).

TABLE 3 | Sampling characteristics and prevalence of highly pathogenic avian influenza H5N1- and H5N6-positive market days of the six surveillance Rounds.

	Rounds						
	1	2	3	4	5	6	Total
Dates	**September 2011–February 2012**	**October 2012–September 2013**	**October 2013–April 2014**	**April 2014–October 2014**	**November 2014–December 2014**	**July 2015–December 2015**	**September 2011–December 2015**
Number of pooled samples	3,952	4,642	3,984	5,301	1,668	2,638	22,185
Number of provinces	30	44	42	44	44	30	48
Number of districts	122	141	135	138	71	58	242
Number of live bird markets	279	152	143	143	77	63	459
Number of days	153	365	212	184	61	183	1,158
Total market days	974	748	624	827	142	146	3,461
Sampling intensity (market days/number of days)	6.4	2.1	3.0	4.5	2.3	0.8	3.0
Observed prevalence H5N1-positive market days (%)	8.5 (41/974)	19.5 (146/748)	15.7 (111/624)	6.8 (56/827)	14.5 (18/142)	10.2 (15/146)	11.2 (387/3,461)
Observed prevalence H5N6-positive market days (%)				0.7 (6/827)	16.2 (23/142)	26.0 (38/146)	6.01 (67/1,115)

In general, sampled provinces were evenly distributed throughout the country although sampling in Rounds 2–4 provided more homogenous coverage of Vietnam than Rounds 1 and 6, with the latter exhibiting a slight north–south emphasis.

Prevalence of H5N1 and H5N6-Positive Market Days

The observed prevalence of H5N1-positive market days varied between rounds (median: 12.35; range: 6.8–19.5%) although this difference was not significant (χ^2 p-value = 0.48) (**Table 3**). The observed prevalence of H5N6-positive market days increased significantly (χ^2 $p < 0.001$) over Rounds 4–6 from 0.7 to 26% (**Table 3**).

Spatiotemporal Distribution of H5N1- and H5N6-Positive Market Days

Province-level Bayes-smoothed prevalence of H5N1-positive market days was spatially heterogeneous in all six Rounds, although it was highest in Rounds 2 and 6 (**Figure 1**). This apparent spatial heterogeneity was supported by both the global and local autocorrelation statistics, which identified significant positive spatial autocorrelation in all rounds except Round 2 (Moran's I p-value > 0.05; **Figure 2**). The positive autocorrelation was characterised by repeated occurrence, in the south of the country, of a cluster of high-risk provinces surrounded by other high-risk provinces, although the size of the cluster varied between Rounds (**Figure 2**). Conversely, northern Vietnam was characterised by low-risk provinces surrounded by other provinces of low risk. However, the north also exhibited periodic recurrent outliers; provinces with a high disease risk but surrounded by others with a low disease risk (**Figure 2**). Hot-spot provinces were identified in all Rounds but the number decreased over time (Rounds 1–3: $n = 4$; Rounds 4–5: $n = 2$; Round 6: $n = 1$) (**Figure 3**). One province, in particular, Ca Mau was identified as a hot-spot province of H5N1-positive market days in four of the six rounds. Unlike H5N1, province-level empirical Bayes-smoothed risk of H5N6-positive market days did not display any significant spatial heterogeneity in any of the Rounds (Moran's I p-value > 0.05) although the level of risk increased between Rounds 4 and 6 (**Figure 4**). In general, the most common pattern was one of outliers; provinces with a high risk of H5N6-positive market days were generally surrounded by low-risk provinces and *vice versa* (**Figure 5**). Two hot-spot provinces were identified in each Round although Quang Ngai was the only province to be identified as a hot spot more than once (Rounds 4 and 5; **Figure 6**).

Risk Factors for H5N1 and H5N6-Positive Market Days

The most robust H5N1 multivariable model, based on the AIC, included six of the thirteen predictor variables; suitability for rice-growing, sampling month, average monthly maximum temperature, river density, travel time to a city and chicken density, and were therefore included in the H5N1 INLA model. The variable "Round" was forced into the model to account for temporal variation in sampling. The CAR model based on these variables had a DIC value of 1,984.31 (H5N1). Inclusion of the spatial random effect "province" improved the fit of the H5N1 model by 8.17%, reducing the DIC to, 1,822.10. Three of the six variables retained in the model were statistically significant, three variables were not deemed significant, due to the odds ratio (OR) 95% credible interval crossing 1. The odds of a market day being positive for H5N1 varied between Rounds. Comparison of OR across months identified the likelihood of market day status being positive to be highest in January and February. The odds of a market day being positive for H5N1 were 3.36 (95% CrI 1.29, 8.36) greater where the average maximum temperature was ≥30.33°C compared with areas where the average maximum temperature was ≤24.47°C (**Table 4**).

In the multivariable analysis, only three of the thirteen predictors were significantly associated with market days being positive for H5N6: river density, human population density and market density. These covariates were taken forward to the H5N6 INLA model. The variable "Round" was forced into the model to account for temporal variation in the sampling. The CAR model based on these variables had a DIC value of 202.36, inclusion of the spatial random effect "province" did not improve the model fit (the DIC

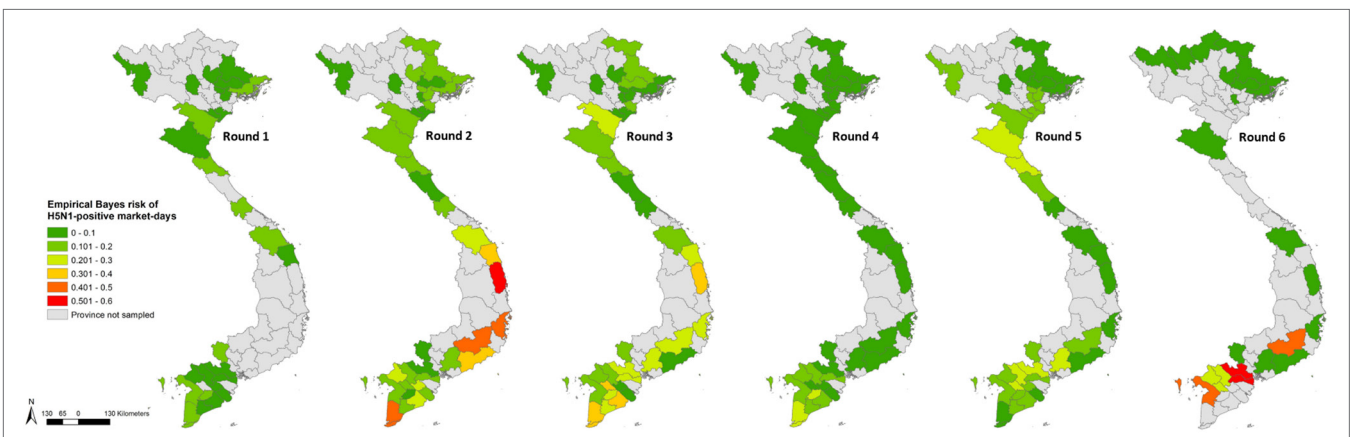

FIGURE 1 | Province-level Bayes risk of highly pathogenic avian influenza H5N1 in Vietnam (Rounds 1–6).

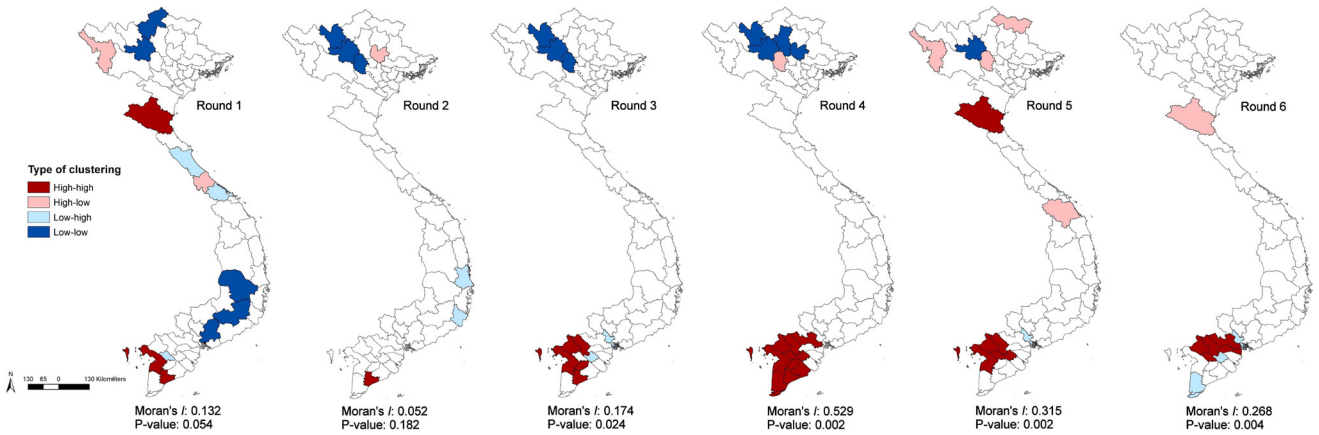

FIGURE 2 | Local Indicators of Spatial Association cluster maps and Moran's *I* statistics of highly pathogenic avian influenza H5N1 Bayes risk for Rounds 1–6.

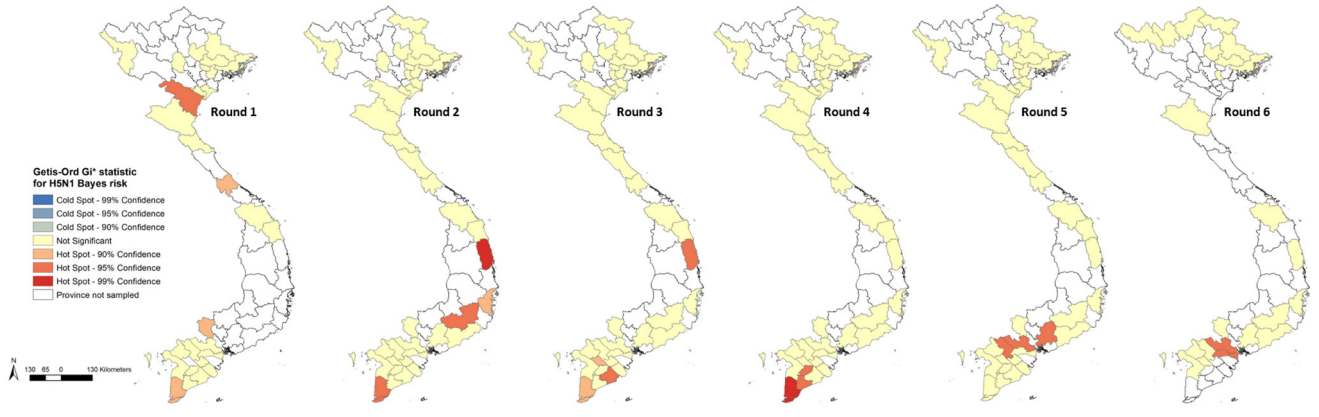

FIGURE 3 | Getis-Ord GI* statistic maps showing hot-spot provinces for highly pathogenic avian influenza H5N1 Bayes risk for Rounds 1–6.

was lowered by 0.03% to 202.29). Therefore, the final model used for H5N6 therefore did not include the spatially varying random effect. The likelihood of a market day being positive for H5N6 was higher with successive Rounds and lower river density (**Table 5**).

Mapping posterior means of the spatially structured random effects for H5N1 showed them to be reasonably homogenously distributed throughout the country, suggesting that there is unexplained variation in most regions, after accounting for the model covariates (**Figure 7**).

DISCUSSION

The results of this study suggest that the epidemiology of H5N1 and H5N6 in Vietnam are very different. Not only do the two strains show different distributions, they are also associated with different risk factors. Whilst the risk of H5N6-positive market days was homogenous across Vietnam, the posterior mean probabilities of H5N1 from the CAR model at the province level showed clear regional differences, with higher probabilities in the southern and central provinces of Vietnam. Similarly, whilst higher risk of H5N6-positive LBMs was associated with lower river density, spatial variation in H5N1 risk was primarily associated with climatic factors.

Collection month was associated with variation in market-day H5N1 risk. The odds of H5N1-positive market days were highest in January and February. No samples collected between January and March were tested for H5N6. Seasonal fluctuations in the proportion of positive market days may be due to a combination of climatic factors and peaks in demand for poultry products. Higher incidence of H5N1 in domestic poultry in central and southern Vietnam has been shown to coincide with an increased demand for poultry products in January and February associated with the Lunar New Year Festival (32). Colder temperatures in winter months have also been proposed to contribute to higher risk of H5N1 due to longer virus survival time in the environment (24, 25).

Higher average maximum temperature was associated with higher risk of market day H5N1 positivity. This factor contributes to the observed north/south risk divide, as average maximum

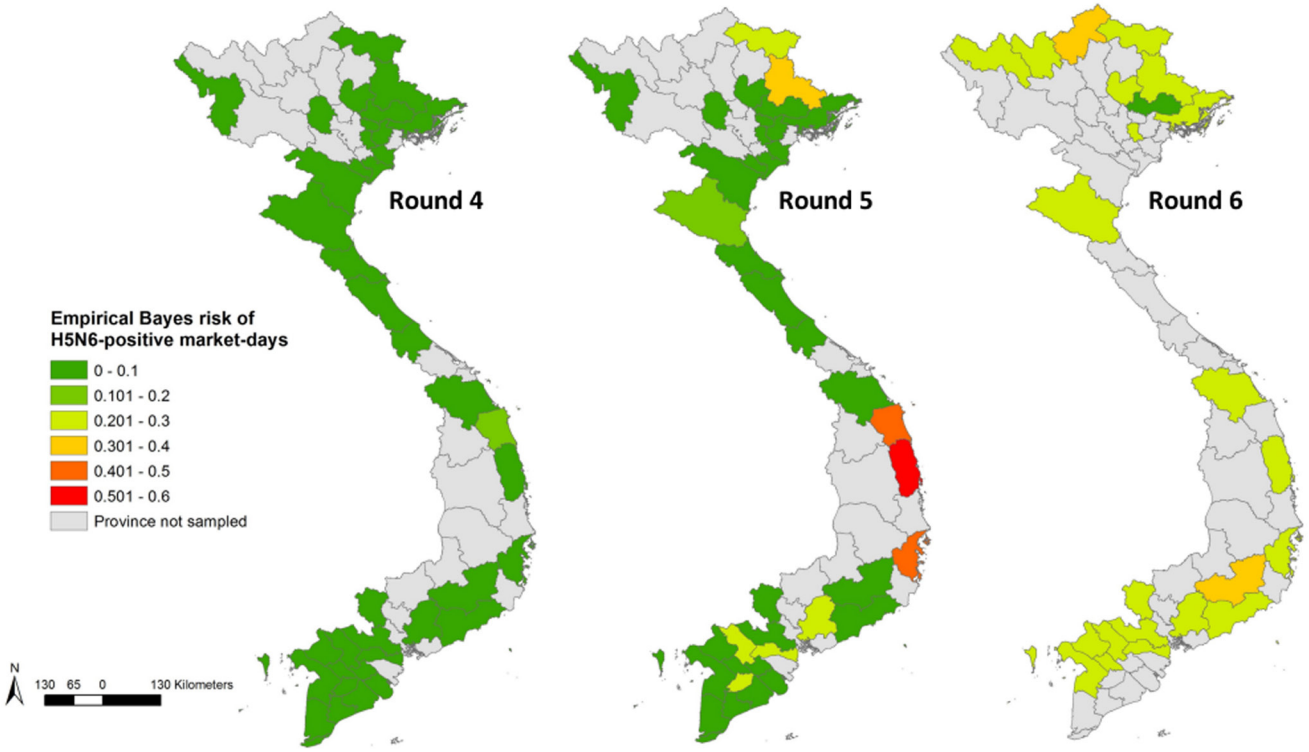

FIGURE 4 | Province-level empirical Bayes risk of highly pathogenic avian influenza H5N6 in Vietnam (Rounds 4–6).

temperatures are higher in southern than northern provinces of Vietnam. However, due to limitations in sampling strategy, with time gaps in surveillance and variation in sampling strategy between years, it is not possible to reliably assess consistency of seasonal patterns over time.

Rice-cropping intensity has previously been associated with H5N1 presence in South East Asia (18). None of the samples collected on the 28 market days in areas with poor suitability for rice production tested positive for H5N1. Similarly, of the six market days sampled for H5N6 in areas with poor suitability for rice production, no samples tested positive. However, a significant association between the risk of H5N1-positive market days and the higher suitability of land for growing rice was not identified in this study, which may be due to the small number of market days sampled in poor rice production areas. Remote sensing data can capture greater resolution compared with traditional census collection, allowing for greater accuracy in assessment of rice-cropping intensity and suitability. This finer scale resolution may improve detection of associations between rice growing and prevalence of HPAI (18).

The purpose of inclusion of variables such as chicken density, duck density, and rice suitability was to capture risk factors at point of production. Consideration must be given to the potential for chicken and duck farms to be located in geographically distant areas from the market (33). Contrary to findings of some previous studies in South East Asia, higher domestic chicken population density and waterfowl density were not found to be associated with increased risk of H5N1-positive market days (8, 17). The production location of poultry from which samples were collected was not obtained during the study. Data used in the models are reflective of the locality of the market, but not necessarily the location of production. Therefore, caution is necessary regarding the interpretation of the association between risk of a market being positive for H5N1 or H5N6 and variables relating to location of production including chicken density, duck density, and the suitability of land for rice production. The prevalence of HPAI at the LBM level will be impacted by the catchment area and the extent of interconnectedness with other LBM through poultry trade. Identification of production location would enable capture of risk associated with production factors with greater accuracy.

Reduced travel time to a major city has been associated with higher risk of H7N9 presence in LBM in Asia (34). Travel time to the nearest city is a measure of accessibility of the LBM, and the increased risk associated with more accessible LBM could be reflective of birds being drawn from more diverse populations, over a larger catchment area and connections with other LBM. In the multivariable INLA model, shorter travel time to the nearest city was not significantly associated with higher H5N1-positive market-day status. This may be due to the highly connected nature of LBM in Vietnam, enabling dissemination even between relatively less well accessed markets (12, 13).

The residual spatial variation in H5N1 market-day risk at the province level indicates that there are unexplained factors contributing to risk that were not included in the model. Vaccination has been used to control HPAI in Vietnam and may have contributed to the spatial and temporal variation in risk,

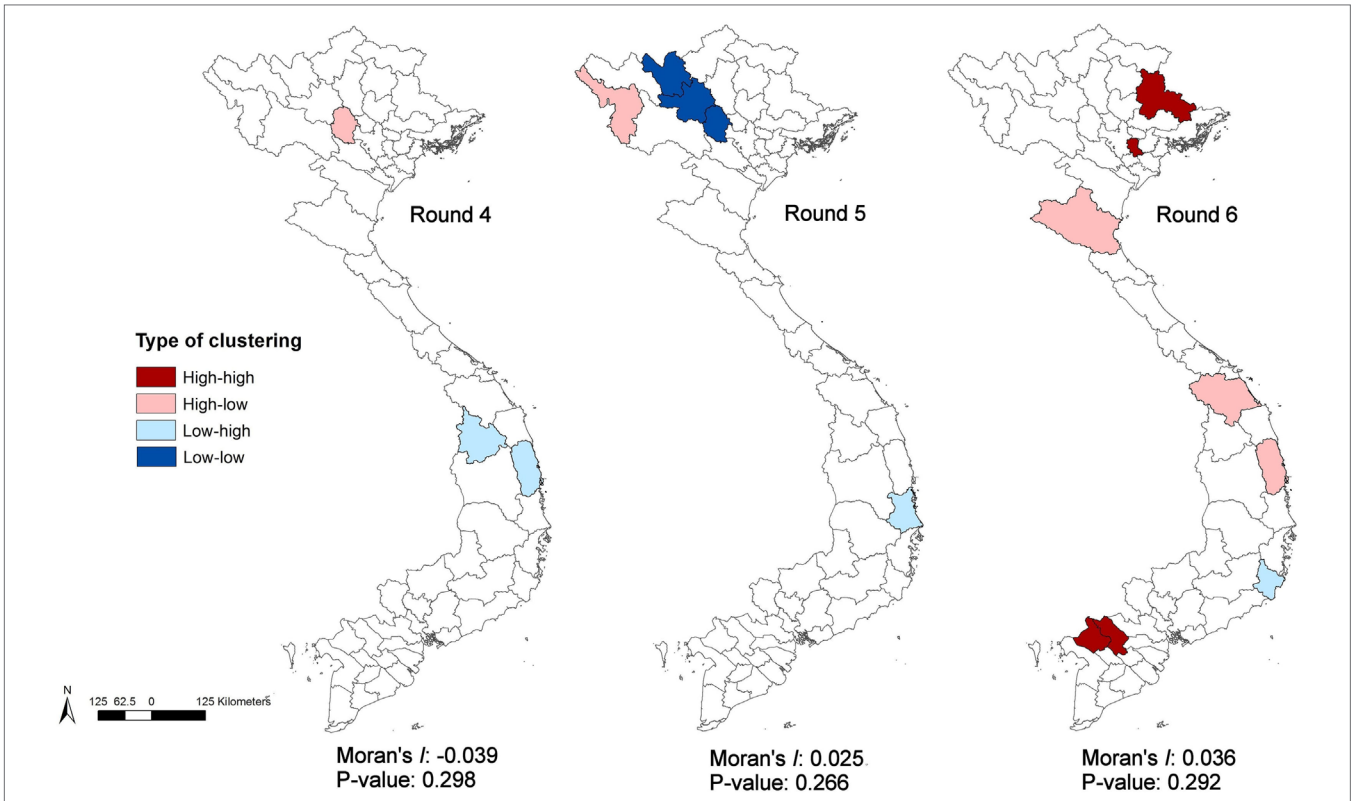

FIGURE 5 | Local Indicators of Spatial Association cluster maps and Moran's *I* statistics of empirical Bayes risk estimates of highly pathogenic avian influenza H5N6 for Rounds 4–6.

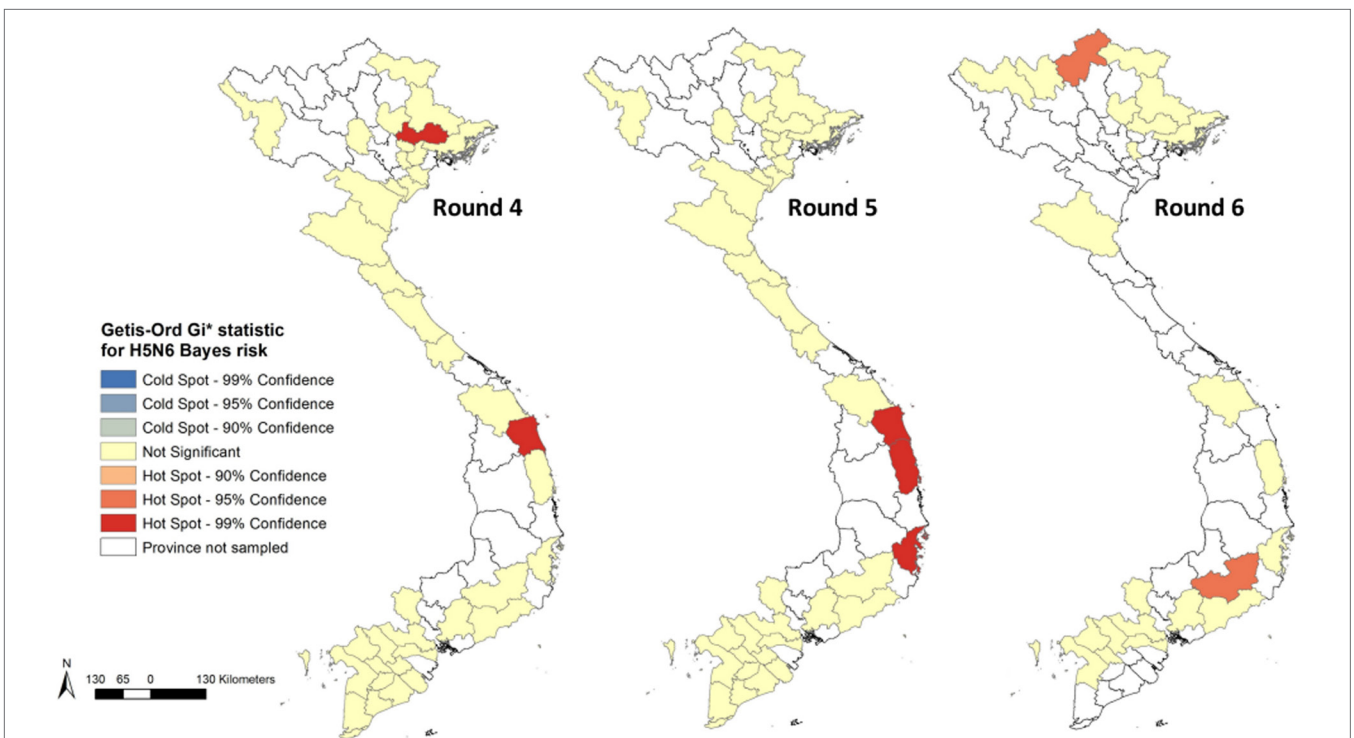

FIGURE 6 | Getis-Ord GI* statistic maps showing hot-spot provinces for highly pathogenic avian influenza H5N6 empirical Bayes risk estimates for Rounds 4–6.

TABLE 4 | Posterior mean coefficients, odds ratios (ORs), and 95% credible intervals (CrI) of spatial and non-spatial conditional autoregressive models of market days positive for highly pathogenic avian influenza H5N1 virus (Vietnam, 2011–2015).

	Coefficient, posterior mean (95% CrI)		OR, posterior mean (95% CrI)	
	Multivariable model (no spatially varying random effect)	**Multivariable model (province as spatially varying random effect)**	**Multivariable model (no spatially varying random effect)**	**Multivariable model (province as spatially varying random effect)**
Suitability for rice growing				
High/moderate	Baseline	Baseline	Baseline	Baseline
Marginal/unsuitable	0.47 (0.15, 0.78)	−0.04 (−0.51, 0.42)	1.60 (1.17, 2.19)	0.96 (0.60, 1.52)
Sampling month				
January	Baseline	Baseline	Baseline	Baseline
February	−0.27 (−0.67, 0.12)	−0.36 (−0.77, 0.05)	0.76 (0.51, 1.13)	0.70 (0.46, 1.05)
March	−0.87 (−1.40, −0.36)	−1.00 (−1.55, −0.47)	0.42 (0.25, 0.70)	0.37 (0.21, 0.63)
April	−1.01 (−1.55, −0.49)	−1.11 (−1.68, −0.57)	0.37 (0.21, 0.62)	0.33 (0.19, 0.57)
May	−1.48 (−2.19, −0.82)	−1.75 (−2.49, −1.06)	0.23 (0.11, 0.44)	0.17 (0.08, 0.35)
June	−0.27 (−1.19, 0.60)	−0.59 (−1.51, 0.29)	0.76 (0.30, 1.83)	0.55 (0.22, 1.34)
July	−0.50 (−1.47, 0.43)	−0.82 (−1.79, 0.12)	0.61 (0.23, 1.54)	0.44 (0.17, 1.13)
August	−0.35 (−1.26, 0.53)	−0.65 (−1.58, 0.26)	0.70 (0.20, 1.70)	0.52 (0.21, 1.30)
September	−0.80 (−1.60, −0.05)	−1.10 (−1.92, −0.33)	0.45 (0.20, 0.95)	0.33 (0.15, 0.72)
October	−1.80 (−3.44, −0.49)	−1.96 (−3.62, −0.62)	0.16 (0.03, 0.61)	0.14 (0.03, 0.54)
November	−0.53 (−0.99, −0.08)	−0.66 (−1.13, −0.20)	0.59 (0.37, 0.92)	0.52 (0.32, 0.82)
December	−0.80 (−1.20, −0.40)	−0.92 (−1.34, −0.51)	0.45 (0.30, 0.67)	0.40 (0.26, 0.60)
Average maximum temperature (°C)				
≤24.47	Baseline	Baseline	Baseline	Baseline
24.48–28.74	0.72 (0.26, 1.20)	0.15 (−0.75, 1.03)	2.06 (1.29, 3.34)	1.17 (0.47, 2.81)
28.75–30.32	1.71 (1.29, 2.15)	1.12 (0.21, 1.99)	5.54 (3.63, 8.62)	3.07 (1.23, 7.32)
≥30.33	1.69 (1.23, 2.16)	1.21 (0.26, 2.12)	5.41 (3.43, 8.71)	3.36 (1.29, 8.36)
River density (km length/km^2)	0.08 (−0.05, 0.21)	−0.05 (−0.29, 0.19)	1.08 (0.95, 1.23)	0.95 (0.75, 1.21)
Travel time (min) to nearest city with population ≥50,000	−0.18 (−0.33, −0.04)	−0.07 (−0.24, 0.08)	0.84 (0.72, 0.96)	0.93 (0.78, 1.09)
Chicken density (heads/km^2)				
<285	Baseline	Baseline	Baseline	Baseline
285–791.3	0.19 (−0.13, 0.51)	0.03 (−0.39, 0.45)	1.21 (0.88, 1.66)	1.03 (0.68, 1.57)
791.4–1,686.1	0.07 (−0.27, 0.41)	0.00 (−0.41, 0.42)	1.07 (0.77, 1.50)	1.00 (0.66, 1.52)
≥1,686.2	−0.44 (−0.86, −0.03)	−0.37 (−0.87, 0.12)	0.64 (0.42, 0.97)	0.69 (0.42, 1.13)
Round				
Round 1	Baseline	Baseline	Baseline	Baseline
Round 2	1.11 (0.76, 1.46)	1.14 (0.76, 1.52)	3.02 (2.13, 4.31)	3.13 (2.14, 4.57)
Round 3	0.85 (0.48, 1.21)	0.85 (0.47, 1.24)	2.33 (1.62, 3.35)	2.35 (1.60, 3.56)
Round 4	−0.12 (−0.85, 0.61)	0.06 (−0.68, 0.81)	0.89 (0.43, 1.85)	1.06 (0.51, 2.25)
Round 5	0.68 (−0.02, 1.33)	0.76 (0.03, 1.44)	1.97 (0.98, 3.78)	2.14 (1.03, 4.23)
Round 6	1.25 (0.18, 2.29)	1.57 (0.47, 2.65)	3.48 (1.19, 9.91)	4.81 (1.60, 14.16)
Model deviance information criterion	1,934.81	1,822.10		

Province included as a spatially varying random effect.

as vaccine coverage has been found to vary with both district and season (35). In addition, the predominant duck breeds may vary between regions and vaccine response of different breeds of domestic ducks to the commercial vaccines has been shown to differ, resulting in shedding continuing in some clinically unaffected, vaccinated ducks (36–38).

Additional market level factors not included in the model have the potential to contribute to between-market variation in the likelihood of a sample testing positive for H5N1 or H5N6. Factors include the number of days per week the market opens, biosecurity measures, length of holding of birds in the LBM, number of birds and stocking density in the LBM, biosecurity, and husbandry on farms producing the poultry for sale (39, 40). Collection of further market level information would enable further improvement of understanding of both H5N1 and H5N6 epidemiology in Vietnam.

Previous studies mapping the spatial distribution of avian influenza in Vietnam have utilised data obtained through passive surveillance (8, 18). In Vietnam, passive surveillance is conducted through farmers or community animal health workers notifying local state vets, with subsequent diagnostic testing and investigation. Positive samples are then reported to the central government. Currently, a low number of outbreaks are reported through passive surveillance (32) due to the reliance on clinical detection of disease, testing, and reporting processes. During this study, samples were collected through active surveillance, with standardised selection criteria across regional areas within each round of sample collection. This approach enables detection of HPAI in subclinical birds and minimises temporal and spatial variation in surveillance effectiveness, enabling more robust identification of regional differences in the prevalence of H5N1 and H5N6 at the level of the LBM. The ongoing active surveillance conducted

TABLE 5 | Posterior mean coefficients, odds ratios (ORs), and 95% credible intervals (CrI) of non-spatial conditional autoregressive models of market days positive for highly pathogenic avian influenza H5N6 virus (Vietnam, 2011–2015).

	Coefficient, posterior mean (95% CrI) Multivariable model (no spatially varying random effect)	OR, posterior mean (95% CrI) Multivariable model (no spatially varying random effect)
River density (km length/km^2)	−0.74 (−1.17, −0.34)	0.48 (0.31, 0.71)
Human population density (heads/km^2)	0.28 (−0.08, 0.61)	1.31 (0.92, 1.84)
Market density (live bird market/10 km^2)		
≤2.8	Baseline	Baseline
2.81–4.64	−0.15 (−1.45, 1.16)	0.86 (0.24, 3.19)
4.65–8.91	0.90 (−0.24 2.14)	2.47 (0.78, 8.52)
≥8.92	0.28 (−1.01, 1.61)	1.32 (0.36, 4.99)
Round		
Round 4	Baseline	Baseline
Round 5	3.37 (2.39, 4.48)	26.06 (9.84, 78.44)
Round 6	3.72 (2.62, 4.92)	40.48 (13.61, 133.16)
Model deviance information criterion	200.94	

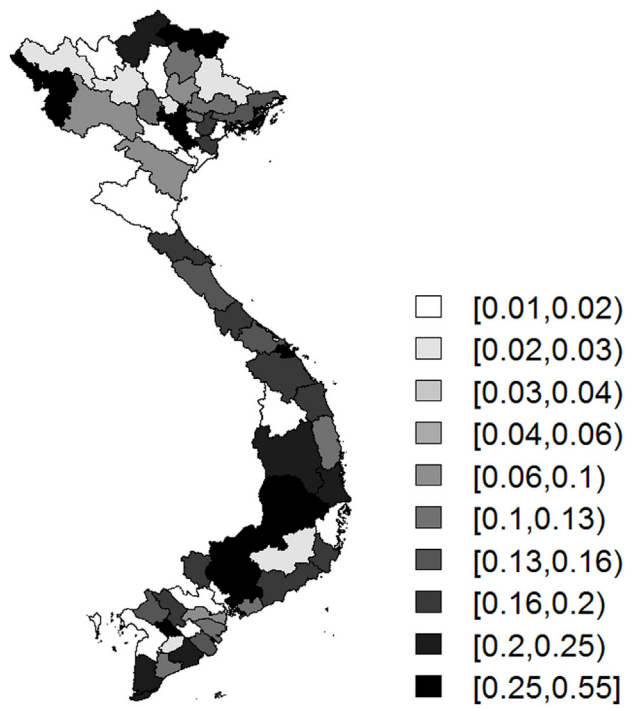

FIGURE 7 | Choropleth map showing the province-level posterior mean probabilities of the spatially structured random effect for H5N1.

in LBM is essential to monitor changes in spatiotemporal distribution patterns and strain evolution. Sampling continues to be focussed upon LBM and provinces where prevalence of infection has previously been detected to be highest.

One of the main limitations of the data analysed in this study is that the sampling strategy was not consistent between Rounds. Sampling at the district level was randomised for Round 1, then from Round 2 onwards the sampling strategy at the district level was to target districts with higher risk of H5N1 infection. The variation in odds of a market-day testing positive for H5N1 or H5N6 between Rounds may reflect temporal variation in risk, however, is augmented by the differences in sampling strategies implemented in different rounds. In addition, the sampling strategy at the level of the LBM was not perfectly sensitive; not all birds were sampled and AI positive birds may have been clustered in only part of an LBM. Due to the potential for under-detection, the observed proportion of HPAI positive market days may be lower than the true proportion of HPAI positive market days. In addition, aggregating market days by province, for reasons of anonymity, will have resulted in some loss of within-province heterogeneity. However, as the provinces with the highest risk were also the smallest (southern) provinces, this loss of information is expected to be comparatively small.

In conclusion, this study suggests that the spatial patterns and risk factors are very different for H5N1 and H5N6 in Vietnam. Whilst H5N1 distribution was spatially heterogeneous with significant clustering of high-risk provinces in the south, H5N6 was homogenously distributed. In addition, the likelihood of H5N1 detection at LBM was primarily associated with climatic factors. The different epidemiology of these two HPAI virus strains in Vietnam suggests the need for different surveillance and control approaches.

ETHICS STATEMENT

Data were generated as part of routine governmental active surveillance monitoring of avian influenza in Vietnam, coordinated by the Department of Animal Health, Ministry of Agriculture and Rural Development in Vietnam. All surveillance activities and protocols were approved by the Vietnamese Department of Animal Health (DAH) Epidemiology Division before implementation.

AUTHOR CONTRIBUTIONS

PL, SN, KI, and PP contributed to the design of the study. KS, KM, AM, DE, GF, and DP contributed to the data analysis plan. AM organised the database. KS, KM, AM, and DE performed the statistical analyses. KM wrote the first draft of the manuscript. KS and AM wrote sections of the manuscript. KS, KM, AM, GF, DP, TV, and MG contributed to interpretation of results. All the authors contributed to manuscript revision, read and approved the submitted version.

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greater disease prevention and control capacity in Vietnam. KM is supported by the Bloomsbury Colleges (University of London) Scholarship and Maynard Endowment. GF and DP receive funding from the BALZAC research programme "Behavioural adaptations in live poultry trading and farming systems and zoonoses control in Bangladesh" (ref: BB/L018993/1). This is one of 11 programmes funded under ZELS, a joint research initiative between Biotechnology and Biological Sciences Research Council (BBSRC), Defence Science and Technology Laboratory (DSTL), Department for International Development (DFID), Economic and Social Research Council (ESRC), Medical Research Council (MRC), and Natural Environment Research Council (NERC): http://www.bbsrc.ac.uk/research/international/zels/. MG is funded by the Belgian FNRS, and this work was partly supported by the NIH grant 1R01AI101028-02A1. RVC reference: PPB_01714.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fvets.2018.00051/full#supplementary-material.

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Low- and High-Pathogenic Avian Influenza H5 and H7 Spread Risk Assessment Within and Between Australian Commercial Chicken Farms

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This study quantified and compared the probability of avian influenza (AI) spread within and between Australian commercial chicken farms via specified spread pathways using scenario tree mathematical modeling. Input values for the models were sourced from scientific literature, expert opinion, and a farm survey conducted during 2015 and 2016 on Australian commercial chicken farms located in New South Wales (NSW) and Queensland. Outputs from the models indicate that the probability of no establishment of infection in a shed is the most likely end-point after exposure and infection of low-pathogenic avian influenza (LPAI) in one chicken for all farm types (non-free range meat chicken, free range meat chicken, cage layer, barn layer, and free range layer farms). If LPAI infection is established in a shed, LPAI is more likely to spread to other sheds and beyond the index farm due to a relatively low probability of detection and reporting during LPAI infection compared to high-pathogenic avian influenza (HPAI) infection. Among farm types, the median probability for HPAI spread between sheds and between farms is higher for layer farms (0.0019, 0.0016, and 0.0031 for cage, barn, and free range layer, respectively) than meat chicken farms (0.00025 and 0.00043 for barn and free range meat chicken, respectively) due to a higher probability of mutation in layer birds, which relates to their longer production cycle. The pathway of LPAI spread between sheds with the highest average median probability was spread via equipment (0.015; 5–95%, 0.0058–0.036) and for HPAI spread between farms, the pathway with the highest average median probability was spread via egg trays (3.70×10^{-5}; 5–95%, 1.47×10^{-6}–0.00034). As the spread model did not explicitly consider volume and frequency of the spread pathways, these results provide a comparison of spread probabilities per pathway. These findings highlight the importance of performing biosecurity practices to limit spread of the AI virus. The models can be updated as new information on the mechanisms of the AI virus and on the volume and frequency of movements shed-to-shed and of movements between commercial chicken farms becomes available.

Keywords: avian influenza, Australia, commercial chickens, H5, H7, scenario trees, partial consequence assessment, spread

INTRODUCTION

The risk of low-pathogenic avian influenza (LPAI) virus spread in Australia is initially dependent on the risk of exposure of commercial chicken farms in this country to LPAI, which has been quantified for New South Wales by Scott et al. (1). After exposure to the virus, the risk of spread is then dependent on infection of the chicken with the virus and establishment of the virus within the flock (2–4). Once established in one flock, LPAI spread within farms (between sheds) and between farms can occur. LPAI infection can be associated with no clinical signs but a range of clinical illness in birds including respiratory disease can also be seen, thereby leading to production losses and decreased welfare (2, 5). For infections with H5 and H7 LPAI viruses, with further virus spread and the subsequent increasing number of infected birds, there is a greater possibility of mutation of the virus to high-pathogenic avian influenza (HPAI). HPAI has very high morbidity and mortality rates in gallinaceous poultry (up to 100%) (5). If mutation does occur, the risk of HPAI spread within and between farms must then be considered.

Factors influencing the success of LPAI or HPAI spread depend heavily on biosecurity actions put into place on the farm. Previous modeling work suggest that bird pickup trucks and feed trucks that move between farms and human movements between sheds were pathways associated with the highest risk of spread of AI. Emphasis to ensure good biosecurity practices associated with these pathways, such as vehicle disinfection and footbaths, was therefore made (6, 7). The timeliness of detection of clinical signs of infected flocks by farmers also plays a significant role in limiting spread of the disease. If the appropriate authority figures are contacted by farmers promptly, management practices can be put into place to limit spread of the virus both within and between farms (2, 8). This is supported by several previous mathematical modeling studies that revealed a reduction in the probability of AI spread to other farms if detection and reporting occurs earlier rather than later in the outbreak and if the detection threshold is lowered or frequent sampling occurs on high-risk farms (9–11).

All seven HPAI outbreaks in Australia to date have had only commercial chicken farms as the index farms; including commercial layer or meat chicken farms, with two outbreaks involving meat chicken breeder farms. Four of the seven HPAI outbreaks involved spread from the index farm to affect the nearby farms (12, 13). In addition, surveillance found evidence of LPAI infection among duck farms in the vicinity for two of the seven HPAI outbreaks, suggesting initial LPAI spread with subsequent mutation (14, 15). The focus on commercial chicken farms in this study is due to the comparatively small threat posed by non-commercial chicken farms to the Australian poultry industry. There is limited contact between non-commercial and commercial chicken farms in Australia. In addition, AI detection on non-commercial chicken farms, as did occur with three of the 12 LPAI cases detected in this country to date, has little impact on the industry, market, and consumers due to the small number of birds to destroy (14–16).

The pathways of spread in the past Australian HPAI outbreaks were suspected based on epidemiological investigations; examples identified include common dead bird pick up and egg transport vehicles among the affected farms (13, 17, 18). However, it is currently unknown for the Australian context which pathways are most likely to cause spread, whether particular farm types are at more risk of spread than others, and the influence biosecurity practices will have on spread. Thus there is a need to quantify and compare the probability of both LPAI and HPAI spread for all types of Australian commercial chicken enterprises, i.e., cage, barn, and free range systems of both layer and meat chicken farms. Further, there is a need to quantify the effect of on-farm preventive actions that can mitigate the risk and impact of future AI outbreak occurrences in Australia.

In response to these needs, the aim of this study was first to estimate the probability of infection and establishment of LPAI virus after one chicken is exposed to the virus using results obtained from Scott et al. (1). Then, potential pathways for LPAI and HPAI spread between sheds and farms on all types of Australian commercial chicken enterprises were identified. A partial consequence assessment was then performed to estimate and compare the probabilities of LPAI and of HPAI spread between sheds and farms with particular focus on the differences in spread via the investigated pathways, without explicit consideration of pathway volume and frequency as insufficient information was available to incorporate consideration of these in this study. Comparison of study results will inform understanding of the most influential pathways of spread of LPAI and HPAI, and of any differences between farm types if these exist. This new knowledge can direct thinking about on-farm biosecurity practices that can be put into place to reduce the potential for AI spread.

MATERIALS AND METHODS

Risk Assessment Model

The overall study used the World Organisation of Animal Health (OIE) risk analysis framework (19) to conduct an exposure and partial consequence assessment in relation to AI for Australian commercial chicken farms. The exposure assessment considered the potential pathways by which chickens situated in a commercial layer or meat chicken farm can be exposed to avian influenza (AI) virus from wild birds. This assessment can be found in the study by Scott et al. (1). The current study focused on a partial consequence assessment, where the risk of spread was determined but the level of consequences following spread was not measured. This assessment considered the pathways by which these viruses can spread between sheds on the same farm and from one farm to other farms. The probability of these pathways occurring was calculated. Such pathways were portrayed using scenario trees (20) and developed using Microsoft Excel (PC/Windows 7, 2010). The probabilities were estimated using Monte Carlo stochastic simulation modeling using the program @RISK 7.0 (Palisade Corporation, USA). Each simulation consisted of 50,000 iterations sampled using the Latin hypercube method with a fixed random seed of one.

Data Sources

Most of the input values used in this model were parameterized using data collected from a survey on commercial chicken

farms in Australia (8, 21). This study defined commercial layer farms as those with more than 1,000 birds, and commercial meat chicken farms as those with more than 25,000 birds. It involved a comprehensive on-farm interview with farmers including questions related to farm management, biosecurity practices, and wild bird presence. In addition, input values were also obtained from scientific literature. An expert opinion workshop was also held to obtain input values that were largely unknown or undescribed in the scientific literature (22).

Survey on Commercial Layer and Meat Chicken Farms in the Sydney Basin Region and South East Queensland

A survey was conducted from mid-2015 with on-farm interviews on 73 commercial chicken farms; nine cage layer, 9 barn layer, 25 free range layer, 15 non-free range meat chicken, and 15 free range meat chicken farms (8, 21). The farms were located in the Sydney basin region in New South Wales (NSW) and in South East Queensland. The Sydney basin region was selected due to the high concentration of both layer and meat chicken farms in this area. However, in this region, free range meat chicken farms are all owned by one of the two large privately owned meat chicken companies in Australia. Therefore, additional farm visits to South East Queensland were conducted to gain more representative data of privately owned meat chicken companies in Australia. The interviews with the farm manager or farm owner involved a comprehensive questionnaire with questions relating to biosecurity practices performed on farm, wild bird and animal presence, general farm information, and farm management. A greater proportion of layer farms and of free range farms were surveyed due to the greater perceived risk of AI occurrence on these farm types. Further details on the survey methodology, including the region and farm selection, questionnaire development, and conduct of the on-farm interviews can be found in the study by Scott et al. (21).

Expert Opinion

Due to many unknowns related to the AI virus, an expert elicitation process was conducted in late 2015 to help inform the parameters of mutation from LPAI to HPAI and farm-to-farm spread pathways; the shed-to-shed spread pathways were informed from a combination of scientific literature and the farm survey. The elicitation process used a modified Delphi technique to gather the information, based on a four-step elicitation process. The process involved the experts completing a questionnaire individually, followed by a discussion of the results at a workshop, and then a reassessment of the questionnaire answers after the workshop. A total of 10 experts who had varying levels of expertise related to the poultry industry, wild bird behavior, and AI virus characteristics, participated in the process. The experts were selected based on their experience in the Australian poultry industries including involvement in the management of HPAI outbreaks in Australia or overseas as well as knowledge on the AI virus and wild birds. The questionnaire included 39 probability questions, and experts were asked to provide a most likely, minimum and maximum estimates of the probabilities and their level of confidence on their estimates. Pert distributions were used to obtain individual expert estimates for each question. The second round of estimates for each question for all experts was then combined using a weighting factor depending on their respective level of expertise relevant to each question, in a discrete distribution. More details on the expert elicitation process and the outcomes of the study can be found in the study by Singh et al. (22).

Statistical Analysis

The statistical program JMP® was used (© 2012 SAS Institute Inc., Cary, NC, USA) to conduct one-way analysis of variance (ANOVA) to analyze the differences between the outcome probabilities from the models for different farm types. The outcome probabilities compared using ANOVA were the outcome probability from 1,000 iterations of each pathway endpoint of the spread scenario tree model simulation for each farm type with each iteration reflecting the situation for one farm at any point in time. A p-value of <0.05 was used to determine statistical significance in these analyses.

Partial Consequence (Spread) Assessment

The partial consequence assessment investigates the pathways of AI virus spread after one bird has been exposed to the virus at any point in time. It provides a comparison of spread probabilities between pathways; however, the volume and frequency of each pathway occurring were not explicitly considered. For shed-to-shed spread, there is consideration of the proportion of farms that perform or have these pathways present in combination with the survival of the virus on these pathways. For farm-to-farm spread, it was assumed that variation between pathways in volume and frequency and in virus survival was considered by experts. From the assumed LPAI exposure of one bird, spread first depends on infection of this bird, and this probability differs between direct or indirect exposure. In addition, spread depends on establishment of the virus within the shed after infection of one individual, which is influenced by the subtype of the virus. Both LPAI and HPAI spread are assessed, where the probability of H5/H7 mutation from LPAI to HPAI is also considered after establishment within a flock. The end-points of this model are exclusive of one another and are as follows: (1) no establishment of the infection; (2) limited LPAI spread; (3) limited HPAI spread; (4) LPAI spread; and (5) HPAI spread.

Limited spread is defined as the spread that would occur even when infection is detected and reported by the farmer. In this situation, although it is assumed that control measures will be put into place to restrict further spread of the virus, spread prior to detection and reporting would be likely to occur due to the routine large volume of activities between both sheds and farms. Supporting this assumption, the number of days required for detection and reporting was estimated using an index function on Microsoft Excel, resulting in a time period of at least 70 days from infection of the first chicken with LPAI to establishment, detection

and reporting by the farmer for all farm types. This estimation considered a reproduction number (*R*) of 1.35, the proportion of birds showing clinical signs, the shed size, and the percentage threshold for LPAI detection and reporting. The calculation of R and the proportion of birds showing clinical signs are presented in the description of the *Establishment of LPAI after infection in one chicken* node in the Supplementary Material. The shed size and percentage threshold for LPAI detection and reporting differ for each farm type and are described by Scott et al. (21). If there is no detection and reporting, the potential pathways by which LPAI and HPAI can spread between sheds and between farms are evaluated for each farm type.

The spread models used to estimate shed-to-shed and farm-to-farm spread are two separate models and are independent of each other. The same input parameters are used in both models with the exception of the last node that considers the different pathways of spread, shed-to-shed and farm-to-farm. The five pathways for spread between sheds are shown in **Figure 1** and the 12 pathways for spread between farms are shown in **Figure 2**, following the nodes "LPAI spread methods" and "HPAI spread methods". The input parameters used are described in **Table 1** and a detailed description of the nodes is provided in the Supplementary Material. The majority of nodes apply to both LPAI and HPAI spread, with some specific to LPAI or HPAI spread only. The specific nodes for LPAI spread are LPAI spread methods shed-to-shed and LPAI spread methods farm-to-farm. The specific nodes for HPAI spread are HPAI clinical signs, detection, and reporting, HPAI spread methods shed-to-shed, and HPAI spread methods farm-to-farm. The probabilities of the different spread pathways were complementary to each other in the spread scenario tree models (e.g., the sum of the probabilities of all pathways occurring equaled one).

Sensitivity Analysis

The Advanced Sensitivity Analysis on the program @RISK 7.0 (Palisade Corporation, USA) was used to determine the effect of input parameters on the model outputs. The input values varied from 0 to 1 in thirds (0, 0.3, 0.6, 1). Each input value of interest was assessed in a simulation of 1,000 iterations while all other input values were fixed to their base value. The model outputs assessed were the probability of LPAI and HPAI spread between both sheds and farms per farm type.

The effect of the following inputs of LPAI and HPAI spread between sheds and farms were investigated: (1) Probability that the H5/H7 LPAI subtype will establish within the flock from one infected chicken *(Prob_Establishment)*; (2) Probability that LPAI established within the flock will mutate to HPAI *(Prob_Mutation)*; (3) Probability that the farmer will detect and report disease to appropriate officials during LPAI establishment *(Prob_LPAI_Detection)*; (4) Probability that HPAI will produce clinical signs with the assumption that the probability of detection is extremely high *(Prob_HPAI_Detection)*.

In addition, the impact of the probability of spread to another shed or farm through any of the pathways considered in this assessment, which is dependent to a high extent on the level of biosecurity implemented on farm, was also investigated. As the probabilities of the different spread pathways were complementary to each other in the spread scenario tree models, each pathway has the same influence on the probability of spread on the sensitivity analysis. As such, only one pathway probability is included in the sensitivity analysis and the generic term *Prob_PathwaySpread* is used.

RESULTS

Probabilities of LPAI and HPAI Spread

Results from the spread models provided the overall probabilities of no establishment of LPAI and of LPAI and HPAI limited spread and LPAI and HPAI spread between both sheds and farms, given one chicken is exposed to LPAI virus from one wild bird in Australia at any point of time. The results are summarized in **Table 2** and **Figure 3**. The pathways involved in calculating these probabilities incorporated the probability of LPAI infection in a chicken after exposure and the probability that the virus is able to spread and establish among chickens within a shed. For all farm types, the most likely end-point after one chicken is exposed and infected with LPAI is no establishment. For each pathotype, the overall probabilities of spread are identical for each farm type between sheds and between farms. The results also show that for all farm types, the probability of limited LPAI spread is lower than that of limited HPAI spread; that LPAI spread is more likely to occur than limited LPAI spread; and that HPAI spread is less likely to occur than limited HPAI spread.

Low-pathogenic avian influenza and HPAI spread occur when the randomly selected values for the beta distribution for the probability of detection and reporting in the spread model are very low or zero. The probabilities of LPAI spread between sheds and farms, although low for all farms, were estimated to be highest in free range farms compared to other farm types. The model estimated a median probability of LPAI spread of 0.068 and 0.059 for free range meat chicken and layer farms, respectively. Among indoor farms, the probability (median; 5–95%) of LPAI spread between sheds and farms is higher in barn meat chicken farms (0.037; 0.015–0.073) when compared to the indoor layer farm types; cage layer (0.027; 0.0028–0.079) and barn layer (0.026; 0.0030–0.071). The probabilities of HPAI spread between sheds and farms are lower than that of LPAI spread for all farm types (**Table 2**).

Probabilities of the Different Spread Pathways

Results of the probability of LPAI and HPAI spread between sheds and farms are summarized in **Figure 4**, which presents the averages of the median, 5% and 95% probability values per pathway among all farm types and provides a comparison of relative probability of spread between pathways that does not explicitly consider the volume and frequency of each respective pathway occurring.

The pathways of spread between sheds were estimated using farm survey data to determine the proportion of farms that would perform or have specific practices or pathways for each farm type. This was combined with scientific literature to determine

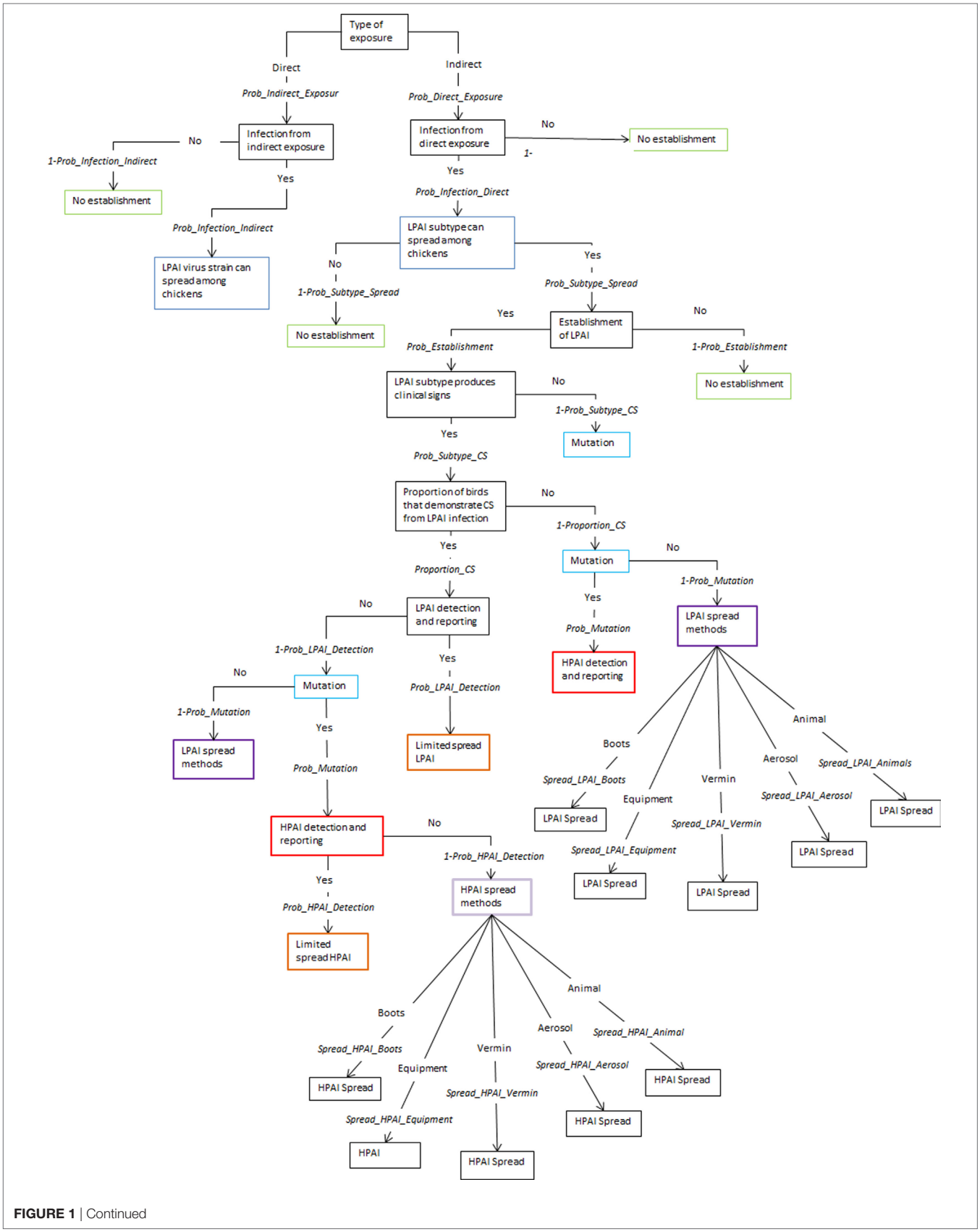

FIGURE 1 | Continued

FIGURE 1 | Scenario tree representing the spread pathways of low-pathogenic and high-pathogenic avian influenza (LPAI and HPAI) viruses between sheds for Australian commercial layer and meat chicken farms. (*Prob_Indirect_Exposure*, *probability of indirect exposure of LPAI virus to a commercial chicken; Prob_Direct_Exposure*, *probability of direct exposure of LPAI virus to a commercial chicken; Prob_Infection_Indirect*, *probability of infection of LPAI after indirect exposure; Prob_Infection_Direct*, *probability of infection of LPAI after direct exposure; Prob_Subtype_Spread*, *probability that the H5/H7 subtype that has infected a chicken is able to spread to other chickens; Prob_Establishment*, *probability that the H5/H7 LPAI subtype will establish within the flock from one infected chicken; Prob_Subtype_CS*, *probability that the LPAI H5/H7 subtype established within the flock is able to produce clinical signs within the flock; Proportion_CS*, *proportion of birds infected with LPAI that will produce clinical signs; Prob_Mutation*, *probability that LPAI established within the flock will mutate to HPAI; Prob_LPAI_Detection*, *probability that the farmer will detect and report disease to appropriate officials during LPAI establishment; Prob_HPAI_Detection*, *probability that HPAI will produce clinical signs with the assumption that the probability of detection is extremely high; Spread_LPAI_Boots*, *probability that shed-to-shed spread of LPAI will occur via the movement of boots; Spread_LPAI_Equipment*, *probability that shed-to-shed spread of LPAI will occur via the movement of equipment; Spread_LPAI_Vermin*, *probability that shed-to-shed spread of LPAI will occur via the movement of vermin such as rats and insects; Spread_LPAI_Aerosol*, *probability that shed-to-shed spread of LPAI will occur via aerosol; Spread_LPAI_Animals*, *probability that shed-to-shed spread of LPAI will occur via the movement of other animals including pets; Spread_HPAI_Boots*, *probability that shed-to-shed spread of HPAI will occur via the movement of boots; Spread_HPAI_Equipment*, *probability that shed-to-shed spread of HPAI will occur via the movement of equipment; Spread_HPAI_Vermin*, *probability that shed-to-shed spread of HPAI will occur via the movement of vermin such as rats and insects; Spread_HPAI_Aerosol*, *probability that shed-to-shed spread of HPAI will occur via aerosol; Spread_HPAI_Animals*, *probability that shed-to-shed spread of HPAI will occur via the movement of other animals including pets*).

the survival of the virus on each of these pathways, and similar volume and frequency for each pathway were assumed. The pathway of LPAI spread between sheds (**Figure 4A**) with the highest average median probability was spread via equipment (0.015; 0.0058–0.036), followed by vermin (0.010; 0.0028–0.023) and then boots (0.0064; 0.00087–0.018). When the results of each farm type were assessed, the pathway of spread via equipment was the pathway with the highest median probability of LPAI spread between sheds for each farm type except free range layer farms. For this farm type, the pathway of LPAI spread between sheds with the highest median probability was spread via vermin (0.019; 0.0022–0.041).

The pathway of HPAI spread between sheds (**Figure 4B**) with the highest average median probability was also spread via equipment (5.76×10^{-5}; 1.90×10^{-6}–0.00057). All farm types except free range layer farms had the pathway of spread via equipment as the pathway with the highest median probability of HPAI spread between sheds. For free range farms, the pathway with the highest median probability was spread via animals (8.93×10^{-5}; 2.57×10^{-6}–0.001) (data not shown in **Figure 4**).

The pathways of spread between farms were estimated from expert opinion which is assumed to have considered variation in volume and frequency and virus survival between pathways. The pathway of LPAI spread between farms (**Figure 4C**) with the highest average median probability was spread via bird pick up systems (0.0072; 0.0019–0.02), followed by egg trays (0.0059; 0.00066–0.017). The latter applies to only layer farm types. When assessing each farm type on its own, the pathway with the highest median probability of LPAI spread between farms was bird pick up systems for both barn and free range meat chicken farm types. Spread via egg trays was the pathway with the highest median probability of LPAI spread between farms for all layer farms.

The pathway of HPAI spread between farms (**Figure 4D**) with the highest average median probability was spread via egg trays (3.70×10^{-5}; 1.47×10^{-6}–0.00034), followed by egg pallets (2.07×10^{-5}; 7.86×10^{-7}–0.00021), bird pick up systems (1.57×10^{-5}; 4.83×10^{-7}–0.00019), and farm workers (1.41×10^{-5}; 4.43×10^{-7}–0.00018). The former two apply to layer farms only. For individual farm types, and similar to that for LPAI, the pathway of HPAI spread between farms with the highest median probability was bird pick up systems for barn and free-range meat chicken farm types. Spread via egg trays was the pathway with the highest median probability of HPAI spread between farms for all layer farms.

Spread Sensitivity Analysis

Figure 5 shows the outputs of the spread sensitivity analysis, which depicts an example of one meat chicken or layer farm type per LPAI (**Figures 5A,B**) or HPAI (**Figures 5C,D**) spread between sheds and farms, as the sensitivity analysis outcomes were similar in proportional increase in value among all farm types. In addition, no difference on the spread sensitivity analyses for spread between sheds and spread between farms was observed.

According to the spread sensitivity analysis, the most influential parameter for LPAI spread between sheds and farms was the probability of establishment (**Figures 5A,B**). When the probability of establishment is increased to 100% (base value 0.47 for all farm types), there is an approximate 2.1 to 2.2-fold increase on the probability of LPAI spread between sheds and farms for all farm types.

The probability of mutation was the most influential parameter affecting the probability of HPAI spread between sheds and farms for all farm types. When this probability is increased to 100% (base value 0.070, 0.070, 0.50, 0.28, 0.30 for barn meat chicken, free range meat chicken, cage layer, barn layer and free range layer farms, respectively), there is at least a 3.5-fold increase on the probability of HPAI spread between both sheds and farms for all farm types (**Figures 5C,D**). The influence of the probability of mutation is most substantial on meat chicken farm types where there is an approximate 17-fold increase on the probability of HPAI spread between both sheds and farms within these farm types. The next most influential parameter on HPAI spread between sheds and farms was the probability of establishment where results obtained were similar to those seen with the LPAI spread sensitivity analysis described above.

The impact of the probability of detection on spread of LPAI and HPAI does not seem to be very significant. When this probability is increased to 100%, there is only an approximate 0.05-fold decrease on the probability of both LPAI (base value between 0.60

and 0.70 for all farm types) and HPAI (base value 0.99 for all farm types) spread between sheds and farms for all farm types.

Investigation of the spread pathways revealed that when the probability of any of these pathways is increased to 100% (base values ranging from 0.00034 to 0.040 and 3.87×10^{-7} and 8.83×10^{-5} for LPAI and HPAI spread, respectively), there is an approximate 1.5 to 2-fold increase on the probability of LPAI and HPAI spread between sheds and between farms for all farm types.

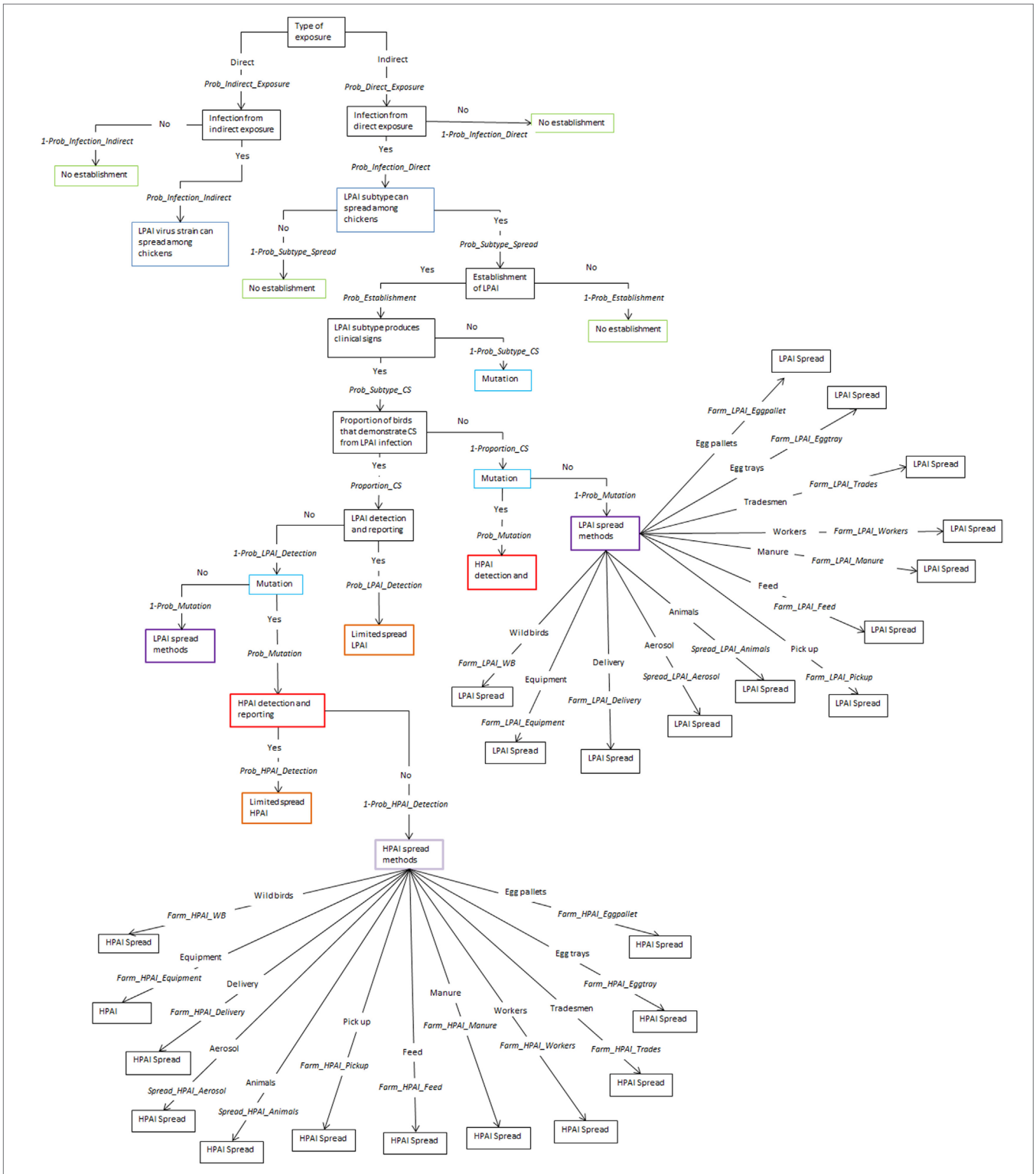

FIGURE 2 | Continued

FIGURE 2 | Scenario tree representing the spread pathways of low-pathogenic and high-pathogenic avian influenza (LPAI and HPAI) viruses between farms for Australian commercial layer and meat chicken farms. (*Prob_Indirect_Exposure*, *probability of indirect exposure of LPAI virus to a commercial chicken; Prob_Direct_Exposure*, *probability of direct exposure of LPAI virus to a commercial chicken; Prob_Infection_Indirect*, *probability of infection of LPAI after indirect exposure; Prob_Infection_Direct*, *probability of infection of LPAI after direct exposure; Prob_Subtype_Spread*, *probability that the H5/H7 subtype that has infected a chicken is able to spread to other chickens; Prob_Establishment*, *probability that the H5/H7 LPAI subtype will establish within the flock from one infected chicken; Prob_Subtype_CS*, *probability that the LPAI H5/H7 subtype established within the flock is able to produce clinical signs within the flock; Proportion_CS*, *proportion of birds infected with LPAI that will produce clinical signs; Prob_Mutation*, *probability that LPAI established within the flock will mutate to HPAI; Prob_LPAI_Detection*, *probability that the farmer will detect and report disease to appropriate officials during LPAI establishment; Prob_HPAI_Detection*, *probability that HPAI will produce clinical signs with the assumption that the probability of detection is extremely high; Farm_LPAI_Equipment*, *probability that farm-to-farm spread of LPAI will occur via the movement of equipment; Farm_LPAI_Aerosol*, *probability that farm-to-farm spread of LPAI will occur via aerosol; Farm_LPAI_Animals*, *probability that farm-to-farm spread of LPAI will occur via the movement of animals including both farm cats and dogs and vermin; Farm_LPAI_WB*, *probability that farm-to-farm spread of LPAI will occur via the movement of wild birds; Farm_LPAI_Delivery*, *probability that farm-to-farm spread of LPAI will occur via the movement of bird delivery transport vehicles; Farm_LPAI_Pickup*, *probability that farm-to-farm spread of LPAI will occur via the movement of dead and live bird pick up vehicles; Farm_LPAI_Feed*, *probability that farm-to-farm spread of LPAI will occur via the movement of feed delivery vehicles; Farm_LPAI_Manure*, *probability that farm-to-farm spread of LPAI will occur via the movement of manure collection systems; Farm_LPAI_Workers*, *probability that farm-to-farm spread of LPAI will occur via the movement of farm workers; Farm_LPAI_Tradesmen*, *probability that farm-to-farm spread of LPAI will occur via the movement of tradesmen such as plumbers and electricians; Farm_LPAI_Eggtray*, *probability that farm-to-farm spread of LPAI will occur via the movement of egg trays; Farm_LPAI_Eggpallet*, *probability that farm-to-farm spread of LPAI will occur via the movement of egg pallets; Farm_HPAI_Equipment*, *probability that farm-to-farm spread of HPAI will occur via the movement of equipment; Farm_HPAI_Aerosol*, *probability that farm-to-farm spread of HPAI will occur via aerosol; Farm_HPAI_Animals*, *probability that farm-to-farm spread of HPAI will occur via the movement of animals including both farm cats and dogs and vermin; Farm_HPAI_WB*, *probability that farm-to-farm spread of HPAI will occur via the movement of wild birds; Farm_HPAI_Delivery*, *probability that farm-to-farm spread of HPAI will occur via the movement of bird delivery transport vehicles; Farm_HPAI_Pickup*, *probability that farm-to-farm spread of HPAI will occur via the movement of dead and live bird pick up vehicles; Farm_HPAI_Feed*, *probability that farm-to-farm spread of HPAI will occur via the movement of feed delivery vehicles; Farm_HPAI_Manure*, *probability that farm-to-farm spread of HPAI will occur via the movement of manure collection systems; Farm_HPAI_Workers*, *probability that farm-to-farm spread of HPAI will occur via the movement of farm workers; Farm_HPAI_Tradesmen*, *probability that farm-to-farm spread of HPAI will occur via the movement of tradesmen such as plumbers and electricians; Farm_HPAI_Eggtray*, *probability that farm-to-farm spread of HPAI will occur via the movement of egg trays; Farm_HPAI_Eggpallet*, *probability that farm-to-farm spread of HPAI will occur via the movement of egg pallets*).

This enabled evaluation of the change in probability of spread with implementation or presence of biosecurity practices that act on these spread pathways.

DISCUSSION

The Probability of Spread

The most likely pathway or outcome after one chicken is exposed and infected with LPAI is no establishment of the infection. This is supported by East et al. (38) where in all 17 samples tested positive for AI antibodies in the sentinel free-range flocks, there was no evidence of chicken-to-chicken transmission. However, these results contrast with work performed at the Australian Animal Health Laboratory (AAHL) where chickens inoculated and subsequently infected with various LPAI subtypes were placed in direct contact with other chickens. All chickens in direct contact with these infected chickens subsequently became infected (23). In addition, the spread model assumes only one chicken is exposed to the virus; it is unknown how many chickens are exposed to the virus and over what time period in an Australian context. Therefore, in order for model validation to occur, sampling of commercial chickens to determine their level of exposure to LPAI must be performed.

The overall probabilities of spread are identical for shed-to-shed and farm-to-farm spread for each farm type and pathotype (presented in **Table 2**), and this is due to the only difference being the specific pathways of spread which are represented in the last node of the scenario tree (**Figures 1** and **2**). The probabilities of LPAI spread between sheds and farms are highest in free range farms. As previously mentioned, the spread model incorporates the probability of LPAI infection after the first bird has been exposed, where this probability is higher after direct exposure compared to indirect exposure. As such, the higher probability of LPAI spread in free range farms is due to exposure of the exposed bird on these farms to more likely be via direct pathways. Among non-free-range farms, the probability of LPAI spread, although similar, is slightly higher in barn meat chicken farms compared to the indoor layer farm types, due to the higher threshold of detection and reporting of sick and dead chickens in meat chicken farms compared to layer farms. The higher threshold provides more opportunity for the virus to spread before it is detected. In contrast, the probability of HPAI spread in meat chicken farms is lower than that of layer farms due to the short-lived nature of meat chicken birds leading to a lower probability of mutation in meat chicken birds compared to layer birds. This is reflected in expert opinion answers which informed the mutation parameter and gave a higher probability of mutation for layer farms compared to meat chicken farms (22).

Relative comparisons of these results to other countries can only be made for countries with similar LPAI and HPAI situations such as Australia, i.e., countries in which LPAI and HPAI are not endemic in poultry and HPAI is not endemic in wild birds. Countries in which HPAI H5N1 is endemic in poultry include Bangladesh, China, Egypt, India, Indonesia, and Vietnam (39). Similarly, comparisons should only be made to those countries that have effective protocols setup to deal with positive detections to limit spread. In Australia, this is written in the Australian Veterinary Emergency Plan (AUSVETPLAN) for avian influenza, which was developed and agreed upon by government and the poultry industry. In the United Kingdom (UK) and United States of America (USA), similar protocols are written in the Notifiable Avian Influenza Disease Control Strategy and HPAI Response Plan (The Red Book), respectively

TABLE 1 | Nodes, parameter estimates, and input values used for the partial consequence assessment estimating the probability of spread of Avian Influenza (AI) viruses from flocks on both layer and meat commercial chicken farms in Australia[a].

Node	Branch of node	Parameter estimates	Input values	Data sources
Parameters that apply to both LPAI and HPAI spread				
1. Type of exposure	Direct Indirect	Probability that exposure to the virus is direct or indirect exposure based on results from the exposure scenario tree (*Prob_Direct_Exposure; Prob_Indirect_Exposure*)	Prob_Direct_Exposure Average of all direct exposure outputs from the three seasons of the respective farm type[a] exposure scenario trees. The following values (median; 5–95%) of *Prob_Direct_Exposure* for each farm type were: Non-free range meat chicken (0.24; 0.095–0.47) Free range meat chicken (0.52; 0.28–0.76) Cage layer (0.36; 0.14–0.60) Barn layer (0.32; 0.10–0.59) Free range layer (0.77; 0.60–0.86) *Prob_Indirect_Exposure* 1. *Prob_Direct_Exposure*	Exposure section of this study (1)
2. Infection from direct exposure	Yes No	Probability of infection from direct exposure to AI virus in one chicken (*Prob_Infection_Direct*) Average of (probability of infection from intranasal inoculation + probability of infection from gastrointestinal inoculation + probability of infection as a direct in-contact animal)	*Probability of infection from intranasal inoculation* (PrIntranasal) Average LPAI H5N2 viral titers in tracheal swabs of Mallard ducks was $10^{3.8}$ EID 50/ml over 6 days post inoculation 26/26 chickens inoculated via intranasal route with $10^{4.69}$ TCID 50/ml H9N2 LPAI became infected 16/18 chickens inoculated via intranasal route with $10^{3.69}$ TCID 50/ml H9N2 LPAI became infected Therefore, 42 (s) of 46 (n) chickens become infected when inoculated via intranasal route with virus concentration similar to what is naturally excreted from upper respiratory tract from ducks PrIntranasal = Beta (s + 1, n − s + 1) *Probability of infection from gastrointestinal inoculation* (PrGIT) Average LPAI H5N2 viral titers in cloacal swabs of Mallard ducks was $10^{2.04}$ EID 50/ml over 5 days post inoculation 1/22 chickens inoculated via gastrointestinal route with $10^{2.69}$ TCID50/ml H9N2 LPAI became infected In natural setting viral titers in duck feces will range considerably, therefore pert distribution used PrGIT = Pert (0, 1/22, 1) *Probability of infection as a direct in-contact animal* (PrContact) 2 in-contact chickens placed directly in-contact with H5N2 LPAI infected chickens (n), 2 became infected (s) PrContact = Beta (s + 1, n − s + 1) *Prob_Infection_Direct* = average (PrIntranasal; PrGIT; PrContact)	Yao et al. (3), Selleck (23), Webster et al. (24)
3. Infection from indirect exposure	Yes No	Probability of infection from indirect exposure to AI virus in one chicken (*Prob_Infection_Indirect*) (Relative likelihood of aerosol exposure × Probability of infection from aerosol + Relative likelihood of all other indirect exposure × Probability of infection from diluted gastrointestinal inoculation)	Relative proportions of the following are taken by summing the two values and dividing each value by the sum: Average of all indirect exposure outputs via aerosol from the three seasons of the respective farm type[a] exposure scenario tree (PropAerosol) Average of all other indirect exposure outputs from the three seasons of the respective farm type[a] exposure scenario tree (PropIndirect) *Probability of infection from aerosol* (PrAerosol) Assume virus concentration in air in realistic scenarios is very low from wild birds 0 (s) of 10 (n) chickens exposed to aerosol virus concentration of $10^{2.69}$ TCID50/ml H9N2 LPAI became infected PrAerosol = Beta (s + 1, n − s + 1) *Probability of infection from gastrointestinal inoculation* (PrGIT) 1/22 chickens inoculated via gastrointestinal route with $10^{2.69}$ TCID 50/ml H9N2 LPAI became infected 0/31 chickens inoculated via gastrointestinal route with $10^{1.69}$ TCID 50/ml H9N2 LPAI became infected Therefore, 1 (s) of 53 (n) chickens become infected when inoculated via gastrointestinal route with diluted virus concentration PrGIT = Beta (s + 1, n − s + 1) *Prob_Infection_Indirect* = (PropAerosol × PrAerosol) + (PropIndirect × PrGIT)	Exposure section on this study (1), Yao et al. (3), Jonges et al. (25)

(*Continued*)

TABLE 1 | Continued

Node	Branch of node	Parameter estimates	Input values	Data sources
4. Low-pathogenic avian influenza (LPAI) subtype can spread among chickens	Yes No	Probability that the H5/H7 subtype is a particular subtype that can spread among chickens once infected in an individual chicken (*Prob_Subtype_Spread*)	Beta (s + 1, n – s + 1) 18 H5/H7 subtypes exist (n), nine have been recorded as AI outbreaks in chickens across the globe and therefore have the ability to spread (s)	FAO EMPRES-i (4)
5. Establishment of LPAI after infection in one chicken	Yes No	Probability that the virus will establish within the flock after infection in one chicken (*Prob_Establishment*)	Uniform (0.423,0.511) Derived from (1 – Probability of extinction) Probability of extinction of infection calculated with a Poisson branching process using a range of reproduction numbers (R) using real outbreak data	Barnes and Glass (26)
6. LPAI subtype leads to clinical signs in chickens after infection	Yes No	Probability that the LPAI subtype infected within the flock is a subtype that produces clinical signs in chickens (*Prob_Subtype_CS*)	Beta (s + 1, n – s + 1) 52 H5/H7 virus subtypes, some repeated, have been inoculated in chickens (n), 24 caused clinical signs in chickens (s)	Spackman et al. (27), Spickler et al. (2)
7. Proportion of chickens that show clinical signs from LPAI infection	Yes No	Estimated proportion of chickens within a flock that show clinical signs after infected with a LPAI subtype capable of producing clinical signs (*Proportion_CS*)	Beta (s + 1, n – s + 1) 23 chickens were inoculated with LPAI viruses of H5/H7 subtypes (n), 6 showed clinical signs (s)	Mo et al. (28), Jones and Swayne (29)
8. LPAI detection and reporting	Yes No	Probability that the farmer will report clinical signs of LPAI to appropriate officials (*Prob_LPAI_Detection*)	Beta (s + 1, n – s + 1) Non-free range meat chicken farms: 50 answers reported from farmers of unusual signs in chickens (n), 31 answers linked to clinical signs caused by LPAI (s) Free range meat chicken farms: 58 answers reported from farmers of unusual signs in chickens (n), 35 answers linked to clinical signs caused by LPAI (s) Cage layer farms: 27 answers reported from farmers of unusual signs in chickens (n), 19 answers linked to clinical signs caused by LPAI (s) Barn layer farms: 30 answers reported from farmers of unusual signs in chickens (n), 21 answers linked to clinical signs caused by LPAI (s) Free range layer farms: 74 answers reported from farmers of unusual signs in chickens (n), 51 answers linked to clinical signs caused by LPAI (s)	Scott et al. (21), Scott et al. (8), Swayne (30)
9. Mutation of LPAI to high-pathogenic avian influenza (HPAI)	Yes No	Probability that LPAI will mutate to HPAI (*Prob_Mutation*)	Results obtained from expert opinion workshop |10 experts responded using a 4-step elicitation process for all questions The question for this node was: "Imagine 100 sheds each of the following operation types where LPAI has recently been established. In how many of these sheds would LPAI mutate to HPAI?" This question was asked for each farm type. The following values (median; 5–95%) for each farm type (where the sum of the yes and no pathways was 1) were: Non-free range meat chicken (0.068; 0–0.21) Free range meat chicken (0.068; 0–0.20) Cage layer (0.49; 0.065–0.93) Barn layer (0.29; 0.054–0.92) Free range layer (0.29; 0.057–0.92)	Singh et al. (22)

(*Continued*)

TABLE 1 | Continued

Node	Branch of node	Parameter estimates	Input values	Data sources
Parameters that are specific to LPAI spread				
10. LPAI methods shed to shed	Boots Equipment Vermin Aerosol Pets	Probability that LPAI will spread between sheds via the following pathways: boots, equipment, vermin, aerosol or pets (*Spread_LPAI_Boots; Spread_LPAI_Equipment; Spread_LPAI_Vermin; Spread_LPAI_Aerosol; Spread_LPAI_Animals)*	*Probability of LPAI spread via boots* Beta (s + 1, n – s + 1) of farm answers (PrBoots) Non-free range meat chicken farms: 1/15 (s/n) answers did not use footbaths Free range meat chicken farms: 1/15 (s/n) answers did not use footbaths Cage layer farms: 7/9 (s/n) answers did not use footbaths Barn layer farms: 3/9 (s/n) answers did not use footbaths Free range layer farms: 6/25 (s/n) answers did not use footbaths Probability of virus presence on boots in one day is 1 as survival is longer than one day on this material *Spread_LPAI_Boots* = (PrBoots) × 1 *Probability of LPAI spread via equipment* Beta (s + 1, n – s + 1) of farm answers (PrEquipment) Non-free range meat chicken farms: 6/11 (s/n) answers do not clean equipment between sheds Free range meat chicken farms: 9/9 (s/n) answers do not clean equipment between sheds Cage layer farms: 7/7 (s/n) answers do not clean equipment between sheds Barn layer farms: 6/7 (s/n) answers do not clean equipment between sheds Free range layer farms: 2/23 (s/n) answers do not clean equipment between sheds Probability of virus presence on equipment in one day is 1 as survival is longer than one day on this material *Spread_LPAI_Equipment* = (PrEquipment) × 1 *Probability of LPAI spread via vermin* Beta (s + 1, n – s + 1) of farm answers (PrVermin) Non-free range meat chicken farms: 24/30 (s/n) answers report vermin inside sheds Free range meat chicken farms: 2/30 (s/n) answers report vermin inside sheds Cage layer farms: 17/18 (s/n) answers report vermin inside sheds Barn layer farms: 17/18 (s/n) answers report vermin inside sheds Free range layer farms: 44/50 (s/n) answers report vermin inside sheds Probability of virus presence/survival in vermin (SurvivalVermin): Beta (s + 1, n – s + 1) 0/12 (s/n) LPAI inoculated rats and 73/171 (s/n) LPAI inoculated fly pools were positive on virus isolation *Spread_LPAI_Vermin* = (PrVermin) × (SurvivalVermin) *Probability of LPAI spread via aerosol* Beta (s + 1, n – s + 1) of farm answers (PrAerosol) Non-free range meat chicken farms: 15/15 (s/n) answers had sheds <60 m from each other Free range meat chicken farms: 15/15 (s/n) answers had sheds < 60m from each other Cage layer farms: 9/9 (s/n) answers had sheds <60 m from each other Barn layer farms: 9/9 (s/n) answers had sheds <60 m from each other Free range layer farms: 25/25 (s/n) answers had sheds <60 m from each other Probability of virus presence/survival in air (SurvivalAerosol): Beta (s + 1, n – s + 1)	Scott et al. (21); Scott et al. (8); Achenbach and Bowen (31); Nielsen et al. (32); Tiwari et al. (33); Jonges et al. (25); Wood et al. (34)

(*Continued*)

TABLE 1 | Continued

Node	Branch of node	Parameter estimates	Input values	Data sources
			0/9 (s/n) air samples tested at < 60m from LPAI infected chicken farms were positive for LPAI virus *Spread_LPAI_Aerosol* = (PrAerosol) × (SurvivalAerosol) *Probability of LPAI spread via animals* Beta (s + 1, n − s + 1) of farm answers (PrAnimals) Non-free range meat chicken farms: 0/15 (s/n) answers allow animals inside sheds Free range meat chicken farms: 2/30 (s/n) answers allow animals inside sheds or range areas Cage layer farms: 6/9 (s/n) answers allow animals inside sheds Barn layer farms: 1/9 (s/n) answers allow animals inside sheds Free range layer farms: 13/50 (s/n) answers allow animals inside sheds or range areas Probability of virus presence on other animals in one day is 1 as virus survival is longer than one day on other animals *Spread_LPAI_Animals* = (PrAnimals) × 1	
11. LPAI spread methods farm to farm	Aerosol Infected wild bird Animals (vermin and pets) Bird delivery transport Bird pick up transport (live and dead) Feed delivery transport Manure collection Farm workers Trades people Shared equipment Egg trays[b] Egg pallets[b]	Probability that LPAI will spread between farms via the following pathways: aerosol, infected wild bird going from one farm to another, other animals including vermin and pets, new bird delivery transport, bird pick up transport both live and dead, feed delivery transport, manure collection, farm workers, trades people such as electricians and plumbers, shared equipment between farms, egg trays[b], egg pallets[b] (*Farm_LPAI_Aerosol; Farm_LPAI_WB; Farm_LPAI_Animals; Farm_LPAI_Delivery; Farm_LPAI_Pickup; Farm_LPAI_Feed; Farm_LPAI_Manure; Farm_LPAI_Workers; Farm_LPAI_Trades; Farm_LPAI_Equipment; Farm_LPAI_Eggtray; Farm_LPAI_Eggpallet*)	Results obtained from expert opinion workshop 10 experts responded using a 4-step elicitation process for all questions The question for this node was: "Imagine 100 LPAI established (farm type)[c] farms. Realistically how many of these will experience LPAI spread to at least one other chicken farm through each of the following pathways?" The values for each pathway and farm type are present in the Supplementary Material	Singh et al. (22)
Parameters that are specific to HPAI spread				
12. HPAI clinical signs, detection and reporting	Yes No	Probability that clinical signs will be shown in chickens infected with HPAI and the probability the farmer will detect and report the disease to appropriate officials (*Prob_HPAI_Detection*)	Beta (s + 1, n − s + 1) 52 chickens were inoculated with HPAI viruses of H7 subtypes (n), 52 showed clinical signs (s) Assume extremely high probability farmer will detect clinical signs of HPAI	Selleck (23)

(*Continued*)

TABLE 1 | Continued

Node	Branch of node	Parameter estimates	Input values	Data sources
13. HPAI spread methods shed to shed	Boots Equipment Vermin Aerosol Pets	Probability that HPAI will spread between sheds via the following pathways: boots, equipment, vermin, aerosol or pets (*Spread_HPAI_Boots; Spread_HPAI_Equipment; Spread_HPAI_Vermin; Spread_HPAI_Aerosol; Spread_HPAI_Animals)*	*Probability of HPAI spread via boots* Beta (s + 1, n – s + 1) of farm answers (PrBoots) Non-free range meat chicken farms: 1/15 (s/n) answers did not use footbaths Free range meat chicken farms: 1/15 (s/n) answers did not use footbaths Cage layer farms: 7/9 (s/n) answers did not use footbaths Barn layer farms: 3/9 (s/n) answers did not use footbaths Free range layer farms: 6/25 (s/n) answers did not use footbaths Probability of virus presence on boots in one day is 1 as survival is longer than 1 day on this material *Spread_HPAI_Boots* = (PrBoots) × 1 *Probability of HPAI spread via equipment* Beta (s + 1, n – s + 1) of farm answers (PrEquipment) Non-free range meat chicken farms: 6/1 (s/n)1 answers do not clean equipment between sheds Free range meat chicken farms: 9/9 (s/n) answers do not clean equipment between sheds Cage layer farms: 7/7 (s/n) answers do not clean equipment between sheds Barn layer farms: 6/7 (s/n) answers do not clean equipment between sheds Free range layer farms: 2/23 (s/n) answers do not clean equipment between sheds Probability of virus presence on equipment in one day is 1 as survival is longer than one day on this material *Spread_HPAI_Equipment* = (PrEquipment) × 1 *Probability of HPAI spread via vermin* Beta (s + 1, n – s + 1) of farm answers (PrVermin) Non-free range meat chicken farms: 24/30 (s/n) answers report vermin inside sheds Free range meat chicken farms: 2/30 (s/n) answers report vermin inside sheds Cage layer farms: 17/18 (s/n) answers report vermin inside sheds Barn layer farms: 17/18 (s/n) answers report vermin inside sheds Free range layer farms: 44/50 (s/n) answers report vermin inside sheds Probability of virus presence/survival in vermin (SurvivalVermin): Beta (s + 1, n – s + 1) 0/516 (s/n) HPAI exposed rats and 41/59 (s/n) HPAI inoculated flies were positive on virus isolation *Spread_HPAI_Vermin* = (PrVermin) × (SurvivalVermin) *Probability of HPAI spread via aerosol* Beta (s + 1, n – s + 1) of farm answers (PrAerosol) Non-free range meat chicken farms: 15/15 (s/n) answers had sheds <150 m from each other Free range meat chicken farms: 15/15 (s/n) answers had sheds <150 m from each other Cage layer farms: 9/9 (s/n) answers had sheds <150 m from each other Barn layer farms: 9/9 (s/n) answers had sheds <150 m from each other Free range layer farms: 25/25 answers had sheds <150 m from each other Probability of virus presence/survival in air (SurvivalAerosol): Beta (s + 1, n – s + 1) 22/90 (s/n) air samples tested at <60 m from HPAI infected chicken farms were positive for HPAI virus *Spread_HPAI_Aerosol* = (PrAerosol) × (SurvivalAerosol) *Probability of HPAI spread via animals*	Scott et al. (21), Scott et al. (8), Tiwari et al. (33), Wood et al. (34), Sawabe et al. (35), Nettles et al. (36), McCluskey (37)

(*Continued*)

TABLE 1 | Continued

Node	Branch of node	Parameter estimates	Input values	Data sources
			Beta (s + 1, n − s + 1) of farm answers (PrAnimals) Non-free range meat chicken farms: 0/15 (s/n) answers allow animals inside sheds Free range meat chicken farms: 2/30 (s/n) answers allow animals inside sheds Cage layer farms: 6/9 (s/n) answers allow animals inside sheds Barn layer farms: 1/9 (s/n) answers allow animals inside sheds Free range layer farms: 13/50 (s/n) answers allow animals inside sheds Probability of virus presence on other animals in one day is 1 as virus survival is longer than one day on other animals *Spread_HPAI_Animals* = (PrAnimals) × 1	
14. HPAI spread methods farm to farm	Aerosol Infected wild bird Animals (vermin and pets) Bird delivery transport Bird pick up transport (live and dead) Feed delivery transport Manure collection Farm workers Trades people Shared equipment Egg trays[b] Egg pallets[b]	Probability that HPAI will spread between farms via the following pathways: aerosol, infected wild bird going from one farm to another, other animals including vermin and pets, new bird delivery transport, bird pick up transport both live and dead, feed delivery transport, manure collection, farm workers, trades people such as electricians and plumbers, shared equipment between farms, egg trays[b], egg pallets[b] (*Farm_HPAI_Aerosol; Farm_HPAI_WB; Farm_HPAI_Animals; Farm_HPAI_Delivery; Farm_HPAI_Pickup; Farm_HPAI_Feed; Farm_HPAI_Manure; Farm_HPAI_Workers; Farm_HPAI_Trades; Farm_HPAI_Equipment; Farm_HPAI_Eggtray; Farm_HPAI_Eggpallet*)	Results obtained from expert opinion workshop 10 experts responded using a four-step elicitation process for all questions The question for this node was: "Imagine 100 HPAI established (farm type)[c] farms. Realistically how many of these will experience HPAI spread to at least one other chicken farm through each of the following pathways?" The values for each pathway and farm type are present in the Supplementary Material	Singh et al. (22)

[a]*A spread scenario tree was performed for all farm types; non-free range meat chicken, free range meat chicken, cage layer, barn layer and free range layer.*

[b]*These pathways applied to layer farms only; cage layer, barn layer, and free range layer.*

[c]*This question was asked for each farm type.*

TABLE 2 | Median (5 and 95 percentiles) probabilities of no establishment and of low-pathogenic avian influenza (LPAI) and high-pathogenic avian influenza (HPAI) spread and limited spread between sheds and farms for the commercial chicken farm types (barn meat chicken, free range meat chicken, cage layer, barn layer, and free range layer) after exposure of one chicken to LPAI from one wild bird in Australia.

Farm type	Median	5%	95%	*F* statistic (degrees of freedom);*P*-value
No establishment				
Barn meat chicken	0.96	0.92	0.98	$F(4,4995) = 990.03$; <0.0001
Free range meat chicken	0.92	0.86	0.96	
Cage layer	0.94	0.89	0.97	
Barn layer	0.95	0.9	0.98	
Free range layer	0.89	0.83	0.93	
Probability of LPAI spread				
Barn meat chicken	0.037	0.015	0.073	$F(4,4995) = 490.61$; <0.0001
Free range meat chicken	0.068	0.033	0.12	
Cage layer	0.027	0.0031	0.079	
Barn layer	0.026	0.003	0.071	
Free range layer	0.059	0.0071	0.12	
Probability of HPAI spread				
Barn meat chicken	2.47×10^{-5}	0	0.00025	$F(4,4995) = 164.01$; <0.0001
Free range meat chicken	4.60×10^{-5}	0	0.00043	
Cage layer	0.00022	1.01×10^{-5}	0.0019	
Barn layer	0.00017	7.33×10^{-6}	0.0016	
Free range layer	0.00037	1.68×10^{-5}	0.0031	
Probability of limited LPAI spread				
Barn meat chicken	0.0032	0.0011	0.008	$F(4,4995) = 515.67$; <0.0001
Free range meat chicken	0.0058	0.0022	0.013	
Cage layer	0.0048	0.0017	0.012	
Barn layer	0.0044	0.0015	0.011	
Free range layer	0.0092	0.004	0.019	
Probability of limited HPAI spread				
Barn meat chicken	0.0044	0.0012	0.013	$F(4,4995) = 624.38$; <0.0001
Free range meat chicken	0.0084	0.0025	0.022	
Cage layer	0.021	0.0044	0.068	
Barn layer	0.016	0.0035	0.063	
Free range layer	0.034	0.0087	0.11	

(40–42). The UK experienced 11 HPAI outbreaks since 2006, all of which were effectively eradicated by destroying all birds on infected premises, comparable to the results of this study which indicate limited HPAI spread to occur more often than HPAI spread (43). However, the USA has experienced more extensive HPAI outbreaks involving dozens of farms, which cost over hundreds of millions of dollars to effectively eradicate. These include the HPAI outbreaks that occurred in Pennsylvania in 1983 and 1984, and the more recent HPAI outbreaks since 2014 that affected more than 10 USA states (44, 45). Suggested factors influencing these extensive HPAI outbreaks in the USA include poor biosecurity between farms, and high levels of exposure to AI virus in poultry farms in general, leading to numerous separate introduction and infection events in addition to spread between sheds and between farms (37).

The Probability of Spread and the Probability of Limited Spread

The spread models revealed that for all farm types, the probability of LPAI spread is greater than the probability of limited LPAI spread. This is because detection and reporting is less likely to occur following LPAI establishment and so control measures are less likely to put into place that will limit LPAI spread. In contrast, the spread models indicate that limited HPAI spread is more likely to occur than HPAI spread due to the high probability farmers will detect and report the changes in morbidity and mortality that follow HPAI establishment in a chicken flock. In general, there is limited information to determine if shed-to-shed spread has occurred on Australian LPAI-infected farms. There is evidence that shed-to-shed spread may have occurred on two farms; specifically chickens in several sheds on one farm were seropositive to LPAI H6N2 in 2006 and LPAI H9N2 was detected in three sheds on a turkey farm in 2012 (46). However, it is also possible that independent introductions and infections occurred on the sheds of these farms instead of spread between sheds. There has only been one incursion to date with evidence of farm-to-farm LPAI spread in Australia; investigation of the 2012 H9N2 incursion identified a second infected turkey farm during trace back surveillance from the first turkey farm. This second turkey farm showed no clinical signs or increased mortality (14). As mentioned, it is very likely that LPAI detections in Australia are underreported due to these being non-clinical LPAI infections which provides credibility to the outputs of the spread model; that the probability of LPAI spread is greater than that of limited LPAI spread.

Most farms in Australia in which HPAI occurred had the virus spread to other sheds within the farm. However, all outbreaks

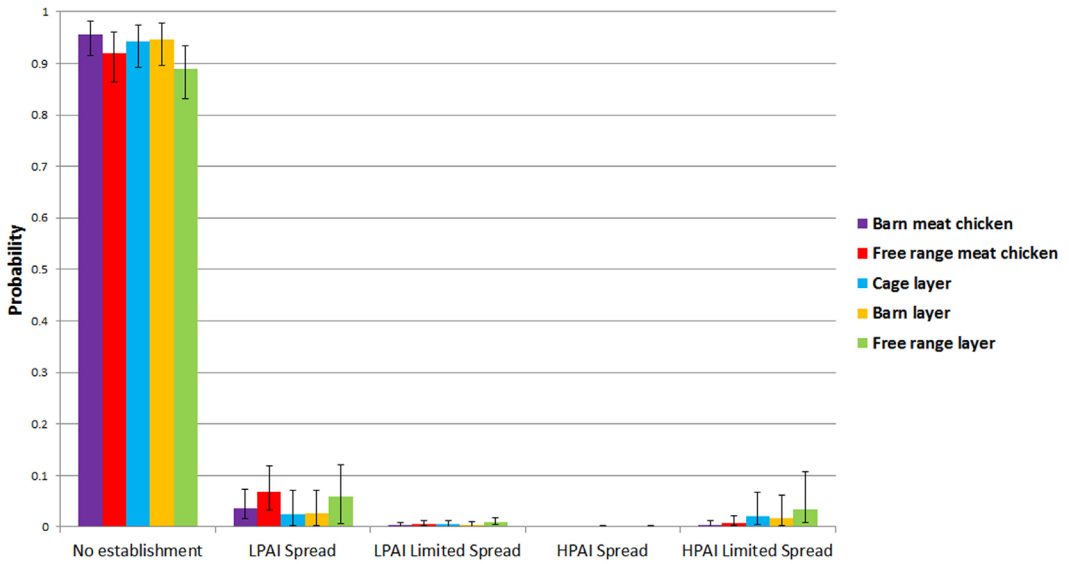

FIGURE 3 | Median (5 and 95 percentiles) probabilities of no establishment and of low-pathogenic avian influenza (LPAI) and high-pathogenic avian influenza (HPAI) spread and limited spread between sheds and farms for the commercial chicken farm types (barn meat chicken, free range meat chicken, cage layer, barn layer, free range layer) after one chicken is exposed to LPAI in Australia.

were effectively controlled via the stamping out procedure and resulting in limited farm-to-farm spread (12, 47, 48). It is likely that the outputs of the spread model which indicate that the probability of HPAI spread is lower than that of limited HPAI spread reflect what has been experienced in Australia; this is easily seen with the farm-to-farm spread model.

The Different Pathways of Spread

The different pathways of LPAI and HPAI spread between sheds have differing probabilities. For LPAI spread between sheds, equipment and vermin were the most likely pathways and aerosol was the least likely pathway. For HPAI spread between sheds, equipment and boots were the most likely pathways and vermin was the least likely pathway. This is largely due to differences in the survival or detection of the virus reported in the literature relevant to these different pathways. LPAI spread via aerosol is regarded as an unlikely pathway in the literature, but detections of HPAI in air samples have been relatively frequently reported, particularly during the 2015 HPAI outbreaks in USA (25, 37). This is likely due to the higher levels of viral replication that occurs in the respiratory tract of birds with HPAI infection compared to LPAI infection (5). The relatively low probability of HPAI spread between sheds via vermin estimated in this study is likely due to how the input parameters in relation to this pathway were calculated. The input parameters were based on several studies where no virus isolation was obtained after exposure of vermin to AI viruses. It is generally been concluded that mice and rats do not play significant roles in the spread of AI virus but insects may (31, 32). In a study where a large number (n = 516) of samples were taken from rodents exposed to HPAI, no positive virus isolations were obtained (36). Similarly, a study where 12 rodents were inoculated with LPAI, no positive virus isolations were identified (31). The feeding of flies with LPAI and HPAI resulted in lower proportions of positive virus isolations from flies fed HPAI compared to LPAI (32, 35). The pathway of shed-to-shed spread via vermin is possibly more significant for LPAI than HPAI.

When considering the results of this study, it must be remembered that the volume and frequency of the different spread pathways between both sheds and farms were not explicitly incorporated in the spread model. For shed-to-shed spread, these pathways were estimated using farm survey data in combination with scientific literature. The farm survey data were used to determine the proportion of farms that would perform or have specific practices or pathways for each farm type and scientific literature was used to determine the probability of survival of the virus on these pathways. It is known that there is a high frequency of daily movements between sheds and if incorporated in the model, may indicate that HPAI spread between sheds is more likely than limited HPAI spread which would actually explain the high incidence of HPAI spread between sheds on farms affected by HPAI outbreaks in Australia (12, 47, 48). This contrasts with the farm-to-farm spread pathways which were informed by expert opinion due to the lack of information in relation to these pathways. Expert understanding and answers of parameters influencing spread by each pathway can be assumed to have included consideration of the volume and frequency of occurrence and the survival of the virus for each pathway.

The output probabilities from the farm-to-farm spread model on the differing pathways of spread largely reflect the expert opinion answers where relatively higher probabilities of farm-to-farm spread were given to pickup trucks, egg trays, and egg pallets. These comparisons can be made from the model output results in **Table 2** and the values in the Supplementary

Material derived from expert opinion that were used to inform the pathways between farms (22). Expert estimates were largely influenced by the previous Australian HPAI outbreaks. An epidemiological investigation of the 2013 HPAI outbreak in Young, NSW suggested that the most likely route of spread of this virus to another farm was the contamination of cardboard egg trays (18). Similarly, a dead bird pick-up vehicle which visited multiple farms was the only identifiable link between farms that were affected by the 1997 HPAI outbreak in Tamworth, NSW (17). This compares with an expert opinion elicitation workshop published in 2011, which estimated the probability of HPAI spread between poultry farms to inform models simulating the transmission and control of HPAI epidemics in the Australian poultry industries. The results of this workshop showed that meat chicken pick up crews followed by slaughter crews, manure collection, and cardboard egg trays were rated as the most likely probabilities of transmission between farms (49). Differences observed between the two expert elicitations could be due to the time difference, as the 2012 and 2013 HPAI outbreaks had not yet occurred when the first expert elicitation was conducted.

Spread Sensitivity Analysis

There were no differences in values or trends on the spread sensitivity analyses of spread between sheds and spread between farms due to the identical structures of the models as described previously. The analyses revealed that the probability of establishment was an important influential parameter on the probability of LPAI and HPAI spread, as well as the probability of mutation on HPAI spread. Although influential, these parameters depend on virus properties and as such cannot be changed by human intervention, and there are large uncertainties associated with these mechanisms (50). Mutation from LPAI to HPAI has particularly large unknowns regarding its probability. A recent review of 42 HPAI outbreaks from 1959 to 2016, most of which involved chickens and turkeys as the initial species, concluded that emergence of HPAI can vary from a few days to a couple of years. It also considered that factors such as poultry age, size of the index farm, and type of farm management do not appear to contribute significantly to HPAI emergence (51). The expert opinion workshop also demonstrated very different estimated probabilities for mutation among the experts (22). The variation of R, which was used to estimate the probability of establishment

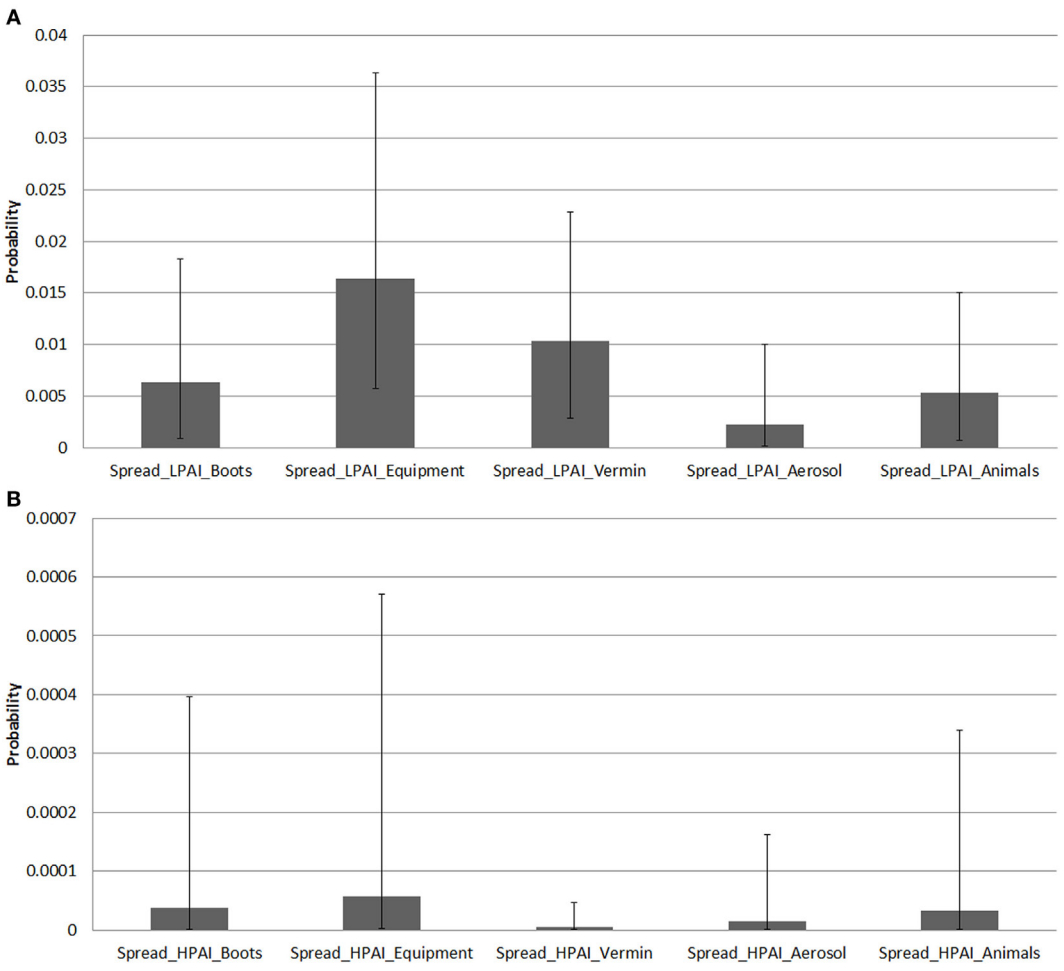

FIGURE 4 | Continued

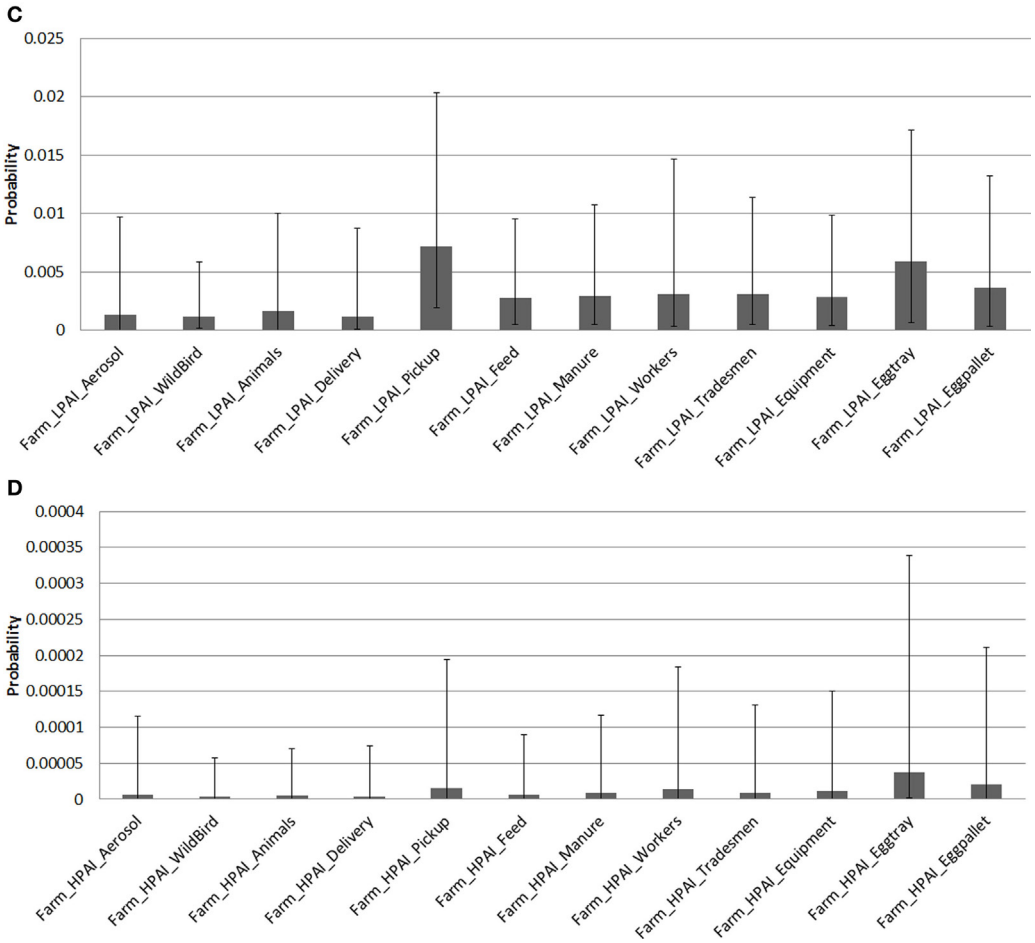

FIGURE 4 | Average median (5 and 95 percentiles) probabilities of low-pathogenic avian influenza (LPAI) and high-pathogenic avian influenza (HPAI) spread pathways between sheds and farms of the commercial chicken farm types (barn meat chicken, free range meat chicken, cage layer, barn layer, free range layer) after one chicken is exposed to LPAI in Australia. **(A)** Average median probabilities of LPAI spread pathways between sheds. *Spread_LPAI_Boots*, *Probability that shed-to-shed spread of LPAI will occur via the movement of boots; Spread_LPAI_Equipment*, *Probability that shed-to-shed spread of LPAI will occur via the movement of equipment; Spread_LPAI_Vermin*, *Probability that shed-to-shed spread of LPAI will occur via the movement of vermin such as rats and insects; Spread_LPAI_Aerosol*, *Probability that shed-to-shed spread of LPAI will occur via aerosol; Spread_LPAI_Animals*, *Probability that shed-to-shed spread of LPAI will occur via the movement of other animals including farm cats and dogs.* **(B)** Average median probabilities of HPAI spread pathways between sheds. *Spread_HPAI_Boots*, *Probability that shed-to-shed spread of HPAI will occur via the movement of boots; Spread_HPAI_Equipment*, *Probability that shed-to-shed spread of HPAI will occur via the movement of equipment; Spread_HPAI_Vermin*, *Probability that shed-to-shed spread of HPAI will occur via the movement of vermin such as rats and insects; Spread_HPAI_Aerosol*, *Probability that shed-to-shed spread of HPAI will occur via aerosol; Spread_HPAI_Animals*, *Probability that shed-to-shed spread of HPAI will occur via the movement of other animals including farm cats and dogs.* **(C)** Average median probabilities of LPAI spread pathways between farms. *Farm_LPAI_Equipment*, *Probability that farm-to-farm spread of LPAI will occur via the movement of equipment; Farm_LPAI_Aerosol*, *Probability that farm-to-farm spread of LPAI will occur via aerosol; Farm_LPAI_Animals*, *Probability that farm-to-farm spread of LPAI will occur via the movement of animals including both farm cats and dogs and vermin; Farm_LPAI_WB*, *Probability that farm-to-farm spread of LPAI will occur via the movement of wild birds; Farm_LPAI_Delivery*, *Probability that farm-to-farm spread of LPAI will occur via the movement of bird delivery transport vehicles; Farm_LPAI_Pickup*, *Probability that farm-to-farm spread of LPAI will occur via the movement of dead and live bird pick up vehicles; Farm_LPAI_Feed*, *Probability that farm-to-farm spread of LPAI will occur via the movement of feed delivery vehicles; Farm_LPAI_Manure*, *Probability that farm-to-farm spread of LPAI will occur via the movement of manure collection systems; Farm_LPAI_Workers*, *Probability that farm-to-farm spread of LPAI will occur via the movement of farm workers; Farm_LPAI_Tradesmen*, *Probability that farm-to-farm spread of LPAI will occur via the movement of tradesmen such as plumbers and electricians; Farm_LPAI_Eggtray*, *Probability that farm-to-farm spread of LPAI will occur via the movement of egg trays; Farm_LPAI_Eggpallet*, *Probability that farm-to-farm spread of LPAI will occur via the movement of egg pallets.* **(D)** Average median probabilities of HPAI spread pathways between farms. *Farm_HPAI_Equipment*, *Probability that farm-to-farm spread of HPAI will occur via the movement of equipment; Farm_HPAI_Aerosol*, *Probability that farm-to-farm spread of HPAI will occur via aerosol; Farm_HPAI_Animals*, *Probability that farm-to-farm spread of HPAI will occur via the movement of animals including both farm cats and dogs and vermin; Farm_HPAI_WB*, *Probability that farm-to-farm spread of HPAI will occur via the movement of wild birds; Farm_HPAI_Delivery*, *Probability that farm-to-farm spread of HPAI will occur via the movement of bird delivery transport vehicles; Farm_HPAI_Pickup*, *Probability that farm-to-farm spread of HPAI will occur via the movement of dead and live bird pick up vehicles; Farm_HPAI_Feed*, *Probability that farm-to-farm spread of HPAI will occur via the movement of feed delivery vehicles; Farm_HPAI_Manure*, *Probability that farm-to-farm spread of HPAI will occur via the movement of manure collection systems; Farm_HPAI_Workers*, *Probability that farm-to-farm spread of HPAI will occur via the movement of farm workers; Farm_HPAI_Tradesmen*, *Probability that farm-to-farm spread of HPAI will occur via the movement of tradesmen such as plumbers and electricians; Farm_HPAI_Eggtray*, *Probability that farm-to-farm spread of HPAI will occur via the movement of egg trays; Farm_HPAI_Eggpallet*, *Probability that farm-to-farm spread of HPAI will occur via the movement of egg pallets.*

in the current study, is significant in previous literature, even within the same pathotype and subtype (49, 52, 53). As there is insufficient knowledge about mutation at present to in any way alter the likelihood of its occurrence, the control of LPAI and HPAI spread is therefore mainly reliant on on-farm biosecurity actions.

The detection and reporting parameters were found not to be a significantly influential parameter on the probability of LPAI and HPAI spread. This is supported by modeling work of Barnes and Glass (26), which demonstrated a high probability that a second shed is already infected with HPAI by the time initial infection is detected and reported using typical daily and weekly mortality rates for all farm types. In addition, the index formula described above used to calculate the number of days from infection in the first chicken to establishment, detection and reporting of LPAI also supports the small influence of detection, and reporting on the overall probability of spread. This formula revealed a long time period of at least 70 days for all farm types; within this time period, it is very possible that spread has already occurred to other sheds or farms due to the high level of movements between sheds and farms on all farm types (54, 55). This compares with previous modeling studies which revealed the high significance of detection and reporting in limiting spread of an AI outbreak. However, these studies assessed different but related factors to detection and reporting; including the impact of changing the detection threshold, performing frequent sampling of farms considered high risk, and ensuring prompt action after detection. In contrast, this study assumed a relatively fixed detection threshold based on farmer answers on unusual clinical signs, and therefore the changing parameter in the sensitivity analysis is simply a change in the proportion of farms that will detect and report at this relatively fixed detection threshold. Considerations to further evaluate the significance of detection and reporting are therefore described below.

The spread pathways on the scenario tree models were complementary to each other where the sum of all LPAI or HPAI spread pathway probabilities of one scenario tree model was one. Therefore, the spread sensitivity analysis could not accurately portray the effects of changing one spread pathway as this would result in complementary changes to the other spread pathways; each spread pathway had the same influence on the probability of spread. This was depicted as "*Prob_PathwaySpread*" which

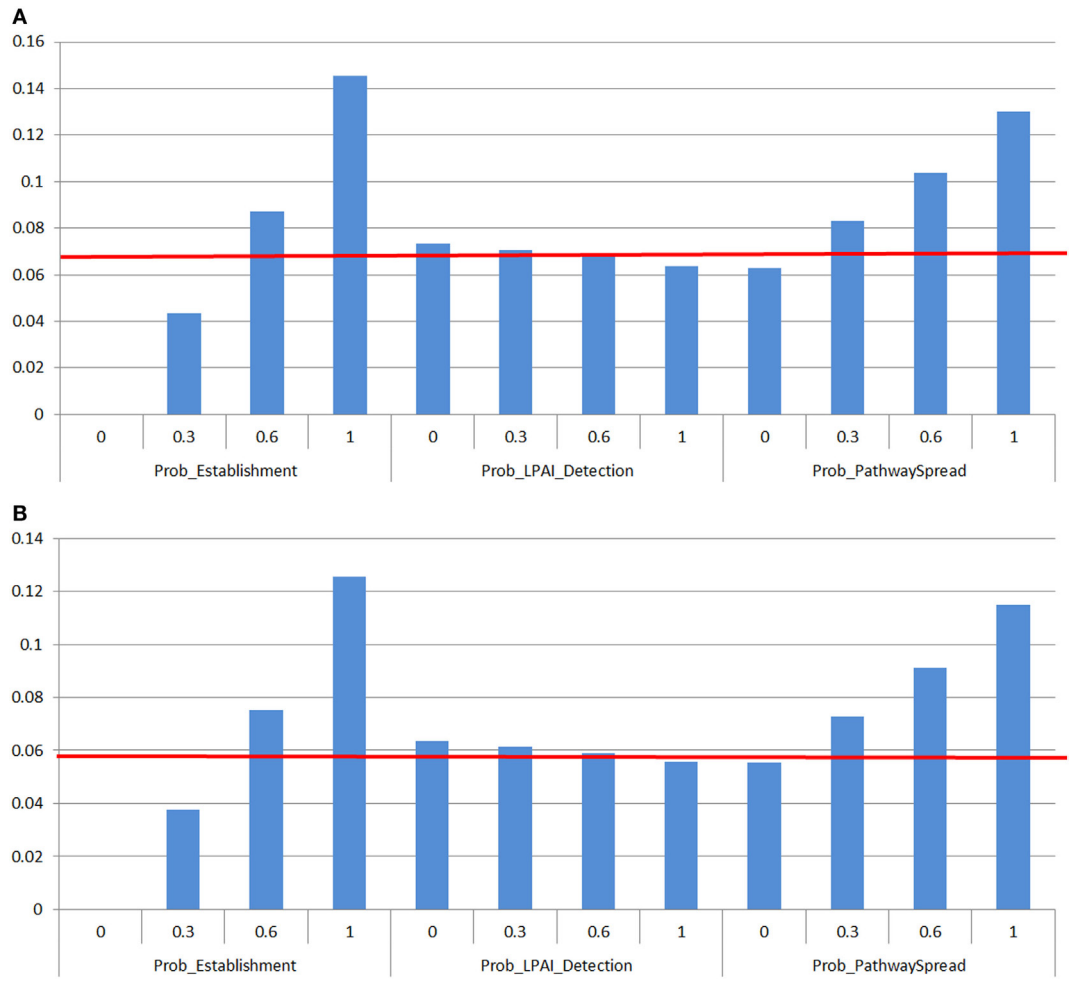

FIGURE 5 | Continued

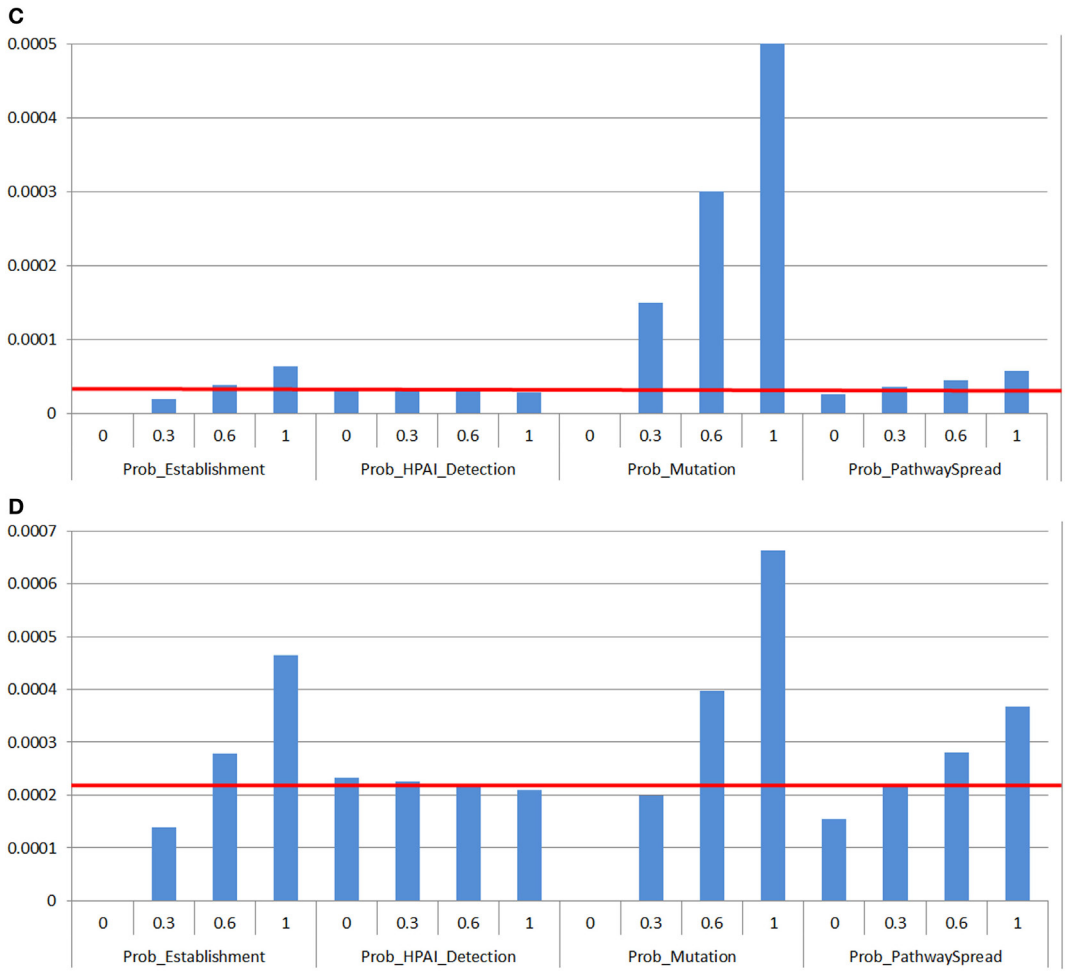

FIGURE 5 | Results of the sensitivity analysis on the spread assessment depicting the change in probability (Y-axis) on the median overall probability of low-pathogenic avian influenza (LPAI) or high-pathogenic avian influenza (HPAI) spread (horizontal line) between sheds on a commercial poultry farm and between commercial poultry farms after exposure of one chicken to low-pathogenic avian influenza (LPAI) virus from wild birds in Australia with changes of certain input variables listed in **Table 1** (*X*-axis). Results were obtained from a simulation of 1,000 iterations using @Risk's Advanced Sensitivity Analysis. The outcomes were similar in proportional increase in value among all farm types so only one example of a meat chicken or layer farm type per LPAI **(A,B)** or HPAI **(C,D)** spread between sheds and farms was used. **(A)** Sensitivity analysis on input parameters affecting the probability of LPAI spread between sheds and farms on free range meat chicken farm types. **(B)** Sensitivity analysis on input parameters affecting the probability of LPAI spread between sheds and farms on free range layer farm types. **(C)** Sensitivity analysis on input parameters affecting the probability of HPAI spread between sheds and farms on barn meat chicken farm types. **(D)** Sensitivity analysis on input parameters affecting the probability of HPAI spread between sheds and farms on cage layer farm types. *Prob_Establishment*, *Probability that the H5/H7 LPAI subtype will establish within the flock from one infected chicken; Prob_Mutation*, *Probability that LPAI established within the flock will mutate to HPAI; Prob_LPAI_Detection*, *Probability that the farmer will detect and report disease to appropriate officials during LPAI establishment; Prob_HPAI_Detection*, *Probability that HPAI will produce clinical signs with the assumption that the probability of detection is extremely high; Prob_PathwaySpread*, *Probability of any one of the spread pathways identified, with consideration of the complementary changes for all other spread pathways that will result given sum of the probabilities of all pathways occurring equaled one.*

represented changing the probability of any one spread pathway and the complementary changes to the probabilities of the other spread pathways. Increasing any spread pathway to 100%, which results in 0% probability of all other spread pathways, resulted in approximate doubling of the overall median probability of either LPAI or HPAI spread. This means if the probability of any pathway is certain to occur, and all other pathways are certain not to occur, the probability of either LPAI or HPAI spread is approximately doubled. In reality all other spread pathways will have a probability greater than zero of occurring. It is therefore expected that in a model where such pathways are not complementary to each other, the cumulative effect of increasing the probabilities of each individual pathway will result in greater than doubling the overall probability of LPAI or HPAI spread. The spread pathways are therefore significant influential parameters on the overall probability of spread and are dependent on biosecurity on the farm. Other highly influential parameters in the spread model such as the probability of establishment and mutation are dependent on the mechanisms of the virus and cannot be changed by human intervention. The importance of improving biosecurity on farms in order to reduce the probability of spread is therefore stressed from these results.

Other Considerations

These results show the large influence people who are not farm workers but regularly visit the farm have on the probability of spread. Such people include egg pallet and tray collectors and bird pick up crews. Consultation among different industry bodies is important to emphasize shared responsibility and agreement to biosecurity codes and guidelines. Further training for both farm workers and people who visit farms in regard to the importance of biosecurity is always beneficial. The integrated nature of the Australian chicken meat industry by a small number of companies allows for this shared responsibility and relative ease of communication across a range of networks. However, this may well be lacking in the Australian layer sector due to the nature of this industry which has a high level of numerous, privately owned farms (55). As new information arises related to the volume and frequency of spread pathways that occur in the Australian commercial chicken industry, as well as further information on the behavior and mechanisms of the AI virus, these can be used to update the input parameters in the spread scenario tree models.

Detection and reporting was not highly influential in this model as this node simply represented the proportion of farms that would detect or report at a relatively fixed detection threshold. However, this study did indicate that spread between sheds is likely to have already occurred before detection. Other factors related to detection and reporting were not assessed and should be considered for future studies, particularly for high-risk farms. These include those factors assessed in previous modeling studies such as; the impact of lowering the detection threshold, frequent sampling of farms considered high risk, and ensuring prompt action after detection (9–11). Frequent sampling can improve knowledge of LPAI transmission which has been demonstrated to be largely unknown in this study particularly in the Australian context. AI surveillance in poultry in Australia is currently not supported by the industry due to the consequences outlined in the AUSVETPLAN associated with H5 or H7 detections (56). Performance of surveillance in some form, such as sampling sentinel flocks or poultry at slaughter and processing should be considered for the Australian poultry industry (38).

Given this model considers and follows the probabilities of exposure quantified by Scott et al. (1), the probabilities estimated in this study can be considered representative for the same region as that of Scott et al. (1); the Sydney basin region. Extrapolating these results to other regions, poultry species or non-commercial chicken farms must be done with caution as differences in the probabilities of exposure may exist. However, the framework of this model can be used to aid in the development of similar risk assessment models for these different farms.

CONCLUSION

This study indicates that the probability of no establishment is the most likely end-point after exposure and infection of LPAI in one chicken. Nodes linked to attributes of the virus, such as the probability of establishment and the probability of mutation, were the most influential factors impacting the probability of LPAI and HPAI spread, respectively. While these cannot be changed by human intervention, some on-farm actions can be performed to potentially reduce the probability of spread. Biosecurity and cleanliness on farms, with particular attention to equipment and egg trays between sheds and farms, respectively, as these were found as the most likely spread pathways, will reduce the probability of spread. The results of this study and that of the exposure risk assessment in Scott et al. (1) help estimate the overall probability of spread and spread pathways of LPAI and HPAI in Australian commercial chicken enterprises. The results also provide guidance to the Australian commercial chicken industry on the importance of farm workers and people who regularly visit farms in performing biosecurity practices, as this is part of a shared responsibility in safeguarding the industry against AI.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Human Ethics Committee of the University of Sydney, Australia with written informed consent from all subjects. The protocol was approved by the the Human Ethics Committee of the University of Sydney, Australia (ethics reference number: 2015/252).

AUTHOR CONTRIBUTIONS

The first author AS was involved with investigation, methodology, writing of the original draft and reviewing and editing. J-AT formed initial conceptualization of the study, and was involved with formal analysis, methodology, project administration, supervision of AS, and reviewing and editing the manuscript. MS was also involved in investigation, methodology, project administration, supervision of AS, and reviewing and editing the manuscript. BB and KG were also involved with initial conceptualization of the study, formal analysis, methodology and provided reviewing and editing. BM, AB and PG were involved with conceptualization, project administration funding/support of the study. PG also provided methodology and supervision. BM provided reviewing and editing. MH-J was heavily involved in formal analysis of the results, conceptualization of the study, methodology, project administration, supervision of AS, and reviewing and editing the manuscript.

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were used for the branching process and spread scenario tree models. Thanks also to the Australian Animal Health Laboratory for providing experimental data on LPAI infections in chickens. The authors also appreciate the experts who attended and completed the expert opinion workshop, of which this data was also used in the spread scenario tree model. Egg and chicken meat companies and producers are also appreciated by the authors for their participation in the farm survey. This research was conducted within the Poultry Cooperative Research Centre (CRC) with support from the CRC and Woolworths Limited. The first author is also a recipient of a Post-graduate Scholarship from the Poultry CRC.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fvets.2018.00063/full#supplementary-material.

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Low Pathogenic Avian Influenza Exposure Risk Assessment in Australian Commercial Chicken Farms

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This study investigated the pathways of exposure to low pathogenic avian influenza (LPAI) virus among Australian commercial chicken farms and estimated the likelihood of this exposure occurring using scenario trees and a stochastic modeling approach following the World Organization for Animal Health methodology for risk assessment. Input values for the models were sourced from scientific literature and an on-farm survey conducted during 2015 and 2016 among Australian commercial chicken farms located in New South Wales and Queensland. Outputs from the models revealed that the probability of a first LPAI virus exposure to a chicken in an Australian commercial chicken farms from one wild bird at any point in time is extremely low. A comparative assessment revealed that across the five farm types (non-free-range meat chicken, free-range meat chicken, cage layer, barn layer, and free range layer farms), free-range layer farms had the highest probability of exposure (7.5×10^{-4}; 5% and 95%, 5.7×10^{-4}—0.001). The results indicate that the presence of a large number of wild birds on farm is required for exposure to occur across all farm types. The median probability of direct exposure was highest in free-range farm types (5.6×10^{-4} and 1.6×10^{-4} for free-range layer and free-range meat chicken farms, respectively) and indirect exposure was highest in non-free-range farm types (2.7×10^{-4}, 2.0×10^{-4}, and 1.9×10^{-4} for non-free-range meat chicken, cage layer, and barn layer farms, respectively). The probability of exposure was found to be lowest in summer for all farm types. Sensitivity analysis revealed that the proportion of waterfowl among wild birds on the farm, the presence of waterfowl in the range and feed storage areas, and the prevalence of LPAI in wild birds are the most influential parameters for the probability of Australian commercial chicken farms being exposed to avian influenza (AI) virus. These results highlight the importance of ensuring good biosecurity on farms to minimize the risk of exposure to AI virus and the importance of continuous surveillance of LPAI prevalence including subtypes in wild bird populations.

Keywords: avian influenza, Australia, commercial chickens, scenario trees, exposure assessment, H5, H7

INTRODUCTION

Low pathogenic avian influenza (LPAI) viruses are naturally circulating in wild birds globally. Birds in the taxonomic orders Anseriformes (waterfowl including ducks and geese) and Charadriiformes (shorebirds including gulls, waders, and auks) constitute the major natural reservoir of LPAI with an approximate prevalence of 2.5 and 0.6%, respectively, in Australia (1). Domestic gallinaceous (e.g., chickens and turkeys) poultry can become infected with LPAI *via* the fecal–oral route whereby poultry consume infectious fecal material from wild birds; either consuming feces directly or indirectly, such as through contaminated water, aerosol, or fomites. Once poultry are infected with LPAI, the virus may then mutate to highly pathogenic avian influenza (HPAI). During HPAI infection, morbidity and mortality rates in gallinaceous poultry are very high (50–89%) and can reach 100% in some flocks (2).

Highly pathogenic avian influenza (AI) is classed as a category 2 emergency animal disease in Australia under the Emergency Animal Disease Response Agreement as it has the potential to cause severe production losses and impact the national economy, and potentially impact human health and/or the environment (3). Australia has experienced seven HPAI outbreaks in poultry farms since 1976. All outbreaks were eradicated using a "stamping out" strategy which involved quarantine and culling of all poultry on infected premises, tracing and surveillance of farms at risk and restriction of movement to reduce spread of the virus (4–6). To date HPAI virus has not been detected in wild birds in Australia (1).

There is concern from experts within the Australian poultry industry about the change in probability of AI outbreak occurrence with the recent consumer driven expansion of free-range poultry farms. There are approximately 800 commercial contract meat chicken grower farms and 300 commercial chicken egg farms currently in Australia (7, 8). Products from commercial chicken farms account for the large majority of the national market; where the top seven meat chicken companies and the top 50 chicken egg producers supply over 95 and 80% of the national chicken meat and eggs consumed in Australia (7–9). Non-commercial chicken farms are small-scale farms that are suspected or proven to have less adoption of biosecurity practices than commercial farms. However, there is limited contact between non-commercial and commercial chicken farms and so the risk of exposure to disease on non-commercial farms may be higher but they do not appear to be a threat to the Australian chicken industry. The cost of a disease outbreak in non-commercial farms would also be less than commercial farms due fewer birds to destroy and a small overall impact on the industry, market and consumers (10). New South Wales (NSW) is the leading state for both egg and chicken meat production; producing 32 and 34% of the national egg and chicken meat volumes, respectively. Queensland and Victoria follow, producing 28 and 27% of the national egg volume, respectively, and 19 and 24% of the national chicken meat volume, respectively (7–9). The highest concentrations of egg farms in the country are in the Greater Sydney (31%) and Hunter regions (20%) (9). Free-range chicken meat production in Australia was regarded as a "cottage industry" in 2006 and now accounts for at least 15% of the total market (7). Similarly, the retail market share of free-range eggs has increased from 10% in 2000 to 50% in 2017 (8). The concern is that the expansion of free-range poultry farms will increase the opportunities of contact between wild birds and domestic poultry in Australia, thus potentially increasing the probability of LPAI exposure in Australian commercial chicken farms.

There are substantial differences in farm design, management, and biosecurity practices among the Australian commercial chicken enterprises, i.e., cage, barn, and free-range systems of both layer and meat chicken farms which can influence wild bird presence on farm (11, 12). In addition, previous work has identified differences in the type of wild birds present on farms of different production types and biosecurity implementation (13). There is a need to quantify and compare the probability of LPAI exposure for all types of Australian commercial chicken enterprises considering these differences. In addition, there is a need to investigate the effect of on-farm preventive actions that can mitigate the risk and impact of future AI outbreak occurrences in Australia. The aim of this study was to consider the potential pathways for LPAI exposure from wild birds to chickens present on all types of commercial chicken enterprises in Australia (non-free-range meat, free-range meat, cage layer, barn layer, free-range layer), and then to conduct a comparative assessment of how likely LPAI exposure from wild birds to chickens would occur via each of the considered pathways and overall for each farm type. The most influential pathways of exposure are also identified, thereby leading to recommendations about on-farm biosecurity practices that could be implemented to minimize these risks.

MATERIALS AND METHODS

Risk Assessment Model

The World Organisation of Animal Health provides a methodological framework for conducting animal health risk analysis (14). Risk assessment is a component of the overall risk analysis methods, which involves an entry, exposure and consequence assessment, and an estimation of the risk. The current study uses an exposure assessment to investigate the potential exposure to AI viruses of domestic chickens raised in commercial chicken properties in Australia. A partial consequence assessment was also conducted and is presented in a subsequent paper (15). The exposure assessment considers all the potential pathways by which chickens located in a commercial layer or meat chicken farm can be exposed to AI virus from wild birds and the probability of these pathways occurring is calculated. Such pathways were portrayed using scenario trees (16) and developed using Microsoft Excel (PC/Windows 7, 2010). The probabilities were estimated using Monte Carlo stochastic simulation modeling using the program @RISK 7.0 (Palisade Corporation, USA) implemented in Microsoft Excel. Each simulation consisted of 50,000 iterations sampled using the Latin hypercube method with a fixed random seed of one.

Data Sources

Most of the input values used in this model were parameterized using data collected from a survey on commercial chicken

farms in Australia (11, 12). The survey defined commercial layer farms as those with more than 1,000 birds and commercial meat chicken farms as those with more than 25,000 birds. It involved a comprehensive on-farm interview with farmers, including questions related to farm management, biosecurity practices, and wild bird presence. Scientific literature was used to estimate input parameters when required.

Survey on Commercial Layer and Meat Chicken Farms in the Sydney Basin Region and South East Queensland

A survey commenced in mid-2015 involving on-farm interviews with the farm manager or farm owner on 73 commercial chicken farms; 15 non-free-range meat, 15 free-range meat, nine cage layer, 9 barn layer, and 25 free-range layer farms. The farms were located in the Sydney basin region in NSW and in South East Queensland. The Sydney basin region was selected due to the high concentration of both layer and meat chicken farms in this area. However in this region, free-range meat chicken farms are all owned by one of two large privately owned meat chicken companies in Australia. Therefore, additional farm visits to South East Queensland were conducted to gain more representative data of privately owned meat chicken companies in Australia. The interviews involved a comprehensive questionnaire which asked questions to the farmers relating to biosecurity practices performed on farm, wild bird and animal presence, general farm information, and farm management. A greater proportion of layer farms and of free-range farms were surveyed due to the greater perceived risk of AI occurrence on these farm types. More details on the methodology of the survey, including the region and farm selection, questionnaire development, and conduct of the on-farm interviews, can be found in Scott et al. (11).

Statistical Analysis

The statistical program JMP® was used (© 2012 SAS Institute Inc., Cary, NC, USA) to conduct one-way analysis of variance (ANOVA) to analyze differences between the outcome probabilities for each of the different farm types. The outcome probabilities compared using ANOVA were the outcome probability from 1,000 iterations of the exposure scenario tree model simulation for each farm type, with each iteration reflecting the situation for one farm at a point in time. A p-value of <0.05 was used to determine statistical significance in these analyses.

Probability of Exposure

The exposure assessment examines all potential pathways by which AI virus can be introduced from wild birds into a commercial layer or meat chicken farm and estimates the probability that a first exposure to a chicken occurs through each of these pathways. Five exposure assessments were performed, one for each farm type: non-free-range meat chicken farms, free-range meat chicken farms, cage layer farms, barn layer farms, and free-range layer farms. Only LPAI viruses were considered due to the fact that HPAI viruses have never been detected from Australian wild birds during surveillance activities (1).

In addition, the models considered differences depending on the season or time of the year, given virus prevalence in wild birds changes during different times of the year. The probability of chickens accessing outdoors in free-range farms also changes during different times of the year due to weather conditions. Therefore, three "seasons" were considered in the exposure assessments; winter (June–August), summer (December–February), and then autumn and spring (March–May and September–November) were combined as one season.

Parameters required in these exposure assessments included (1) the probability of wild bird presence in different areas of the commercial chicken farm; (2) the probability of wild birds being infected and excreting LPAI viruses; and (3) parameters in relation to the management and biosecurity practices performed on the farm that would increase or reduce the probability of exposure. The main pathways of exposure considered in these assessments were the following six pathways: (1) direct exposure in a shed; (2) direct exposure around feed storage areas; (3) indirect exposure through fomites or vectors; (4) indirect exposure through aerosol; (5) indirect exposure through contaminated water inside; (6) indirect exposure through contaminated water outside sheds; and (7) direct exposure on the range.

For the purpose of this model, direct exposure is defined as physical contact between a wild bird and a commercial chicken or direct contact between a commercial chicken and wild bird feces. By contrast, indirect exposure is defined as a commercial chicken coming into contact with the virus through a medium, i.e., through water, fomites, or vectors. Fomites include boots and equipment contaminated with wild bird feces and are subsequently in contact with chickens through movement. Biological vectors may become infected with the virus, most notably insects, mice, and rats, and may shed the virus in the presence of chickens or be consumed by chickens. Mechanical vectors, such as dogs and cats, can also present the virus to chickens through movement only.

The overall probability of exposure represents the probability of a first exposure to a domestic chicken by one wild bird in each farm type, irrespective of the pathway of exposure. This probability was calculated by summing the outcome probability of all the pathways that lead to exposure for each farm type. In addition, the overall probability of direct and indirect exposure was calculated by summing the outcome probabilities of the direct (pathways 1, 2, and 7) and indirect (pathways 3, 4, 5, and 6) pathways, respectively, which lead to exposure for each farm type.

The models estimate the probability of exposure posed by a single wild bird at any point in time. This outcome probability of exposure is then used to estimate the expected number of exposures considering a range of the number of wild birds that could visit a property at any point in time and using binomial distributions. The standard prevalence of LPAI, at approximately 2.5, 0.6, and 0.5% for waterfowl, shorebirds, and other bird types, respectively, of which a subset are H5 and H7 subtypes, was used for these binomial distributions (1). The prevalence of LPAI in waterfowl and the proportion of waterfowl on the farm was then also changed in the model to assess the expected number of exposures in potential worst-case scenarios: (1) 100% waterfowl proportion and no change in waterfowl LPAI prevalence; (2)

80% waterfowl proportion and 20% waterfowl LPAI prevalence; (3) 100% waterfowl proportion and 10% waterfowl LPAI prevalence; (4) 50% waterfowl proportion and 20% waterfowl LPAI prevalence; and (5) 50% waterfowl proportion and 10% waterfowl LPAI prevalence. The distributions assume that all wild birds are independent, have the same probability of being infected, and those infected are always infective.

Tables 1–5 provide a description of the nodes and input parameters of the scenario trees used for the exposure assessments for non-free-range meat chicken farms, free-range meat chicken farms, cage layer farms, barn layer farms, and free-range layer farms, respectively. Cage and barn layer farms are referred to as non-free-range layer farms and have the same scenario tree structure. Similarly, free-range layer and free-range meat chicken farms also have the same scenario tree structure. Therefore, the scenario trees used for non-free-range layer farms, non-free-range meat chicken farms, and free-range layer and meat chicken farms are depicted in **Figures 1–3**, respectively. Nodes and input parameters related to the range areas are specific for free-range farm types, and the nodes for pathways in which surface water is used are specific for layer farm types and free-range meat chicken farms. The pathway (6) indirect exposure through contaminated water outside sheds does not exist for non-free-range meat chicken farms, and pathway (7) direct exposure on the range does not exist for non-free-range meat chicken and layer farms. Detailed and further descriptions of the nodes are provided in the supplementary information.

Sensitivity Analysis

The Advanced Sensitivity Analysis tool of the program @RISK 7.0 (Palisade Corporation, USA) was used to determine the impact of changes in the input parameters on the model outputs.

The effect of the following inputs on exposure were investigated: (1) proportion of waterfowl on property (*Prop_WF*); (2) proportion of waterfowl on waterbodies (*WB_WF*); (3) proportion of waterfowl in feed storage areas (*F_WF*); (4) proportion of waterfowl on the range (*R_WF*); (5) Farm use of surface water (*Surface_Water_Used*); (6) water inside the chicken shed is treated (*Water_Inside_Treated*); (7) water outside the chicken shed is treated (*Water_Outside_Treated*); (8) escapee chickens from the shed or range (*Escape*); and (9) indirect exposure to the virus (*Indirect*). The influence of the prevalence of LPAI (*Prev_WF)* in waterfowl was also investigated separately.

The values for the input parameters included in the sensitivity analysis were varied from 0 to 1 in thirds (0, 0.3, 0.6, 1), with 1,000 iterations used for each of the values included, while all other input values were fixed to their base value. The model outputs assessed were the overall probability of exposure to LPAI across the three seasons per farm type.

RESULTS

Probability of Direct and Indirect Exposure

The probability of a first LPAI exposure to a chicken on a commercial chicken farm being exposed from a wild bird present on the farm at any point in time through the pathways considered in this assessment was estimated to be extremely low for all farm types (**Table 6**). The assessment estimates a median (5–95%) overall probability of LPAI exposure on commercial free-range layer farms to be 7.5×10^{-4} (5.7×10^{-4}–1.0×10^{-4}); this being the highest probability among all farm types. The farm type with the lowest estimated overall probability of LPAI exposure was the barn layer farm type (3.0×10^{-4}; 1.4×10^{-4}–5.8×10^{-4}).

When the type of LPAI exposure was considered, direct exposure had the highest probability of causing first exposure to a domestic chicken among free-range farm types, with the lowest being reported for barn layer farms (**Table 6**). By contrast, the probability of indirect exposure was highest in non-free-range farm types.

To assess the influence of flock size of the farm on the probability of exposure, the overall probability of exposure of each farm type was multiplied by hypothetical numbers of sheds on the property, as each shed can be considered independent in the exposure models. Five and 10 sheds were used, and results are shown in **Table 6**.

Estimated Number of Exposures According to Volume of Wild Birds

Results from the binomial distributions are shown in **Table 7** and **Figure 4**. According to the model, a considerable number of wild birds are required for exposure to occur across all farm types. The output distributions indicate that for all farm types, except free-range layer farms, when 1,000 wild birds are present at any point in time around the farm, only on 5 of 100 farms (or scenarios) would one exposure occur, indicating that in 94.9% of farms, exposure would not occur. For free-range layer farms, only 100 wild birds are required to be present to expect a similar exposure output. In instances where 1,000 birds visit at any point in time on free-range layer farms, on 50 out of 100 farms (or scenarios), one LPAI exposure would occur based on the median of one in **Table 7**.

The number of exposures was assessed by changing the proportion of wild birds that are waterfowl and the LPAI prevalence of waterfowl, with some scenarios representing worst-case scenarios (with high proportion of waterfowl present among the wild birds on farm and with elevated LPAI prevalence among the wild birds on farm). Waterfowl may make up a considerable proportion of wild birds on the property during specific events such as drought and, similarly, the prevalence of LPAI in waterfowl may increase with population dynamics, such as an increase in immune-naive juvenile birds. The impact of these scenarios on the number of expected exposures is shown in **Table 7**. The largest number of exposures is seen when the proportion of waterfowl is increased to 80% and the prevalence increased to 20%. For all farm types, other than free-range layer farms, at least one exposure would occur when 50 wild birds are present at the property, and this would occur on 5 out of 100 farms (or scenarios). Only 10 wild birds are required for an exposure to occur in these circumstances for free-range layer farms.

TABLE 1 | Nodes, parameter estimates, and input values used for the exposure assessment estimating the probability of exposure of commercial chickens on non-free-range meat chicken farms in Australia (specifically in the Sydney basin region and South East Queensland) to low pathogenic avian influenza (LPAI) from wild birds.

Node	Branch of node	Parameter estimates	Input values	Data sources
1. Type of wild bird on farm property	Waterfowl Shorebirds Other	Proportion of answers from farmers that reported the respective wild bird type on their property (*Prop_WF; Prop_SH; Prop_O*)	Beta ($s + 1$, $n - s + 1$) 48 answers of wild birds on the property (n); 16 answers of waterfowl; 7 answers of shorebirds; and 25 answers of other wild birds	Scott et al. (11, 13)
2. Prevalence of LPAI in wild birds	Yes No	Probability of the different wild bird types; waterfowl, shorebirds, or other, being infected with LPAI of H5 or H7 subtype in winter, summer, and autumn/spring (*Prev_WF_Winter; Prev_WF_Summer; Prev_WF_AuSp; Prev_SH_Winter; Prev_SH_Summer; Prev_SH_AuSp; Prev_O_Winter; Prev_O_Summer; Prev_O_AuSp*)	Beta ($s + 1$, $n - s + 1$) multiplied by the proportion of H5 and H7 of total positive influenza A samples in New South Wales (NSW) for the seasons winter, summer, and autumn/spring Information on the values for waterfowl and shorebirds that informed the Beta distributions for the three seasons and the proportion of influenza A samples that are H5 and H7 subtypes can be obtained by contacting the corresponding author 1,552 other bird types samples (n), 1 positive other bird type sample (s); this Beta distribution used for all three seasons	Grillo et al. (1) Hansbro et al. (17) NSW NAIWB Surveillance unpublished data (2016)
3. Respective wild bird type has been reported inside chicken sheds	Yes No	Proportion of farms that witnessed the respective wild bird type inside chicken sheds on the farm. The data suggest the probability for waterfowl and shorebirds inside sheds is close to 0 and, therefore, a Pert distribution is used for these wild bird types (*Sheds_WF; Sheds_SH; Sheds_O*)	*Sheds_WF* = Pert (0, 0, 0.05) *Sheds_SH* = Pert (0, 0, 0.05) *Sheds_O* = Beta ($s + 1$, $n - s + 1$) 15 non-free-range meat chicken farms surveyed; 7 reported seeing other wild bird types in sheds	Scott et al. (11, 13)
4. Respective wild bird type has been reported in other locations on the farm	Waterbodies Feed storage	Proportion of answers from farmers that witnessed the respective wild bird type in the respective areas (*WB_WF, F_WF; WB_SH, F_SH; WB_O, F_O*)	Beta ($s + 1$, $n - s + 1$) 16 answers of waterfowl in other locations (n); 13 answers of waterfowl in waterbodies; and 3 answers of waterfowl in feed storage areas 7 answers of shorebirds in other locations (n); 6 answers of shorebirds in waterbodies; and 1 answer of shorebirds in feed storage areas 18 answers of other wild bird types in other locations (n); 9 answers of other bird types in waterbodies; and 9 answers of other bird types in feed storage areas	Scott et al. (11, 13)
5. Aerial transmission of LPAI from wild birds to domestic chickens	Yes No	Probability of LPAI introduction *via* aerial dispersion from wild birds on waterbodies to chickens on farm (*Aerosol_WB*)	Beta ($s + 1$, $n - s + 1$) 12 air samples tested at less than 100 m from 83 LPAI (H5N2)-infected swans (n), 0 positive air samples obtained	Jonges et al. (18)
6. Surface water is used for chickens	Yes No	Proportion of farms that use surface water for the chicken farm (*Surface_Water_Used*)	Beta ($s + 1$, $n - s + 1$) 15 farms surveyed (n), 1 farm used surface water for farm	Scott et al. (11, 13)
7. Water inside chicken sheds is treated	Yes No	Proportion of answers from farmers that treat water inside the chicken sheds (*Water_Inside_Treated*)	Beta ($s + 1$, $n - s + 1$) 32 answers of water use inside chicken sheds (n), 28 answers of water treated inside chicken sheds	Scott et al. (11, 13)
8. Chickens have escaped the shed	Yes No	Proportion of farms that have reported chickens unintentionally outside of the shed (*Escape*)	Beta ($s + 1$, $n - s + 1$) 15 farms surveyed (n), 0 farms reported chickens escaped shed	Scott et al. (11, 13)
9. Other indirect routes that can lead to LPAI introduction	Yes No	Probability that chickens will be exposed to LPAI virus *via* other indirect methods; boots, mice/rats, insects, and pets combined into one probability (*Indirect*) (Probability of exposure from boots + mice/rats + insects + pets)	*Probability of exposure from boots* (PrBoots) 1/25 answers did not use footbaths avian influenza (AI) virus survival on boots is 3/6 days, considered high probability of exposure PrBoots = (1/25) × [Uniform (0.7, 1)] *Probability of exposure from mice/rats* (PrMice) 10/25 answers had mice/rats in sheds	Scott et al. (11, 13) Achenbach and Bowen (19) Nielsen et al. (20) Tiwari et al. (21) Nazir et al. (22)

(*Continued*)

TABLE 1 | Continued

Node	Branch of node	Parameter estimates	Input values	Data sources
			12 mice inoculated (*n*), 0 positive on virus isolation	
			PrMice = (10/25) × [Beta (*s* + 1, *n* − *s* + 1)]	
			Probability of exposure from insects (PrInsects)	
			14/25 answers had insects in sheds	
			171 insects tested (*n*), 73 positive on virus isolation	
			PrInsects = (14/25) × [Beta (*s* + 1, *n* − *s* + 1)]	
			Probability of exposure from pets (PrPets)	
			0/25 answers allowed pets in sheds	
			AI virus survival on feces is 2/6 days, considered moderate probability of exposure	
			PrBoots = (1/25) × [Uniform (0.3, 0.5)]	

Exposure Assessment in the Three Seasons

The estimated probabilities of a chicken on commercial chicken farms being exposed to LPAI virus from wild birds at any point in time during the three seasons previously defined (winter, autumn/spring, and summer) are summarized in **Table 8**. The results show that the overall probability of exposure differs between the different seasons and the season influence also differs between farm types. While the median overall probability of exposure to LPAI virus is highest in winter for free-range layer farms, this probability is highest in autumn/spring for non-free-range meat chicken, cage layer, and barn layer farm types. No difference between winter and autumn/spring was observed for free-range meat chicken farms. For all farm types, the lowest median overall probability of LPAI virus exposure is in summer.

Exposure Sensitivity Analysis

According to the exposure sensitivity analysis, the most influential parameters were the proportion of waterfowl among wild birds on the property and the probability of waterfowl being on the feed storage areas (**Figure 5**). When the proportion of waterfowl among wild birds on the property becomes 100% (base value between 0.28 and 0.34 for all farm types), which can occur during circumstances such as drought, there is an approximate 2.8-fold increase in the probability of LPAI exposure for all farm types. A similar increase in the probability of LPAI exposure is obtained when the probability of waterfowl being on feed storage areas is increased to 100%. On free-range farms (**Figures 5A,B**), waterfowl on the range was the third most influential parameter. When the probability of waterfowl on the range is increased to 100% (base value 0.50 and 0.28 for free-range meat chicken and layer farms, respectively), an approximate 1.7-fold increase in the probability of LPAI exposure occurs. On barn layer farm types, the treatment of water inside the shed is also an important influential parameter. If the probability of water inside sheds being appropriately treated is decreased to 0% (base value 0.92 for barn layer farms), there is an approximate 2.4-fold increase in the probability of LPAI exposure. For other farm types, the impact of water treatment is not as significant. Escapee chickens from the sheds or the range was another influential parameter; if the probability of this occurring is increased to 100% (base value 0.042, 0.042, 0.45, 0.067, and 0.46 for non-free-range meat chicken, free-range meat chicken, cage layer, barn layer and free-range layer farms, respectively), an approximate 1.7-fold increase in the probability of LPAI exposure occurs for all farm types. The other indirect routes parameter (includes boots, mice/rats, insects, farm cats, or dogs) was slightly less influential, with an approximate 1.5-fold increase in the probability of LPAI exposure if the probability of these pathways occurring increases to 100% (base value of 0.28–0.43 for all farm types). The proportion of waterfowl on waterbodies, the use of surface water, and the treatment of water outside of sheds were found to be the least influential parameters in the exposure probability for all farm types.

Sensitivity analysis on the prevalence of LPAI in waterfowl was also conducted separately as this parameter has a profound influence on the probability of LPAI exposure. It was found

TABLE 2 | Nodes, parameter estimates, and input values used for the exposure assessment estimating the probability of exposure of commercial chickens on free-range meat chicken farms in Australia (specifically in the Sydney basin region and South East Queensland) to low pathogenic avian influenza (LPAI) from wild birds.

Node	Branch of node	Parameter estimates	Input values	Data sources
1. Type of wild bird on farm property	Waterfowl Shorebirds Other	Proportion of answers from farmers that reported the respective wild bird type on their property (*Prop_WF; Prop_SH; Prop_O*)	Beta ($s + 1$, $n - s + 1$) 36 answers of wild birds on the property (n); 12 answers of waterfowl; 2 answers of shorebirds; and 22 answers of other wild birds	Scott et al. (11, 13)
2. Prevalence of LPAI in wild birds	Yes No	Probability of the different wild bird types; waterfowl, shorebirds, or other, being infected with LPAI of H5 or H7 subtype in winter, summer, and autumn/spring (*Prev_WF_Winter; Prev_WF_Summer; Prev_WF_AuSp; Prev_SH_Winter; Prev_SH_Summer; Prev_SH_AuSp; Prev_O_Winter; Prev_O_Summer; Prev_O_AuSp*)	Beta ($s + 1$, $n - s + 1$) multiplied by the proportion of H5 and H7 of total positive influenza A samples in New South Wales (NSW) for the seasons winter, summer, and autumn/spring Information on the values for waterfowl and shorebirds that informed the Beta distributions for the 3 seasons and the proportion of influenza A samples that are H5 and H7 subtypes can be obtained by contacting the corresponding author 1,552 other bird types samples (n), 1 positive other bird type sample (s); this Beta distribution used for all three seasons	Grillo et al. (1) Hansbro et al. (17) NSW NAIWB Surveillance unpublished data (2016)
3. Respective wild bird type has been reported inside chicken sheds	Yes No	Proportion of farms that witnessed the respective wild bird type inside chicken sheds on the farm. The data suggest the probability for waterfowl and shorebirds inside sheds is close to 0 and, therefore, a Pert distribution is used for these wild bird types (*Sheds_WF; Sheds_SH; Sheds_O*)	*Sheds_WF* = Pert (0, 0, 0.05) *Sheds_SH* = Pert (0, 0, 0.05) *Sheds_O* = Beta ($s + 1$, $n - s + 1$) 15 farms surveyed; 11 reported seeing other wild bird types in sheds	Scott et al. (11, 13)
4. Respective wild bird type has been reported in other locations on the farm	Waterbodies Feed storage	Proportion of answers from farmers that witnessed the respective wild bird type in the respective areas (*WB_WF, F_WF; WB_SH, F_SH; WB_O, F_O*)	Beta ($s + 1$, $n - s + 1$) 20 answers of waterfowl in other locations (n); 14 answers of waterfowl in waterbodies; 2 answers of waterfowl in feed storage areas; and 4 answers of waterfowl on the range 4 answers of shorebirds in other locations (n); 2 answers of shorebirds in waterbodies; 0 answer of shorebirds in feed storage areas, and 2 answers of shorebirds on the range 37 answers of other wild bird types in other locations (n); 10 answers of other bird types in waterbodies; 12 answers of other bird types in feed storage areas; and 15 answers of other bird types on the range	Scott et al. (11, 13)
5. Suitable weather conditions for range access	Yes No	Probability that the weather conditions for seasons winter, summer, and autumn/spring are suitable for farmers to allow chickens on the range; when conditions are dry, between 17 and 28 C and there is no severe weather (*Range_Winter, Range_Summer, Range_AuSp*) (Probability of suitable temperature + dry conditions + no severe weather)	Beta ($s + 1$, $n - s + 1$) Winter: 13,248 winter hours recorded (n), 1,555 winter hours >17°C; 1,755 winter hours where precipitation >1 mm; 114 severe weather events in NSW, 1 severe weather events in Sydney basin in winter Summer: 13,248 summer hours recorded (n), 6,231.5 summer hours <28°C; 8,098.5 summer hours where precipitation >1 mm; 114 severe weather events in NSW, 64 severe weather events in Sydney basin in summer Autumn/Spring: 26,352 autumn/spring hours recorded (n), 9,338.5 autumn/spring hours >17 C and <28 C; 3,960.5 autumn/spring hours where precipitation >1 mm; 114 severe weather events in NSW, 49 severe weather events in Sydney basin in autumn/spring	Bureau of Meterology (23)
6. Birds are a suitable age for range access	Yes No	Proportion of the chicken's lifetime in which they are allowed onto the range (*Age*)	Beta ($s + 1$, $n - s + 1$) Average age at flock depopulation 43.25 days (n), age allowed outside 21 days	Scott et al. (11, 13)
7. Birds actually go onto the range	Yes No	Proportion of flock that actually leave shed and use the range as reported by farmers (*Use_Range*)	Average of 15 Beta functions ($s + 1$, $n - s + 1$) Total flock proportion 100 (n); proportion of flock that use range (9 varying answers)	Scott et al. (11, 13)

(*Continued*)

TABLE 2 | Continued

Node	Branch of node	Parameter estimates	Input values	Data sources
8. Aerial transmission of LPAI from wild birds to domestic chickens	Yes No	Probability of LPAI introduction *via* aerial dispersion from wild birds on waterbodies to chickens on farm (*Aerosol_WB*)	Beta ($s + 1$, $n - s + 1$) 12 samples tested at less than 100 m from 83 LPAI (H5N2) infected swans (n), 0 positive air samples obtained	Jonges et al. (18)
9. Surface water is used for chickens	Yes No	Proportion of answers from farmers that use surface water for the chicken farm (*Surface_Water_Used*)	Beta ($s + 1$, $n - s + 1$) 15 farms surveyed (n), 2 answers used surface water for farm	Scott et al. (11, 13)
10. Locations surface water is used for	Inside shed Outside shed	Proportion of answers from farmers that use surface water for inside the shed versus outside the shed (*Water_Inside_Used, Water_Outside_Used*)	Beta ($s + 1$, $n - s + 1$) 6 answers of surface water use (n), 4 answers use surface water inside shed, and 2 answers use surface water outside shed	Scott et al. (11, 13)
11. Water inside chicken sheds is treated	Yes No	Proportion of answers from farmers that treat water inside the chicken sheds (*Water_Inside_Treated*)	Beta ($s + 1$, $n - s + 1$) 34 answers of water use inside chicken sheds (n), 34 answers of water treated inside chicken sheds	Scott et al. (11, 13)
12. Water outside chicken sheds is treated	Yes No	Proportion of answers from farmers that treat water used outside the shed (*Water_Outside_Treated*)	Beta ($s + 1$, $n - s + 1$) 9 answers of water use outside chicken sheds (n), 8 answers of water treated outside chicken sheds	Scott et al. (11, 13)
13. Chickens have escaped the shed or range area	Yes No	Proportion of farms that have reported chickens unintentionally outside of the shed or range area (*Escape*)	Beta ($s + 1$, $n - s + 1$) 15 farms surveyed (n), 0 farms reported chickens escaped shed or range area	Scott et al. (11, 13)
14. Other indirect routes that can lead to LPAI introduction	Yes No	Probability that chickens will be exposed to LPAI virus via other indirect methods; boots, mice/rats, insects and pets combined into one probability (*Indirect*) (Probability of exposure from boots + mice/rats + insects + pets)	*Probability of exposure from boots* (PrBoots) 1/28 answers did not use footbaths AI virus survival on boots is 3/6 days, considered high probability of exposure PrBoots = (1/28) × [Uniform (0.7, 1)] *Probability of exposure from mice/rats* (PrMice) 12/28 answers had mice/rats in sheds 12 mice inoculated (n), 0 positive on virus isolation PrMice = (10/25) × [Beta ($s + 1$, $n - s + 1$)] *Probability of exposure from insects* (PrInsects) 13/28 answers had insects in sheds 171 insects tested (n), 73 positive on virus isolation PrInsects = (13/28) × [Beta ($s + 1$, $n - s + 1$)] *Probability of exposure from pets* (PrPets) 2/28 answers allowed pets in sheds on range area AI virus survival on feces is 2/6 days, considered moderate probability of exposure PrBoots = (2/28) × [Uniform (0.3, 0.5)]	Scott et al. (11, 13) Henzler et al. (24) Achenbach and Bowen (19) Nielsen et al. (20) Tiwari et al. (21) Nazir et al. (22)

TABLE 3 | Nodes, parameter estimates and input values used for the exposure assessment estimating the probability of exposure of commercial chickens on cage layer farms in Australia (specifically in the Sydney basin region and South East Queensland) to low pathogenic avian influenza (LPAI) from wild birds.

Node	Branch of node	Parameter estimates	Input values	Data sources
1. Type of wild bird on farm property	Waterfowl Shorebirds Other	Proportion of answers from farmers that reported the respective wild bird type on their property (*Prop_WF; Prop_SH; Prop_O*)	Beta ($s + 1$, $n - s + 1$) 30 answers of wild birds on the property (n); 9 answers of waterfowl; 2 answers of shorebirds; and 19 answers of other wild birds	Scott et al. (11, 13)
2. Prevalence of LPAI in wild birds	Yes No	Probability of the different wild bird types; waterfowl, shorebirds or other, being infected with LPAI of H5 or H7 subtype in winter, summer and autumn/spring (*Prev_WF_Winter; Prev_WF_Summer; Prev_WF_AuSp; Prev_SH_Winter; Prev_SH_Summer; Prev_SH_AuSp; Prev_O_Winter; Prev_O_Summer; Prev_O_AuSp*)	Beta ($s + 1$, $n - s + 1$) multiplied by the proportion of H5 and H7 of total positive influenza A samples in New South Wales (NSW) for the seasons winter, summer, and autumn/spring Information on the values for waterfowl and shorebirds that informed the Beta distributions for the 3 seasons and the proportion of influenza A samples that are H5 and H7 subtypes can be obtained by contacting the corresponding author 1,552 other bird types samples (n), 1 positive other bird type sample (s); this Beta distribution used for all three seasons	Grillo et al. (1) Hansbro et al. (17) NSW NAIWB Surveillance unpublished data (2016)
3. Respective wild bird type has been reported inside chicken sheds	Yes No	Proportion of farms that witnessed the respective wild bird type inside chicken sheds on the farm. The data suggests the probability for waterfowl and shorebirds inside sheds is close to 0 and, therefore, a Pert distribution is used for these wild bird types (*Sheds_WF; Sheds_SH; Sheds_O*)	*Sheds_WF* = Pert (0, 0, 0.05) *Sheds_SH* = Pert (0, 0, 0.05) *Sheds_O* = Beta ($s + 1$, $n - s + 1$) 9 farms surveyed; 5 reported seeing other wild bird types in sheds	Scott et al. (11, 13)
4. Respective wild bird type has been reported in other locations on the farm	Waterbodies Feed storage	Proportion of answers from farmers that witnessed the respective wild bird type in the respective areas (*WB_WF, F_WF; WB_SH, F_SH; WB_O, F_O*)	Beta ($s + 1$, $n - s + 1$) 9 answers of waterfowl in other locations (n); 9 answers of waterfowl in waterbodies; and 0 answers of waterfowl in feed storage areas 2 answers of shorebirds in other locations (n); 2 answers of shorebirds in waterbodies; and 0 answer of shorebirds in feed storage areas 14 answers of other wild bird types in other locations (n); 6 answers of other bird types in waterbodies; and 8 answers of other bird types in feed storage areas	Scott et al. (11, 13)
5. Aerial transmission of LPAI from wild birds to domestic chickens	Yes No	Probability of LPAI introduction via aerial dispersion from wild birds on waterbodies to chickens on farm (*Aerosol_WB*)	Beta ($s + 1$, $n - s + 1$) 12 samples tested at less than 100 m from 83 LPAI (H5N2) infected swans (n), 0 positive air samples obtained	Jonges et al. (18)
6. Surface water is used for chickens	Yes No	Proportion of farms that use surface water for the chicken farm (*Surface_Water_Used*)	Beta ($s + 1$, $n - s + 1$) 9 farms surveyed (n), 2 farm used surface water for farm	Scott et al. (11, 13)
7. Locations surface water is used for	Inside shed Outside shed	Proportion of answers from farmers that use surface water for inside the shed versus outside the shed (*Water_Inside_Used, Water_Outside_Used*)	Beta ($s + 1$, $n - s + 1$) 3 answers of surface water use (n), 1 answers use surface water inside shed, and 2 answers use surface water outside shed	Scott et al. (11, 13)
8. Water inside chicken sheds is treated	Yes No	Proportion of answers from farmers that treat water inside the chicken sheds (*Water_Inside_Treated*)	Beta ($s + 1$, $n - s + 1$) 18 answers of water use inside chicken sheds (n), 17 answers of water treated inside chicken sheds	Scott et al. (11, 13)
9. Water outside chicken sheds is treated	Yes No	Proportion of answers from farmers that treat water used outside the shed (*Water_Outside_Treated*)	Beta ($s + 1$, $n - s + 1$) 5 answers of water use outside chicken sheds (n), 2 answers of water treated outside chicken sheds	Scott et al. (11, 13)
10. Chickens have escaped the shed	Yes No	Proportion of farms that have reported chickens unintentionally outside of the shed (*Escape*)	Beta ($s + 1$, $n - s + 1$) 9 farms surveyed (n), 1 farms reported chickens escaped shed	Scott et al. (11, 13)

(*Continued*)

TABLE 3 | Continued

Node	Branch of node	Parameter estimates	Input values	Data sources
11. Other indirect routes that can lead to LPAI introduction	Yes	Probability that chickens will be exposed to LPAI virus via other indirect methods; boots, mice/rats, insects and pets combined into one probability (*Indirect*) (Probability of exposure from boots + mice/rats + insects + pets)	*Probability of exposure from boots* (PrBoots) 7/30 answers did not use footbaths AI virus survival on boots is 3/6 days, considered high probability of exposure PrBoots = (7/30) × [Uniform (0.7, 1)] *Probability of exposure from mice/rats* (PrMice) 8/30 answers had mice/rats in sheds 12 mice inoculated (*n*), 0 positive on virus isolation PrMice = (10/25) × [Beta ($s + 1$, $n - s + 1$)] *Probability of exposure from insects* (PrInsects) 9/30 answers had insects in sheds 171 insects tested (*n*), 73 positive on virus isolation PrInsects = (9/30) × [Beta ($s + 1$, $n - s + 1$)] *Probability of exposure from pets* (PrPets) 6/30 answers allowed pets in sheds AI virus survival on feces is 2/6 days, considered moderate probability of exposure PrBoots = (6/30) × [Uniform (0.3, 0.5)]	Scott et al. (11, 13) Henzler et al. (24) Achenbach and Bowen (19) Nielsen et al. (20) Tiwari et al. (21) Nazir et al. (22)
	No			

from the sensitivity analysis there is an approximate threefold to fourfold increase in the probability of LPAI exposure across the farm types when the LPAI prevalence in wild waterfowl is increased to 20%.

DISCUSSION

This study comparatively estimates the probability of a first exposure of a chicken to LPAI viruses from wild birds present on different types of commercial chicken enterprises in Australia. The probabilities estimated in this study can be considered representative for the Sydney basin region as weather information and the majority of on-farm surveys conducted are specific to this region. In addition, all of the LPAI wild bird prevalence data used in this study was from the Sydney basin region, where most samples were collected from the Lower Hunter region which was considered part of the Sydney basin region in the survey by Scott et al. (11). Generalizing these probabilities to commercial chicken farms in other regions of Australia, non-commercial chicken farms or farms with poultry species other than chickens must be done with caution as differences in farm design and management and biosecurity practices exist as well as differences in weather conditions and LPAI wild bird prevalence in different regions (1, 23, 25). Further research is required to confidently quantify the risk of exposure to commercial chicken farms in other regions, to non-commercial chicken farms and other poultry farms.

Probability of LPAI Exposure

The probabilities of exposure estimated in this study apply to commercial chicken farms according to the definition implemented in the on-farm survey conducted by Scott et al. (11) as this survey provided data that informed most input parameters. This survey included layer and meat chicken farm which house more than 1,000 or 25,000 chickens, respectively. Thus, the model outputs in this study apply to these flock sizes. There is epidemiological evidence that large flock sizes may be at greater risk of HPAI introduction compared to small flock sizes (26). There is limited information to suggest that this is also true for LPAI introduction, but it is logical to acknowledge that large flock sizes have more animal contacts which may increase the risk of LPAI exposure. This study assessed the influence of flock size on the overall probability by considering the number of sheds on the property and demonstrated that more sheds on a property lead to greater probabilities of exposure.

Overall, the probability of a first exposure to LPAI from wild birds at any point in time is extremely low for all farm types; however, the highest probability of exposure is seen among free-range layer farms, with this probability being over two times higher than for the other farm types. These results are in agreement with a study conducted by Gonzales et al. (27), which reported a rate of introduction of LPAI virus 13 times higher in outdoor layer farms when compared to indoor layer farms in the Netherlands. It has been indicated that the most efficient means of introduction of AI into commercial poultry is through direct contact with infected birds (28). Free-range farms have access to the outdoors where direct exposure to wild birds is more likely to occur compared to

TABLE 4 | Nodes, parameter estimates, and input values used for the exposure assessment estimating the probability of exposure of commercial chickens on barn layer farms in Australia (specifically in the Sydney basin region and South East Queensland) to low pathogenic avian influenza (LPAI) from wild birds.

Node	Branch of node	Parameter estimates	Input values	Data sources
1. Type of wild bird on farm property	Waterfowl Shorebirds Other	Proportion of answers from farmers that reported the respective wild bird type on their property (*Prop_WF; Prop_SH; Prop_O*)	Beta ($s + 1$, $n - s + 1$) 26 answers of wild birds on the property (n); 7 answers of waterfowl; 2 answers of shorebirds; and 17 answers of other wild birds	Scott et al. (11, 13)
2. Prevalence of LPAI in wild birds	Yes No	Probability of the different wild bird types; waterfowl, shorebirds or other, being infected with LPAI of H5 or H7 subtype in winter, summer, and autumn/spring (*Prev_WF_Winter; Prev_WF_Summer; Prev_WF_AuSp; Prev_SH_Winter; Prev_SH_Summer; Prev_SH_AuSp; Prev_O_Winter; Prev_O_Summer; Prev_O_AuSp*)	Beta ($s + 1$, $n - s + 1$) multiplied by the proportion of H5 and H7 of total positive influenza A samples in New South Wales (NSW) for the seasons winter, summer, and autumn/spring Information on the values for waterfowl and shorebirds that informed the Beta distributions for the 3 seasons and the proportion of influenza A samples that are H5 and H7 subtypes can be obtained by contacting the corresponding author 1,552 other bird types samples (n), 1 positive other bird type sample (s); this Beta distribution used for all three seasons	Grillo et al. (1) Hansbro et al. (17) NSW NAIWB Surveillance unpublished data (2016)
3. Respective wild bird type has been reported inside chicken sheds	Yes No	Proportion of farms that witnessed the respective wild bird type inside chicken sheds on the farm. The data suggests the probability for waterfowl and shorebirds inside sheds is close to 0 and, therefore, a Pert distribution is used for these wild bird types (*Sheds_WF; Sheds_SH; Sheds_O*)	*Sheds_WF* = Pert (0, 0, 0.05) *Sheds_SH* = Pert (0, 0, 0.05) *Sheds_O* = Beta ($s + 1$, $n - s + 1$) 9 barn layer farms surveyed; 5 reported seeing other wild bird types in sheds	Scott et al. (11, 13)
4. Respective wild bird type has been reported in other locations on the farm	Waterbodies Feed storage	Proportion of answers from farmers that witnessed the respective wild bird type in the respective areas (*WB_WF, F_WF; WB_SH, F_SH; WB_O, F_O*)	Beta ($s + 1$, $n - s + 1$) 7 answers of waterfowl in other locations (n); 7 answers of waterfowl in waterbodies; 0 answers of waterfowl in feed storage areas 2 answers of shorebirds in other locations (n); 2 answers of shorebirds in waterbodies; 0 answer of shorebirds in feed storage areas 12 answers of other wild bird types in other locations (n); 4 answers of other bird types in waterbodies; 8 answers of other bird types in feed storage areas	Scott et al. (11, 13)
5. Aerial transmission of LPAI from wild birds to domestic chickens	Yes No	Probability of LPAI introduction via aerial dispersion from wild birds on waterbodies to chickens on farm (*Aerosol_WB*)	Beta ($s + 1$, $n - s + 1$) 12 samples tested at less than 100 m from 83 LPAI (H5N2) infected swans (n), 0 positive air samples obtained	Jonges et al. (18)
6. Surface water is used for chickens	Yes No	Proportion of farms that use surface water for the chicken farm (*Surface_Water_Used*)	Beta ($s + 1$, $n - s + 1$) 9 farms surveyed (n), 3 farms used surface water for farm	Scott et al. (11, 13)
7. Locations surface water is used for	Inside shed Outside shed	Proportion of answers from farmers that use surface water for inside the shed versus outside the shed (*Water_Inside_Used, Water_Outside_Used*)	Beta ($s + 1$, $n - s + 1$) 6 total answers of surface water use water is used for (n), 5 answers for inside the shed, 1 answer for outside the shed	Scott et al. (11, 13)
8. Water inside chicken sheds is treated	Yes No	Proportion of answers from farmers that treat water inside the chicken sheds (*Water_Inside_Treated*)	Beta ($s + 1$, $n - s + 1$) 19 answers of water use inside chicken sheds (n), 18 answers of water treated inside chicken sheds	Scott et al. (11, 13)
9. Water outside chicken sheds is treated	Yes No	Proportion of answers from farmers that treat water used outside the shed (*Water_Outside_Treated*)	Beta ($s + 1$, $n - s + 1$) 1 answers of water use outside chicken sheds (n), 1 answers of water treated outside chicken sheds	Scott et al. (11, 13)

(*Continued*)

TABLE 4 | Continued

Node	Branch of node	Parameter estimates	Input values	Data sources
10. Chickens have escaped the shed	Yes No	Proportion of farms that have reported chickens unintentionally outside of the shed (*Escape*)	Beta ($s + 1$, $n - s + 1$) 9 farms surveyed (n), 0 farms reported chickens escaped shed	Scott et al. (11, 13)
11. Other indirect routes that can lead to LPAI introduction	Yes No	Probability that chickens will be exposed to LPAI virus via other indirect methods; boots, mice/rats, insects and pets combined into one probability (*Indirect*) (Probability of exposure from boots + mice/rats + insects + pets)	*Probability of exposure from boots* (PrBoots) 3/21 answers did not use footbaths AI virus survival on boots is 3/6 days, considered high probability of exposure PrBoots = (3/21) × [Uniform (0.7, 1)] *Probability of exposure from mice/rats* (PrMice) 8/21 answers had mice/rats in sheds 12 mice inoculated (n), 0 positive on virus isolation PrMice = (8/21) × [Beta ($s + 1$, $n - s + 1$)] *Probability of exposure from insects* (PrInsects) 9/21 answers had insects in sheds 143 insects tested (n), 114 positive on virus isolation PrInsects = (9/21) × [Beta ($s + 1$, $n - s + 1$)] *Probability of exposure from pets* (PrPets) 1/21 answers allowed pets in sheds AI virus survival on feces is 2/6 days, considered moderate probability of exposure PrBoots = (1/21) × [Uniform (0.3, 0.5)]	Scott et al. (11, 13) Henzler et al. (24) Achenbach and Bowen (19) Nielsen et al. (20) Tiwari et al. (21) Nazir et al. (22)

indoor farms, and this is in agreement with results presented here. However, during on-farm surveys, it was found that Australian free-range meat chickens are relatively restricted in their access to the outdoors, which is determined by their age and suitable outside weather conditions (11). Most Australian meat chickens are processed when they reach only 50 days, whereas layer chickens are kept in production until around 70–80 weeks (11, 25).

There have been a total of 15 confirmed LPAI cases in Australian poultry since 1976 (29, 30). These cases include LPAI detections of various subtypes, including outbreaks and single bird detections, in Australian poultry. These detections have been a result of passive surveillance (diagnostic submissions), active surveillance (during HPAI outbreaks) and incidental findings not associated with disease. Most have occurred in domestic flocks of ducks, with five incidents in combined chicken and duck farms. In addition, breeder birds were involved in several incidents, with two detections in breeder duck farms, two in breeder chicken farms, and two in mixed breeder and meat duck farms. Four cases occurred in meat poultry farms (two turkey and two duck farms). LPAI has never been detected on a meat chicken farm or on a single-species commercial egg layer enterprise (29). The exposure model considers single-species commercial chicken farms only. Therefore, of all LPAI detections that have occurred in Australia so far, comparisons with the model results can only be made with the two LPAI detections that occurred in breeder chicken farms. Breeder chicken farms are essentially equivalent to barn layer chicken farms and usually have good biosecurity (25). However, the exposure model suggests barn layers have the lowest probability of overall LPAI exposure compared to all farm types. As well as good biosecurity, breeder chicken farms tend to also have close flock health monitoring, as the LPAI detections that occurred were during outbreak investigation related to a drop in production performance (29). It is very likely LPAI detections in Australia are underreported as LPAI infections can be non-clinical, especially in ducks (29). This study found that information on AI virus characteristics and behavior, especially in an Australian context, is extremely scarce.

To best validate these models, routine sampling of Australian commercial chicken farms for LPAI should be conducted. According to the Australian Veterinary Emergency Plan for AI (31), farms with positive detections of H5 or H7 AI virus via cloacal or oropharyngeal swabs must be depopulated and quarantine measures put into place. Given the current depopulation policy, the introduction of financial incentives or encouragement from industry is required to convince farmers to participate in active surveillance sampling. Voluntary participation in routine surveillance as part of a farm accreditation program can also be considered (32). As an alternative to this sampling approach, serological surveys can also be used as occurs in the Netherlands, where all poultry farms were tested for evidence of seroconversion at least once a year, with outdoor layer farms being tested three to four times per year. These data were used to estimate the introduction rates between different farm types (27). Serological sampling has also been performed in Australia but in small, sentinel free-range flocks located near waterfowl habitat and far from commercial chicken enterprises. Results from this sampling showed an extremely low introduction rate; from 2,000

TABLE 5 | Nodes, parameter estimates and input values used for the exposure assessment estimating the probability of exposure of commercial chickens on free-range layer farms (specifically in the Sydney basin region and South East Queensland) in Australia to low pathogenic avian influenza (LPAI) from wild birds.

Node	Branch of node	Parameter estimates	Input values	Data sources
1. Type of wild bird on farm property	Waterfowl Shorebirds Other	Proportion of answers from farmers that reported the respective wild bird type on their property (*Prop_WF; Prop_SH; Prop_O*)	Beta ($s + 1$, $n - s + 1$) 140 answers of wild birds on the property (n); 44 answers of waterfowl; 33 answers of shorebirds; 63 answers of other wild birds	Scott et al. (11, 13)
2. Prevalence of LPAI in wild birds	Yes No	Probability of the different wild bird types; waterfowl, shorebirds or other, being infected with LPAI of H5 or H7 subtype in winter, summer, and autumn/spring (*Prev_WF_Winter; Prev_WF_Summer; Prev_WF_AuSp; Prev_SH_Winter; Prev_SH_Summer; Prev_SH_AuSp; Prev_O_Winter; Prev_O_Summer; Prev_O_AuSp*)	Beta ($s + 1$, $n - s + 1$) multiplied by the proportion of H5 and H7 of total positive influenza A samples in New South Wales (NSW) for the seasons winter, summer, and autumn/spring Information on the values for waterfowl and shorebirds that informed the Beta distributions for the 3 seasons and the proportion of influenza A samples that are H5 and H7 subtypes can be obtained by contacting the corresponding author 1,552 other bird types samples (n), 1 positive other bird type sample (s); this Beta distribution used for all three seasons	Grillo et al. (1) Hansbro et al. (17) NSW NAIWB Surveillance unpublished data (2016)
3. Respective wild bird type has been reported inside chicken sheds	Yes No	Proportion of farms that witnessed the respective wild bird type inside chicken sheds on the farm. The data suggests the probability for waterfowl and shorebirds inside sheds is close to 0 and, therefore, a Pert distribution is used for these wild bird types (*Sheds_WF; Sheds_SH; Sheds_O*)	*Sheds_WF* = Pert (0, 0, 0.05) *Sheds_SH* = Pert (0, 0, 0.05) *Sheds_O* = Beta ($s + 1$, $n - s + 1$) 25 farms surveyed; 13 reported seeing other wild bird types in sheds	Scott et al. (11, 13)
4. Respective wild bird type has been reported in other locations on the farm	Waterbodies Feed storage	Proportion of answers from farmers that witnessed the respective wild bird type in the respective areas (*WB_WF, F_WF; WB_SH, F_SH; WB_O, F_O*)	Beta ($s + 1$, $n - s + 1$) 44 answers of waterfowl in other locations (n); 23 answers of waterfowl in waterbodies; 9 answers of waterfowl in feed storage areas, 12 answers of waterfowl on the range 33 answers of shorebirds in other locations (n); 12 answers of shorebirds in waterbodies; 9 answer of shorebirds in feed storage areas, 12 answers of shorebirds on the range 50 answers of other wild bird types in other locations (n); 14 answers of other bird types in waterbodies; 16 answers of other bird types in feed storage areas, 20 answers of other bird types on the range	Scott et al. (11, 13)
5. Suitable weather conditions for range access	Yes No	Probability that the weather conditions for seasons winter, summer, and autumn/spring are suitable for farmers to allow chickens on the range; when conditions are dry, between 17 and 28 C and there is no severe weather (*Range_Winter, Range_Summer, Range_AuSp*) (Probability of suitable temperature + dry conditions + no severe weather)	Beta ($s + 1$, $n - s + 1$) Winter: 13,248 winter hours recorded (n), 1,555 winter hours > 17C; 1,755 winter hours where precipitation > 1mm; 114 severe weather events in NSW, 1 severe weather events in Sydney basin in winter Summer: 13,248 summer hours recorded (n), 6,231.5 summer hours <28°C; 8,098.5 summer hours where precipitation >1 mm; 114 severe weather events in NSW, 64 severe weather events in Sydney basin in summer Autumn/Spring: 26,352 autumn/spring hours recorded (n), 9,338.5 autumn/spring hours >17°C and <28°C; 3,960.5 autumn/spring hours where precipitation >1 mm; 114 severe weather events in NSW, 49 severe weather events in Sydney basin in autumn/spring	Bureau of Meterology (23)
6. Birds are a suitable age for range access	Yes No	Proportion of the chicken's lifetime in which they are allowed onto the range (*Age*)	Beta ($s + 1$, $n - s + 1$) Average age at flock depopulation 87.32 weeks (n), average age allowed outside 22.94 weeks	Scott et al. (11, 13)
7. Birds actually go onto the range	Yes No	Proportion of flock that actually leave shed and use the range as reported by farmers (*Use_Range*)	Average of 25 Beta functions ($s + 1$, $n - s + 1$) Total flock proportion 100 (n); proportion of flock that use range (25 varying answers)	Scott et al. (11, 13)

(*Continued*)

TABLE 5 | Continued

Node	Branch of node	Parameter estimates	Input values	Data sources
8. Aerial transmission of LPAI from wild birds to domestic chickens	Yes No	Probability of LPAI introduction via aerial dispersion from wild birds on waterbodies to chickens on farm (*Aerosol_WB*)	Beta ($s + 1$, $n - s + 1$) 12 air samples tested at less than 100 m from 83 LPAI (H5N2) infected swans (n), 0 positive air samples obtained	Jonges et al. (18)
9. Surface water is used for chickens	Yes No	Proportion of answers from farmers that use surface water for the chicken farm (*Surface_Water_Used*)	Beta ($s + 1$, $n - s + 1$) 25 farms surveyed (n), 6 answers used surface water for farm	Scott et al. (11, 13)
10. Locations surface water is used for	Inside shed Outside shed	Proportion of answers from farmers that use surface water for inside the shed versus outside the shed (*Water_Inside_Used, Water_Outside_Used*)	Beta ($s + 1$, $n - s + 1$) 22 answers of surface water use (n), 12 answers use surface water inside shed, 10 answers use surface water outside shed	Scott et al. (11, 13)
11. Water inside chicken sheds is treated	Yes No	Proportion of answers from farmers that treat water inside the chicken sheds (*Water_Inside_Treated*)	Beta ($s + 1$, $n - s + 1$) 50 answers of water use inside chicken sheds (n), 48 answers of water treated inside chicken sheds	Scott et al. (11, 13)
12. Water outside chicken sheds is treated	Yes No	Proportion of answers from farmers that treat water used outside the shed (*Water_Outside_Treated*)	Beta ($s + 1$, $n - s + 1$) 17 answers of water use outside chicken sheds (n), 14 answers of water treated outside chicken sheds	Scott et al. (11, 13)
13. Chickens have escaped the shed or range area	Yes No	Proportion of farms that have reported chickens unintentionally outside of the shed or range area (*Escape*)	Beta ($s + 1$, $n - s + 1$) 25 farms surveyed (n), 21 farms reported chickens escaped shed or range area	Scott et al. (11, 13)
14. Other indirect routes that can lead to LPAI introduction	Yes No	Probability that chickens will be exposed to LPAI virus via other indirect methods; boots, mice/rats, insects and pets combined into one probability (*Indirect*) (Probability of exposure from boots + mice/rats + insects + pets)	*Probability of exposure from boots* (PrBoots) 6/63 answers did not use footbaths AI virus survival on boots is 3/6 days, considered high probability of exposure PrBoots = (6/63) × [Uniform (0.7, 1)] *Probability of exposure from mice/rats* (PrMice) 19/63 answers had mice/rats in sheds 12 mice inoculated (n), 0 positive on virus isolation PrMice = (10/25) × [Beta ($s + 1$, $n - s + 1$)] *Probability of exposure from insects* (PrInsects) 25/63 answers had insects in sheds 171 insects tested (n), 73 positive on virus isolation PrInsects = (25/63) × [Beta ($s + 1$, $n - s + 1$)] *Probability of exposure from pets* (PrPets) 13/63 answers allowed pets in sheds on range area AI virus survival on feces is 2/6 days, considered moderate probability of exposure PrBoots = (13/63) × [Uniform (0.3, 0.5)]	Scott et al. (11, 13) Henzler et al. (24) Achenbach and Bowen (19) Nielsen et al. (20) Tiwari et al. (21) Nazir et al. (22)

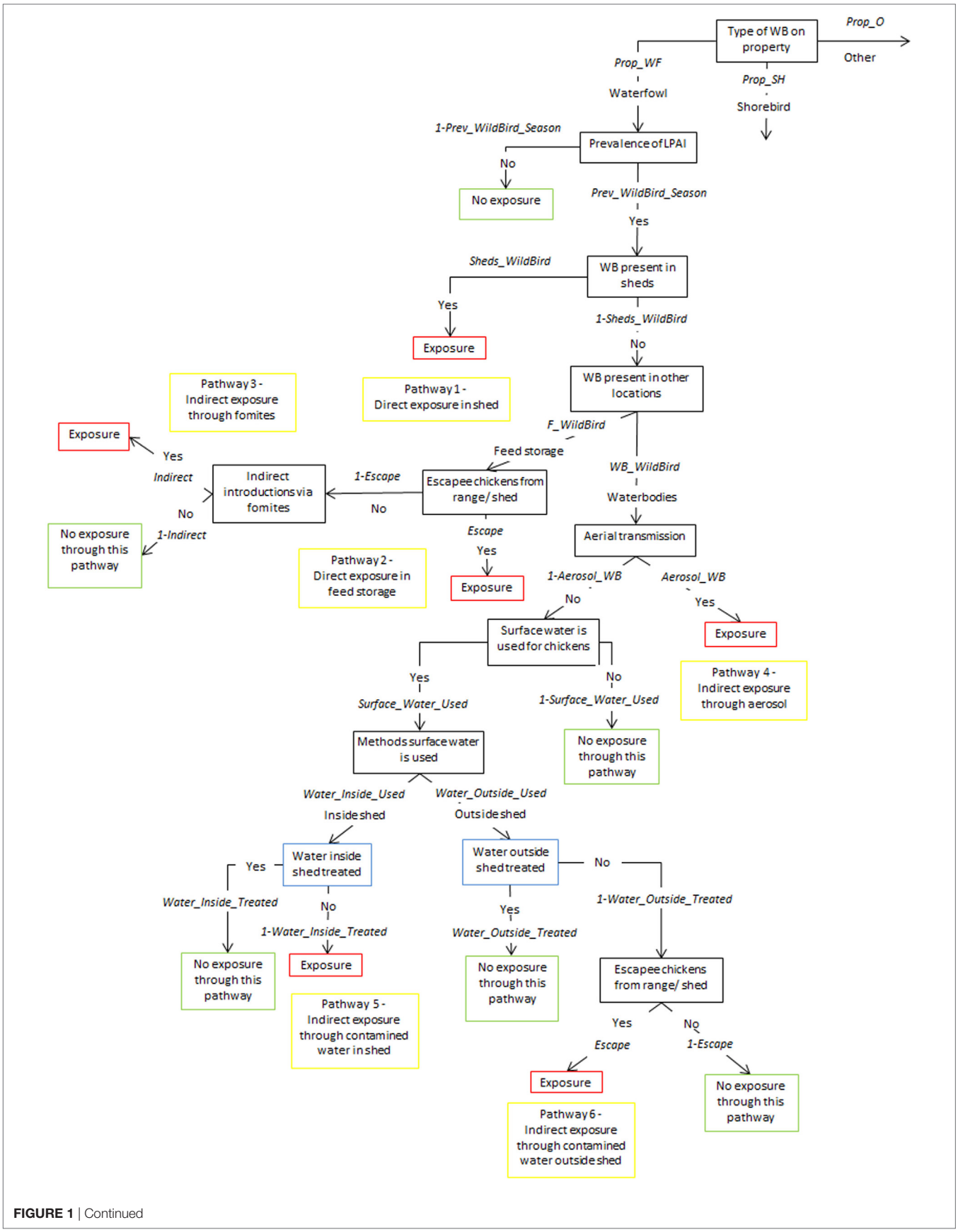

FIGURE 1 | Continued

FIGURE 1 | Scenario tree representing the exposure of chickens on non-free-range layer farms to low pathogenic avian influenza (LPAI) viruses from wild birds in Australia (*Prop_WF*, proportion of waterfowl answers reported on property, *Prop_SH*, proportion of shorebird answers reported on property, *Prop_O*, proportion of other bird types reported on property, *Prev_WildBird_Season*, prevalence of LPAI of the respective wild bird type (waterfowl, shorebird, or other) in the respective season (winter, summer, or autumn/spring), *Sheds_WildBird*, proportion of farms that reported the respective wild bird type (waterfowl, shorebird, or other) inside chicken sheds on the farm, *F_WildBird*, proportion of answers that witnessed the respective wild bird type (waterfowl, shorebird, or other) in feed storage areas, *WB_WildBird*, proportion of answers that witnessed the respective wild bird type (waterfowl, shorebird, or other) in waterbodies on/near the farm, *Escape*, proportion of farms that reported chickens escaping from shed, *Indirect*, probability of the occurrence of other indirect methods that can introduce LPAI (boots, mice/rats, insects, farm cats, or dogs), *Aerosol_WB*, probability of LPAI exposure from aerial dispersion of virus from wild birds on waterbodies, *Surface_Water_Used*, proportion of answers that surface water is used for chicken farm, *Water_Inside_Used*, proportion of answers that surface water is used inside sheds, *Water_Outside_Used*, proportion of answers that surface water is used outside sheds, *Water_Inside_Treated*, proportion of answers that treat water used inside sheds, *Water_Outside_Treated*, proportion of answers that treat water used outside sheds).

samples collected over 8 years, 0.85% (17) samples tested positive for AI antibodies and 4.35% (87) were uncertain. The number of H5 and H7 subtypes was not determined in the study (33). Although useful, this information cannot be confidently applied to commercial chicken enterprises due to stark differences in the number of birds in a flock, management practices, and farm locations.

Probability of Direct and Indirect LPAI Exposure

The differences in the probability of direct and indirect exposure between free-range and non-free-range farms are likely due to the definitions of exposures types used in this model. Direct exposure is more likely to occur when chickens have access to the outdoors and, as such, exposure to the virus in non-free-range farms is more likely to occur through indirect pathways. Biosecurity refers to actions to prevent the introduction and spread of infectious agents. In relation to poultry enterprises this refers to practices, such as the use of foot baths, treatment of water, disinfection of equipment between sheds, and vermin control (34). It was found during on-farm surveys that non-free-range meat chicken farms were usually older farms with relatively poorer biosecurity compared to free-range meat chicken farms (12). This relative lack in biosecurity contributed to the highest median probability of indirect exposure occurring in non-free-range meat chicken farms compared to the rest of the farm types. This in combination with the relative restriction to the outdoors in free-range meat chicken farms lead to the higher overall probability of LPAI exposure in non-free-range meat chicken farms compared to free-range meat chicken farms. Biosecurity was also relatively lacking in cage layer farms compared to other farm types, where layer chickens were reported to escape the sheds to the feed storage areas and wild birds reported to be inside sheds (11, 12). This explains the relatively high probability of both direct and indirect LPAI exposure in cage layer farms compared to other farm types.

Another major introduction route implicated for LPAI is the contamination of drinking water for chickens with infective wild bird feces. At least half of all Australian HPAI outbreaks so far are likely to have been associated with the introduction of LPAI *via* contaminated drinking water (4, 35). However, on-farm survey results showed a high level of water treatment across all farm types. The treatment methods identified in the on-farm surveys were deemed adequate to deactivate LPAI, due to the fragile nature and short persistence of AI viruses in the environment (21). Therefore, the use of surface water is not a highly influential parameter, also depicted in the sensitivity analyses, due to the high proportion of water treatment among all farm types. Overall, the treatment of water inside and outside sheds were not found to be significantly influential parameters. In general, it was found that water treatment inside sheds was more influential in the indoor, non-free-range farms compared to free-range farms due to the limited opportunities of direct exposure in indoor farm types.

The exposure sensitivity analysis revealed that the most influential parameters were related to waterfowl presence on the farm; particularly the proportion of waterfowl among wild birds on the property, waterfowl around feed storage areas, and waterfowl on the range. Waterfowl on waterbodies was not a highly influential parameter due to the high proportion of farms that treat surface water, as previously mentioned, and the low probability of aerosol transmission of LPAI from wild waterfowl on waterbodies to commercial chickens (18). However, waterbodies are an attractant for waterfowl and artificial waters, such as dams are used extensively by waterfowl (36) and it is expected that waterfowl on waterbodies in proximity to farms will move to feed storage areas or the range of the farm. To effectively reduce the probability of LPAI exposure to Australian commercial chickens, efforts must be considered to ethically and effectively deter waterfowl from chicken farms. However, farm dams play an important role in water supply and irrigation in Australian agriculture and so the removal of open water sources can be of a great detriment to the farmer (37). In addition, covering open water sources and netting ranges are cost prohibitive (38). Recommendations from a critical review on the deterrence of wild waterfowl from Australian poultry production areas include maintaining optimal grass height, preventing grass going to seed, improving drainage on range areas and around sheds, and prompt cleaning of feed spills around feed storage areas. Other sophisticated recommendations include the development of a 24/7 waterfowl monitoring system on farm and then trialing a range of cost-effective radar-activated on-demand auditory, visual, or physical deterrent systems (38).

Volume of Wild Birds on the Probability of LPAI Exposure

In addition to the presence of waterfowl in different areas of the farm, the actual number of waterfowl present as well as the prevalence of LPAI in waterfowl are highly influential on the potential number of exposures occurring. The 1994 H7N2

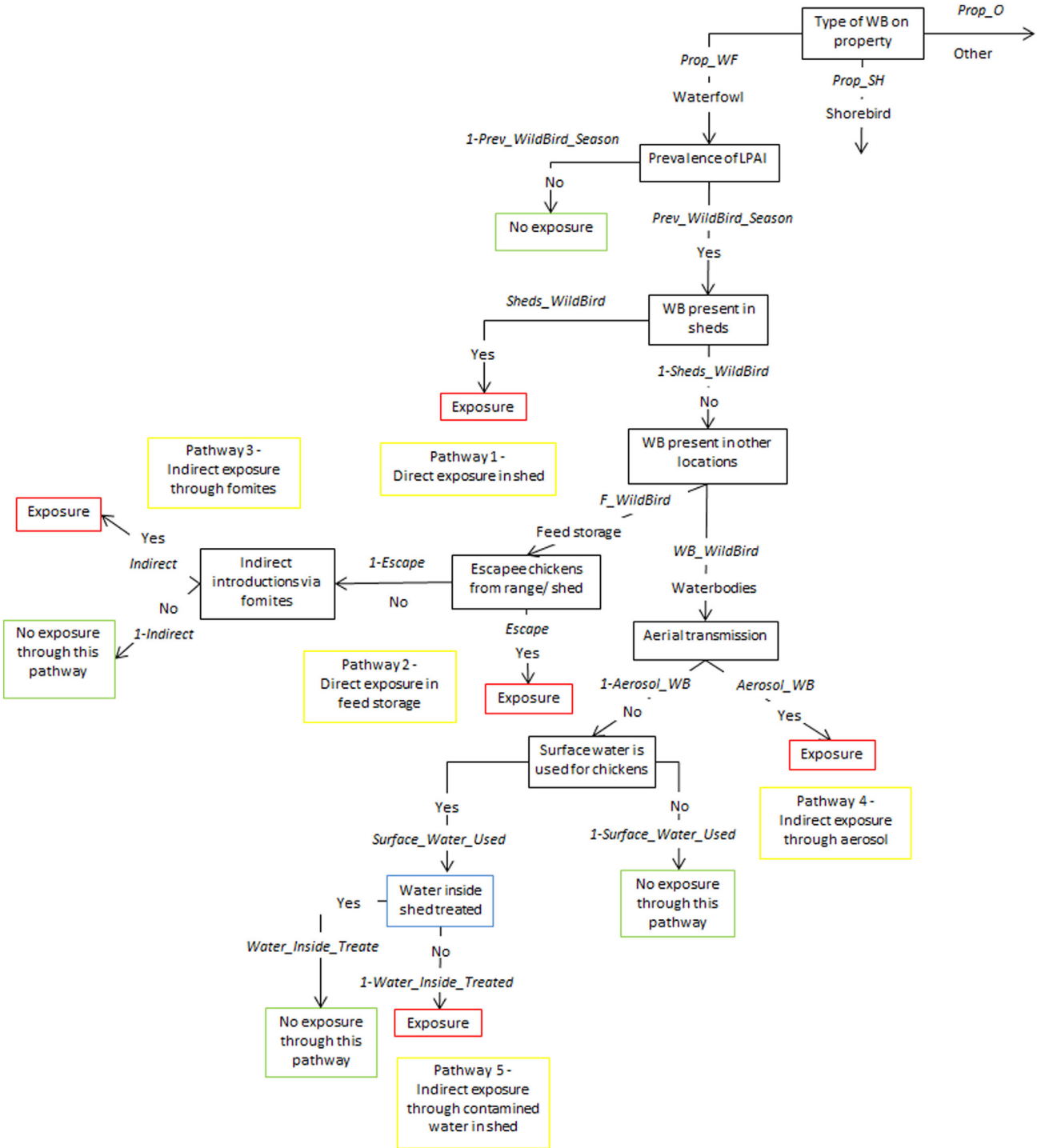

FIGURE 2 | Scenario tree representing the exposure of chickens on non-free-range meat chicken farms to low pathogenic avian influenza (LPAI) viruses from wild birds in Australia (*Prop_WF*, proportion of waterfowl answers reported on property, *Prop_SH*, proportion of shorebird answers reported on property, *Prop_O*, proportion of other bird types reported on property, *Prev_WildBird_Season*, prevalence of LPAI of the respective wild bird type (waterfowl, shorebird, or other) in the respective season (winter, summer, or autumn/spring), *Sheds_WildBird*, proportion of farms that reported the respective wild bird type (waterfowl, shorebird, or other) inside chicken sheds on the farm, *F_WildBird*, proportion of answers that witnessed the respective wild bird type (waterfowl, shorebird, or other) in feed storage areas, *WB_WildBird*, proportion of answers that witnessed the respective wild bird type (waterfowl, shorebird, or other) in waterbodies on/near the farm, *Escape*, proportion of farms that reported chickens escaping from shed, *Indirect*, probability of the occurrence of other indirect methods that can introduce LPAI (boots, mice/rats, insects, farm cats, or dogs), *Aerosol_WB*, probability of LPAI exposure from aerial dispersion of virus from wild birds on waterbodies, Surface_ Water_Used, proportion of answers that surface water is used for chicken farm, *Water_Inside_Treated*, proportion of answers that treat water used inside sheds).

outbreak in Lowood, Queensland is a classic example of both Australian waterfowl movements and the impact of the number of waterfowl in a property. The outbreak occurred during severe drought and a river that constituted one border for the farm as well as a small dam near the entrance of the chicken sheds had attracted a large population of wild birds prior to the subsequent outbreak. LPAI was speculated to be introduced to the flock through contaminated drinking water (4). Currently, there is no available data that accurately estimates the number of wild birds that visit Australian commercial chicken farms over a certain

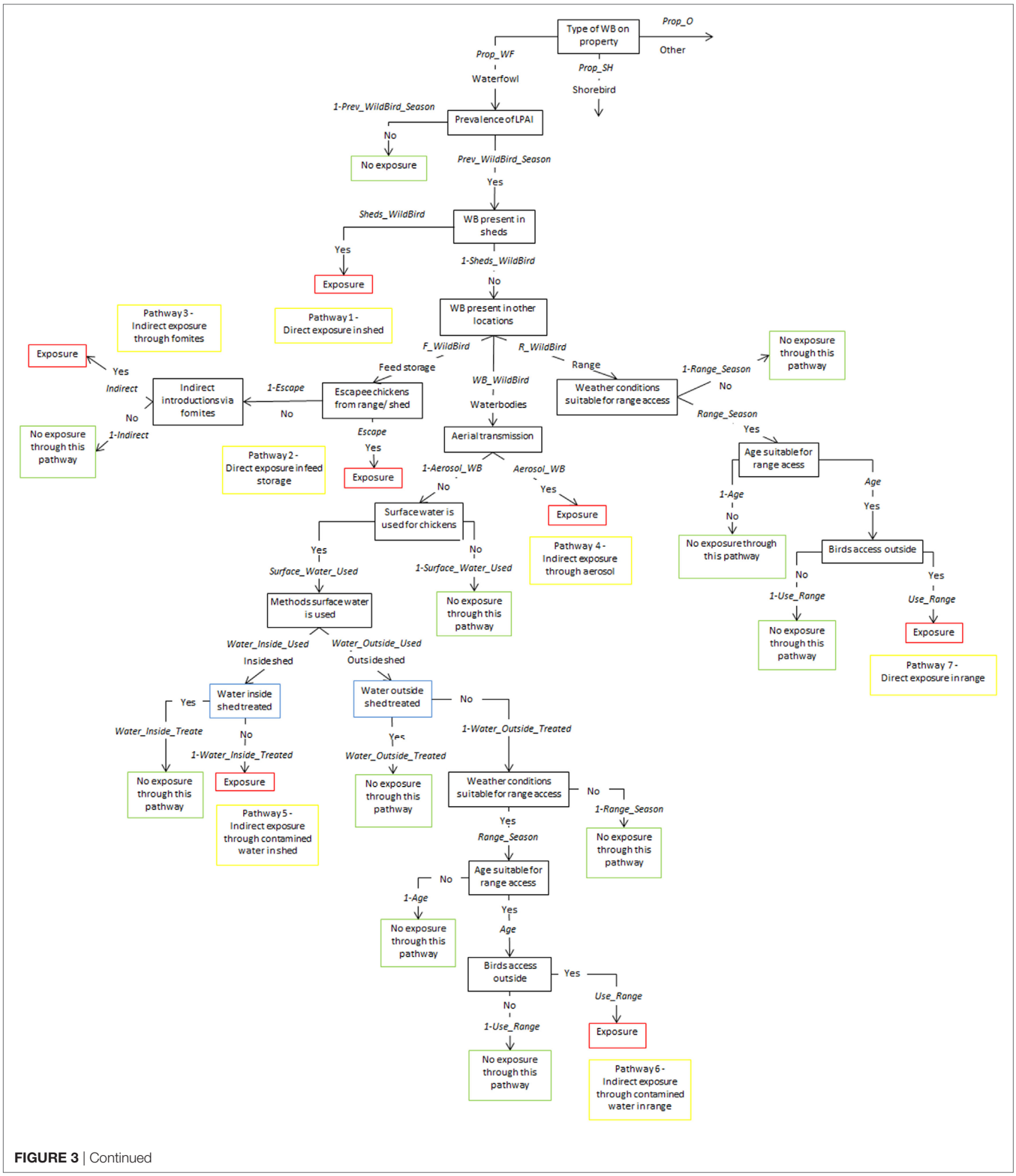

FIGURE 3 | Continued

FIGURE 3 | Scenario tree representing the exposure of chickens on free-range layer and meat chicken farms to low pathogenic avian influenza (LPAI) viruses from wild birds in Australia (*Prop_WF*, proportion of waterfowl answers reported on property, *Prop_SH*, proportion of shorebird answers reported on property, *Prop_O*, proportion of other bird types reported on property, *Prev_WildBird_Season*, prevalence of LPAI of the respective wild bird type (waterfowl, shorebird, or other) in the respective season (winter, summer, or autumn/spring), *Sheds_WildBird*, proportion of farms that reported the respective wild bird type (waterfowl, shorebird, or other) inside chicken sheds on the farm, *F_WildBird*, proportion of answers that witnessed the respective wild bird type (waterfowl, shorebird, or other) in feed storage areas, *WB_WildBird*, proportion of answers that witnessed the respective wild bird type (waterfowl, shorebird, or other) in waterbodies on/near the farm, *R_WildBird*, proportion of answers that witnessed the respective wild bird type (waterfowl, shorebird, or other) on the range, *Escape*, proportion of farms that reported chickens escaping from shed and from range, *Indirect*, probability of the occurrence of other indirect methods that can introduce LPAI (boots, mice/rats, insects, farm cats, or dogs), *Aerosol_WB*, probability of LPAI exposure from aerial dispersion of virus from wild birds on waterbodies, Surface_Water_Used, proportion of answers that surface water is used for chicken farm, *Water_Inside_Used*, proportion of answers that surface water is used inside sheds, *Water_Outside_Used*, proportion of answers that surface water is used outside sheds, *Water_Inside_Treated*, proportion of answers that treat water used inside sheds, *Water_Outside_Treated*, proportion of answers that treat water used outside sheds, *Range_Season*, probability that weather conditions are suitable for chickens to access range, *Age*, proportion of chicken's lifetime in which they are allowed onto range, *Use_range*, proportion of flock that actually leave shed and use range).

TABLE 6 | Median (5 and 95 percentiles) probabilities of direct and indirect exposure of a chicken on the commercial chicken farm types (non-free-range meat chicken, free-range meat chicken, cage layer, barn layer, free-range layer) to low pathogenic avian influenza (LPAI) viruses for the first time at any point in time from wild birds in Australia (specifically in the Sydney basin bioregion and South East Queensland).

Exposure and farm type	Median	5%	95%	*F*statistic (degrees of freedom); *p*-value
Overall probability of exposure (direct and indirect)				
Non-free-range meat chicken	0.00037	0.00020	0.00064	$F(4,4995) = 1812.63$; <0.0001
Free-range meat chicken	0.00032	0.00018	0.00057	
Cage layer	0.00032	0.00015	0.00063	
Barn layer	0.00030	0.00014	0.00058	
Free-range layer	0.00075	0.00057	0.00010	
Probability of direct exposure				
Non-free-range meat chicken	8.68×10^{-5}	3.153×10^{-5}	0.00019	$F(4,4995) = 8927.21$; <0.0001
Free-range meat chicken	0.00016	8.45×10^{-5}	0.00030	
Cage layer	0.00011	3.81×10^{-5}	0.00025	
Barn layer	8.82×10^{-5}	3.00×10^{-5}	0.00022	
Free-range layer	0.00056	0.00043	0.00073	
Probability of indirect exposure				
Non-free-range meat chicken	0.00027	0.00014	0.00053	$F(4,4995) = 235.78$; <0.0001
Free-range meat chicken	0.00016	5.72×10^{-5}	0.00036	
Cage layer	0.00020	7.76×10^{-5}	0.00047	
Barn layer	0.00019	7.46×10^{-5}	0.00045	
Free-range layer	0.00017	9.38×10^{-5}	0.00036	
Overall probability of exposure (direct and indirect—5 sheds on the property)				
Non-free-range meat chicken	0.00185	0.001	0.0032	$F(4,4995) = 1878.45$; <0.0001
Free-range meat chicken	0.0016	0.0009	0.00285	
Cage layer	0.0016	0.00075	0.00315	
Barn layer	0.0015	0.0007	0.0029	
Free-range layer	0.00375	0.00285	0.0005	
Overall probability of exposure (direct and indirect—10 sheds on the property)				
Non-free-range meat chicken	0.0037	0.002	0.0064	$F(4,4995) = 1878.45$; <0.0001
Free-range meat chicken	0.0032	0.0018	0.0057	
Cage layer	0.0032	0.0015	0.0063	
Barn layer	0.003	0.0014	0.0058	
Free-range layer	0.0075	0.0057	0.001	

time period. Wildlife camera trapping work conducted by Scott et al. (13) demonstrated an average of 17 wild bird sightings a week. This is very likely an underestimate as the cameras did not capture the whole farm area. However, this data can be extrapolated, and it can be said that approximately 17 wild birds a week is equivalent to approximately 1,000 wild birds a year. Therefore, the number of exposures estimated in this study for 1,000 wild birds present at one point in time could indicate the cumulative expected number of exposures that can occur in one year. Accurate information of wild bird numbers can be obtained from manual wild bird farm surveys or the development of a 24/7 wild bird monitoring system on farm as was stated as a recommendation for wild waterfowl deterrence previously (38).

The Effects of Season on the Probability of LPAI Exposure

The probability of the first exposure to LPAI virus for a chicken on an Australian commercial chicken farm was found to be lowest in summer for all farm types. The highest probability was estimated to be in winter for chickens on free-range layer

TABLE 7 | Number of low pathogenic avian influenza (LPAI) virus exposures that would occur given a number of wild birds (*n*) and changes in the overall probability of LPAI exposure (*p*) with changes in the proportion of wild birds on the farm that are waterfowl and the prevalence of LPAI in waterfowl for the commercial chicken farm types (non-free-range meat chicken, free-range meat chicken, cage layer, barn layer, free-range layer) at any point in time out of 100 scenarios (or farms) using binomial distributions.

Waterfowl proportion		**Standard**			**100%**			**80%**			**100%**			**50%**			**50%**		
Waterfowl LPAI prevalence		**Standard**			**Standard**			**20%**			**10%**			**20%**			**10%**		
Farm type	**Number of wild birds**	**Median**	**5%**	**95%**	**Median**	**5%**	**95%**	**Median**	**5%**	**95%**	**Median**	**5%**	**95%**	**Median**	**5%**	**95%**	**Median**	**5%**	**95%**
Non-free-range meat chicken	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	50	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	1
	100	0	0	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1
	1,000	0	0	2	1	0	3	3	0	7	2	0	5	2	0	5	1	0	3
Free-range meat chicken	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	50	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0
	100	0	0	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1
	1,000	0	0	1	1	0	3	2	0	7	1	0	5	2	0	5	1	0	3
Cage layer	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	50	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0
	100	0	0	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1
	1,000	0	0	2	1	0	3	2	0	7	1	0	5	2	0	5	1	0	3
Barn layer	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	50	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0
	100	0	0	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1
	1,000	0	0	1	1	0	3	2	0	7	1	0	5	1	0	5	1	0	3
Free-range layer	10	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	50	0	0	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1
	100	0	0	1	0	0	1	0	0	2	0	0	2	0	0	2	0	0	1
	1,000	1	0	2	2	0	5	6	2	11	4	1	8	4	1	8	2	0	5

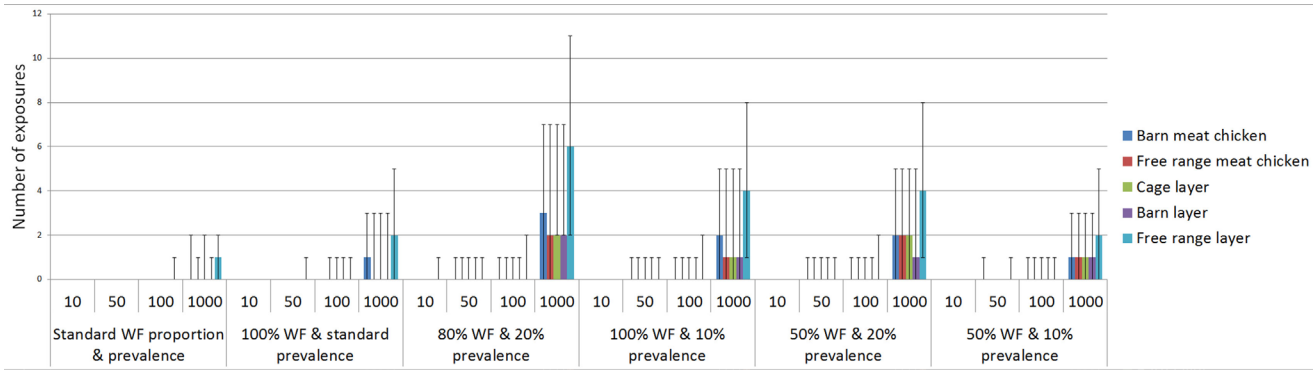

FIGURE 4 | Number of low pathogenic avian influenza (LPAI) virus exposures that would occur given a number of wild birds (*n*) and changes in the overall probability of LPAI exposure (*p*) with changes in the proportion of wild birds on the farm that are waterfowl and the prevalence of LPAI in waterfowl for the commercial poultry farm types (non-free-range meat chicken, free-range meat chicken, cage layer, barn layer, free-range layer) at any point in time out of 100 scenarios (or farms) using binomial distributions; WF, waterfowl.

TABLE 8 | Median (5 and 95 percentiles) overall probabilities of exposure (direct and indirect) of a chicken on the commercial chicken farm types (non-free-range meat chicken, free-range meat chicken, cage layer, barn layer, free-range layer) to low pathogenic avian influenza (LPAI) viruses for the first time at any point in time during the three defined seasons; winter (June–August); summer (December–February); and autumn and spring (March–May and September–November); from wild birds in Australia (specifically in the Sydney basin bioregion and South East Queensland).

Farm type	Median	5%	95%	*F*statistic (degrees of freedom); *p*-value
Winter				
Non-free-range meat chicken	0.00044	0.00024	0.00079	$F(4,4995) = 2327.39$; <0.0001
Free-range meat chicken	0.00039	0.00022	0.00068	
Cage layer	0.00038	0.00017	0.00077	
Barn layer	0.00035	0.00016	0.00070	
Free-range layer	0.00102	0.00076	0.0014	
Summer				
Non-free-range meat chicken	0.00019	0.00010	0.00034	$F(4,4995) = 403.78$; <0.0001
Free-range meat chicken	0.00018	9.06×10^{-5}	0.00035	
Cage layer	0.00018	8.09×10^{-5}	0.00036	
Barn layer	0.00017	7.56×10^{-5}	0.00033	
Free-range layer	0.00030	0.00020	0.00049	
Autumn/Spring				
Non-free-range meat chicken	0.00046	0.00026	0.00082	$F(4,4995) = 1525.98$; <0.0001
Free-range meat chicken	0.00039	0.00023	0.00069	
Cage layer	0.00040	0.00018	0.00079	
Barn layer	0.00036	0.00017	0.00072	
Free-range layer	0.00093	0.00069	0.0012	

farms and autumn/spring for the rest of the farms, except for free-range meat chicken farms which reported no difference between winter and autumn/spring. However, there were minor differences in the probabilities of exposure for all farm types between winter and autumn/spring overall. Among previous HPAI outbreaks in Australia, one occurred in winter (July), four in autumn and spring (May, October, and November), and two in summer (December and January). The three latest outbreaks that occurred in Tamworth (1997), Maitland (2012), and Young (2013) occurred in October or November (4, 39, 40). The mechanisms of mutation from LPAI to HPAI are poorly understood and difficult to predict. In some overseas outbreaks, LPAI viruses have been detected in domestic poultry weeks or months prior to the subsequent HPAI virus outbreaks (41). It could be speculated for the Australian HPAI outbreaks that occurred in summer, when the probability of LPAI exposure is estimated to be lowest compared to the other seasons, which introduction of the virus occurred during spring, the virus then circulated within the flock for months and mutation subsequently occurring in summer (42). On the other hand, Fusaro et al. (43) demonstrated that some H7 LPAI subtypes detected in Italy can mutate quickly in order to adapt to the new host species.

The seasonal variations in the probability of exposure are influenced by the wild bird LPAI prevalence data and the guidelines on outside weather conditions that determine whether or not chickens are provided access to the range. The overall prevalence of LPAI in Australian wild waterfowl at any point in time is approximately 2.5%. Seasonal effects on the prevalence of LPAI in wild birds within NSW do not appear to fluctuate as greatly as in the northern hemisphere (17). There is evidence to suggest that the fluctuation of wild bird LPAI prevalence in Australia is more dependent on rainfall patterns and bird movements, abundance and breeding particularly in Australian waterfowl (44, 45). In the northern hemisphere, there is generally a low prevalence of LPAI in winter, an increase in viral prevalence in summer, followed by a peak in prevalence in autumn (46, 47). This contrasts with NSW data which reveals a high prevalence of LPAI in winter and autumn/spring and a low prevalence in summer (17). In the northern hemisphere, the increased prevalence in summer is thought to be due to the progressive influx of immunonaïve juvenile waterfowl to the population, following breeding in spring (48). In Australia, the breeding seasons and movements

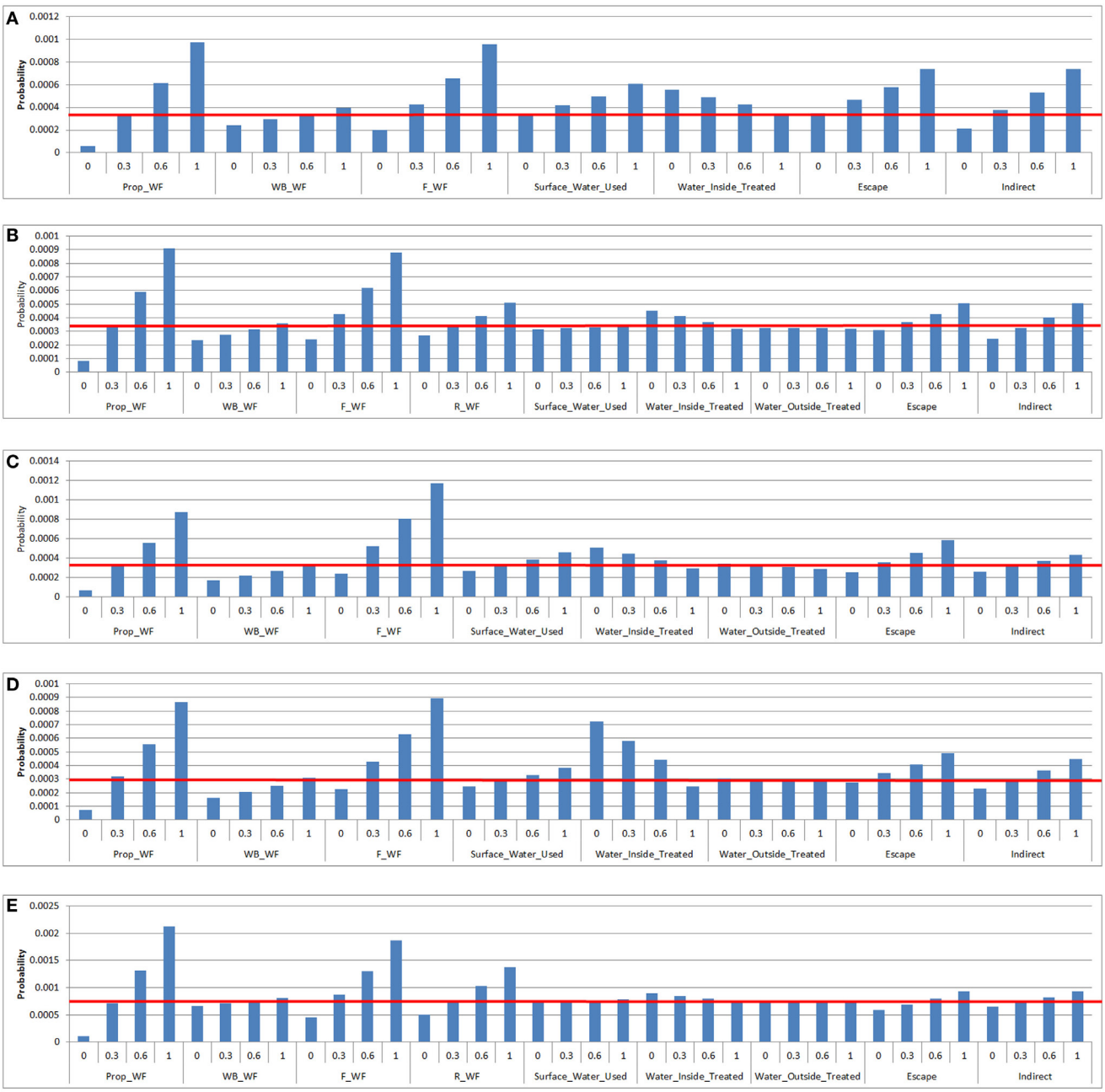

FIGURE 5 | Results of the sensitivity analysis on the exposure assessment depicting the change in probability (*Y*-axis) on the median overall probability of exposure (horizontal line) of a chicken on a commercial chicken farm to low pathogenic avian influenza (LPAI) virus from wild birds in Australia with changes of certain input variables listed in **Tables 4** and **5** (*X*-axis). Results were obtained from a simulation of 1,000 iterations using @Risk's Advanced Sensitivity Analysis. [**(A)** = non-free-range meat chicken; **(B)** = free-range meat chicken; **(C)** = cage layer; **(D)** = barn layer; **(E)** = free-range layer]; Prop_WF, proportion of waterfowl reported on property, WB_WF, proportion of responses that witnessed waterfowl in waterbodies on/near the farm, F_WF, proportion of responses that witnessed waterfowl in feed storage areas, R_WF, proportion of responses that witnessed waterfowl on the range, Surface_Water_Used, proportion of responses that use surface water for the chicken farm, Water_Inside_Treated, proportion of responses that treat water used inside sheds, Water_Outside_Treated, proportion of responses that treat water used outside sheds, Escape, proportion of farms that reported chickens escaping from shed [and from range for **(B)** and **(E)**], Indirect, probability of the occurrence of other indirect methods that can introduce LPAI (boots, mice/rats, insects, farm cats, or dogs).

of waterfowl are less predictable; many populations are nomadic, which contrasts with the waterfowl populations in the northern hemisphere which are well known for their annual migrations over long distances. Movements and breeding of Australian waterfowl are instead largely determined by the distribution of surface water and rainfall (49, 50). A high

prevalence of LPAI may occur during periods of waterfowl congregation, such as during droughts. A particular example that supports this point is the 1994 H7N3 HPAI outbreak that occurred in Queensland, Australia, which took place during a period of severe drought. Water used for the farm was drawn from a river on the periphery of the farm and had attracted a large population of wild birds. This likely greatly increased the probability of LPAI exposure to the farm and lead to the HPAI outbreak (4).

Birds in the families Scolopacidae and Charadriidae (shorebirds and waders) do undergo annual migrations over long distances and visit Australasia (49). In the northern hemisphere, the arrival of migrant birds to the resident population coincides with the peak LPAI prevalence in autumn. Migrating birds may be more susceptible to infection from long distance flights and/or relatively low immune resistance to locally circulating LPAI strains compared to resident birds (48). These shorebirds are more likely to become infected with local Australian LPAI subtypes rather than bring exotic strains of the virus into Australia; the probability of the latter occurring was previously estimated to be extremely low (51).

Conclusion

There are still many uncertainties related to the mechanisms of the LPAI virus introduction and exposure, particularly in Australian commercial chicken farm settings. However, the results of this study have used the best data available at this time. The results suggest that chickens on commercial free-range layer farms have approximately double the risk of LPAI exposure compared to other farm types. The probability of direct exposure is also more likely in both free-range layer and meat chicken farms compared to the other farm types. Moreover, the probability of LPAI exposure seems to be lower in summer compared to all other seasons and this is influenced by the prevalence of LPAI in wild birds and the weather conditions in which free-range chickens are allowed to go on the range. The proportion of waterfowl on the farm and the presence of waterfowl on the range and feed storage areas are the most influential parameters on the probability of exposure. These results highlight the importance of good biosecurity on farms, providing insight regarding the on-farm actions that can reduce the risk of LPAI exposure such as those related to waterfowl deterrence. In addition, the importance of continuous surveillance of Australian wild bird populations to monitor LPAI prevalence and subtypes is highlighted, as this can help predict future introductions and outbreaks. The need of further research in AI virus properties, particularly in an Australian context is also highlighted.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Human Ethics Committee of the University of Sydney, Australia with written informed consent from all subjects. The protocol was approved by the Human Ethics Committee of the University of Sydney, Australia (ethics reference number: 2015/252).

AUTHOR CONTRIBUTIONS

The first author ABS was involved in investigation, methodology, writing of the original draft, and reviewing and editing. J-AT formed initial conceptualization of the study, and was involved in formal analysis, methodology, project administration, supervision of ABS, and reviewing and editing the manuscript. MS was also involved in investigation, methodology, project administration, supervision of ABS, and reviewing and editing the manuscript. BB and KG were also involved in initial conceptualization of the study, formal analysis, methodology, and provided reviewing and editing. BM, AB, and PG were involved in conceptualization and project administration funding/support of the study. PG also provided methodology and supervision. BM provided reviewing and editing. MH-J was heavily involved in formal analysis of the results, conceptualization of the study, methodology, project administration, supervision of ABS, and reviewing and editing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fvets.2018.00068/full#supplementary-material.

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Linking Supply Chain Governance and Biosecurity in the Context of HPAI Control in Western Java: A Value Chain Perspective

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Despite extensive efforts to control the highly pathogenic avian influenza (HPAI), it remains endemic in Western Java, Indonesia. To understand the limited effectiveness of HPAI control measures, it is important to map the complex structure of the poultry sector. The governance of the poultry value chain in particular, could play a pivotal role, yet there is limited information on the different chain governance structures and their impacts on HPAI control. This article uses value chain analysis (VCA), focusing on an in-depth assessment of governance structures as well as transaction cost economics and quantitative estimates of the market power of different chain actors, to establish a theoretical framework to examine biosecurity and HPAI control in the Western Java poultry chain. During the research, semi-structured interviews were conducted with key value-chain stakeholders, and the economic performance of identified actors was estimated. Results indicated the co-existence of four different poultry value chains in West Java: the integrator chain, the semi-automated slaughterhouse chain, the controlled slaughter-point chain, and the private slaughter-point chain. The integrator chain was characterized by the highest levels of coordination and a tight, hierarchical governance. In contrast, the other three types of value chains were less coordinated. The market power of the different actors within the four value chains also differed. In more integrated chains, slaughterhouses held considerable market power, while in more informal value chains, market power was in the hands of traders. The economic effects of HPAI and biosecurity measures also varied for the identified actors in the different value chains. Implementation of biosecurity and HPAI control measures was strongly related to the governance structure of the chain, with interactions between different chains and governance structures accentuating the risk of HPAI. Our findings highlight that a proper understanding of the chain governance structure is vital to improve the effectiveness of HPAI control measures, by making the interventions more specific and fit-for-purpose given the incentive structures present in different chains.

Keywords: value chain analysis, HPAI, chain governance, diversity of transactions, transaction cost economics, biosecurity

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INTRODUCTION

Highly pathogenic avian influenza (HPAI) H5N1 is an important endemic disease in Indonesia (1, 2). HPAI outbreaks negatively affect public health but also food safety, social wellbeing and the broader economy. HPAI has been difficult to control in Indonesia for a variety of reasons. These include the limited capacity for pre-requisite programs that address HPAI prevention, limited disease surveillance activities, and low levels of public health regulation. Likewise, the Indonesian government has had difficulties implementing its planned HPAI control programs. For instance, vaccination at the farm level has not been effectively implemented (3), while surveillance activities for HPAI through the Participatory Disease Surveillance and Response program have had only limited success (4, 5). There were many cases of under-reporting of HPAI due to farmer fears for mandatory culling without proper compensation (6). Efforts to apply biosecurity measures in both the small-scale commercial (termed "sector 3" by the Food and Agriculture Organization (FAO) of the United Nations) and backyard (sector 4) poultry farms were largely unsuccessful. The proximity and mutual interaction of both types of smallholder poultry systems often reinforce disease dynamics and perpetuate recurrent "infection cycles" of HPAI (6).

An important underlying reason for the failure of HPAI control programs in Indonesia lies in the organizational and institutional structure of the poultry sector (7). The structure of rearing and selling poultry comprises all activities and interactions from farmer to consumer, and in Indonesia, that structure is complex with multiple links and interactions. Although the Indonesian poultry sector consists of a number of different value chains, there is a noteworthy lack of understanding of the structure of the existing value chains, the nature of value chain links and interactions, and how the poultry sector structure affects efforts to control HPAI. As noted by Rich and Perry (8), "weak" links in the chain can compromise control efforts at other stages, and as such, it is crucial to identify the incentives and pressures that drive these actors to work in "sub-optimal" ways from a disease control standpoint (even if economically rational). Therefore, understanding poultry value chain structures and their influence on HPAI control is important to develop incentives that drive chain actors to implement control measures. This can be achieved by employing value chain analysis (VCA) to analyze the marketing and governance structure of value chains.

An often overlooked aspect of the value chain is its governance structure, defined as the mechanisms that drive the coordination of transactions between actors. Value chains can be tightly governed through contracts or vertical integration where demands for quality or other product attributes are necessary. By contrast, transactions in traditional chains are simply governed by price and availability. Insight into the governance structure further reveals the power relations, which can be expressed in terms of diversity of transactions. When transactions are coordinated by a dominant chain actor, the ability of, or incentives for certain actors to comply with disease control will be affected.

Given our interest in linking VCA results to the control of HPAI, we used transaction cost economics (TCE) to relate governance to biosecurity practices (9). Which type of governance minimizes transaction costs depends on the relationship-specific investments (asset specificity) (9). Investments in biosecurity are one form of asset-specificity. In the case of HPAI control, these investments can be seen as risk mitigation practices that bind partners into tighter forms of coordination and improve incentives to control disease. In Indonesia, biosecurity investments and practices vary across different forms of value chain governance. Differences in biosecurity practices cause different risks of HPAI incursion within and between poultry chains. Moreover, where multiple types of value chain governance co-exist, there could be a variety of market and governance failures that spill over across different chains, driving the endemicity of HPAI. Since dominant actors may have a more significant role in the control of HPAI, we need to identify those actors that govern the chain. One approach to identify the dominant actor is by evaluating the chains' economic performance and the distribution of profits over the various actors within the value chain (10). A proper understanding of the poultry value chain and its governance is vital to drive improved adoption of HPAI control strategies of different value chain stakeholders (8).

Research applying VCA in the context of animal diseases has emphasized the importance of the value chain perspective to evaluate livestock disease management strategies. VCA provides information on the flow of materials, resources, commodities, and value-adding activities between the different parts of the value chain (e.g., (7, 11–15). In the context of HPAI in Indonesia, research adopting a value chain perspective has been limited. Existing literature includes study chronicling the HPAI situation on Java (16); a case study of HPAI in Bogor (17); a qualitative risk assessment of HPAI (6); a study examining the alignment of poultry sector actors with avian influenza control in Indonesia (18); and a study identifying risk factors of HPAI (5). These research outputs from the International Livestock Research Institute (ILRI) and the FAO highlight the complexity of different poultry value chains in Indonesia, but do not provide an in-depth assessment of governance structures or the diversity of transactions with respect to HPAI control. Sudarman et al. (17) come closest in this regard, but their focus is more holistic, zooming on the chain rather than on governance as such.

The objective of this study is to assess the complexity of poultry value chain structures and their influence on HPAI control in Western Java, paying particular attention to the relationship between value chain structures, actors, governance, and economic performance. The study focuses on relations across different types of actors and does not explore the horizontal links within different chain nodes or public governance. The study provides an in-depth discussion of the poultry chain that explains critical control points for HPAI and where policy can more effectively intervene taking the complexity of the marketing chain into account. More detailed information about governance and transaction diversity in Western Java will improve our understanding of the poultry value chain, and the role governance plays in shaping economic motivations and behavior of value chain actors. Thus, such information can be used to incentivize all actors to participate in fit-for-purpose HPAI control strategies in Western Java.

ANALYTICAL AND THEORETICAL FRAMEWORK

To understand the diversity of transactions and governance structures of the poultry value chain, we used three complementary approaches. We first performed a value chain analysis (VCA), following Kaplinsky and Morris(2001), and applied it in an animal health context as in Rich and Wanyoike(11). This was followed by an analysis where we linked governance typologies to biosecurity practices (9, 10, 19). Finally, from the first two approaches, we derived quantitative estimates of economic performance (10). The results from these three approaches were combined to assess the risk factors of HPAI introduction and transmission, and the consequences of HPAI in the absence of government intervention.

First, VCA was used to construct the network of input-output relationships of the poultry supply chain. VCA tools allow practitioners to create a value chain map for the traditional and modern channels describing the actors and the nature of value chain governance structures. Value-chains represent the various processes involved in producing goods in the supply chain based on the notion of value-added at the production level. Once a value chain map has been identified, other approaches can be used together with VCA to obtain more insight into the poultry chain.

Second, governance structures were classified through the typology of Gereffi et al. (19). This typology illustrates the diversity of transactions triggered by the dominant actor's needs, shifting the degree of coordination, the capabilities in the supply base, the ability to codify transactions and the complexity of transactions in the value chain. In this typology, Gereffi et al. (19) identified five types of governance structures based on the degree of transaction coordination between value chain actors. The most loosely coordinated mode of governance is through markets, i.e., on the basis of price and availability. A modular form of governance involves customization of a product by a seller to a buyer without any other form of explicit coordination. Relational governance involves transactions facilitated through specific relationships and mutual dependence between buyers and sellers (e.g., family ties). Captive governance typically involves the direct coordination of transactions by the buyer through contracts and the provision of inputs and technical support. Captive governance is often required when product specifications are exacting, necessitating tighter control of transactions by the buyer to ensure quality control. Finally, vertical integration involves transactions taking place solely within one organization or firm to ensure compliance with internal processes, rather than taking the risk of working with independent suppliers.

Using insights from TCE, we identified how different types of value chain governance patterns influence biosecurity practices. TCE helps to justify the rationale associated with different types of coordination (governance) mechanisms (20). The underlying assumption of the TCE approach is that the actors will choose the governance form that minimizes transaction costs. Three aspects of transaction cost underpin these decisions: the level of asset specificity, the level of uncertainty, and the frequency of transaction. Asset specificity refers to the degree of relationship-specific investments made by two parties to facilitate their transactions. Investments that are highly specific are unlikely to be productively re-used for other purposes, serving to bind actors more closely together. In such cases, tighter forms of coordination, such as contracts or vertical integration are required to protect those investments. Similarly, as the level of uncertainty (risk) and the transaction frequency (e.g., the intensity of exchange, number of times the same transactions take place) increase, greater coordination and tighter governance structures may be necessary. We posit that different types of biosecurity practices in different chains may be influenced by the coordination mechanisms associated with the governance structure of the value chain.

Third, we estimated economic performance via VCA to quantify the value added for each channel. Kaplinsky and Morris (10) define power as the ability of one party "to force other parties to take particular actions" or "to be deaf to demands of others". Our power estimation used the value chain structure to estimate chain conduct in terms of price and quantity decisions. The estimated profits and the profitability were used as a measure of economic performance. Economic performance is an essential parameter to understand the pattern of returns as part of distributional outcomes in the value chain, showing the added value (output value minus input costs) for each link of the chain (10). The share of chain value added can be an indicator of a firm's power, but qualitative indicators can be more relevant. Chain actors with a relatively high economic performance (profitability) can be seen as actors with a relatively high market power. They are able to exploit high prices and/or create barriers to entry (21). Knowledge about the share of chain value added can support other indicators that analyze power asymmetries such as the market structures (the number of buyers versus the number of sellers), the degree of dependence between buyers and sellers, and the characterization of the governance structures.

Finally, we assessed the risk factors of HPAI introduction and transmission and the consequences of failure to control HPAI in the chain. We looked at the enabling conditions generated under the different forms of value chain governance. Four factors can be used to identify the risk of HPAI introduction and transmission in relation with the value chain map, governance structure and the implementation of biosecurity: (1) the number of actors involved (22), (2) the frequency of contacts with a possible source (22, 23), (3) the number of links within the chain stages (13) and (4) the contact structure in the poultry chain (5, 13, 14, 22, 23). These four factors can be assessed based on the value chain map, the governance typologies present in each chain, and how they relate to the biosecurity practices in place.

MATERIALS AND METHODS

Table 1 shows the relation between the theoretical framework and the data collection process, and provides details on the specific actors interviewed during the study. We carried out three workshops, seven site visits and 26 in-depth interviews with several key value chain stakeholders, to assess the governance and biosecurity practices in the different identified poultry value chains. The data collected during the early phases of our research were validated in later steps. This enabled us to make a thorough assessment of governance in the poultry value chain,

TABLE 1 | Data collection and respondents.

Approaches	Steps	Data Collected	Interviewed Actors
Value Chain Map	Workshop 1 (focus group discussions) in December 2013	• Actors • Production systems • Input, output, cost, price	• 4 high-level representatives of large integrated companies, • the chairman of slaughter house association representative (ARPHUIN)
	Workshop 2 (focus group discussions) in December 2013	• Actors • Production systems • Input, output, cost, price	• 2 representatives of a small semi-automated slaughterhouse in Bogor • the chairman of the union of farmer association (GOPAN)
Value chain governance typology, TCE	Site visits in December 2013	• Actors • Production systems • Biosecurity • Chain governance • TCE	• 1 poultry farm, • 1 collecting farm, • 1 integrator slaughterhouse, • 2 semi-automated slaughterhouses, • 1 slaughter-point/wet market, • 1 specialty store
	In-depth interviews 1 in January 2014	• Actors • Production systems • Biosecurity practices • Chain governance • TCE	• 2 representatives of the banking sectors, • 2 government officials, • 2 representatives of farmer associations, • 1 representative of traders • 1 representative of a traditional private slaughter-point associations • 1 integrator slaughterhouse, • 2 semi-automated slaughterhouses
Value chain governance typology, TCE (validation)	In-depth interviews 2 in January 2014	• Actors • Production systems • Biosecurity practices • Chain governance • TCE	• the Chairman of the Poultry Farmer Association (PINSAR) • the Chairman of the Federation of the Indonesian Poultry Society (FMPI). • 1 representative of academia
	In-depth interviews 3 in September to November 2015	• Actors • Production systems • Biosecurity practices • Chain governance • TCE	• 1 representative of the banking sectors, • 2 government officials, • 2 representatives of farmer associations, • 1 representative of a traditional private slaughter-point associations • 1 integrator slaughterhouse, • 3 semi-automated slaughterhouses • 2 specialty stores
Value chain economic performance (quantitative estimates of the market power)	Workshop 3 (focus group discussions) in in March 2015	• Actors • Production systems • Inputs per stage • Outputs per stage • Costs per stage • Prices per stage • Simulations	• 2 consultants • 4 government officials • 3 semi-automated slaughterhouses • 1 representative automated slaughter house • 1 representative of Farmer Associations (PINSAR) • 1 representative of the union of farmer association (GOPAN)

as compared to more conventional VCA studies. The interviews were based on semi-structured questionnaires. We specified the questions for the typology according to Gereffi et al. (19) on the degree of coordination, the capabilities in the supply base, the ability to codify transactions and the complexity of transactions. Questions regarding TCE were aimed at three aspects: the level of asset specificity, the level of uncertainty, and the frequency of transactions within each chain. We interviewed the respondents about biosecurity practices based on the FAO poultry biosecurity guidelines.

The different workshops also provided information about (1) actor roles in coordination mechanisms such as the setting of product and process standards(for biosecurity and food safety); (2) the monitoring of performance, environmental standards, labor standards and conformance to ISO and HACCP standards; and (3) the different roles of actors in the implementation of sanctions whenever the performance of other actors within their chain does not meet the pre-specified requirements.

The key value chain stakeholders interviewed in this study were the Federation of the Indonesian Poultry Society (FMPI) (the only organization uniting all poultry actors in the region), the slaughter-house association (ARPHUIN) that represents the modern chain, the Union of Farmers Association (GOPAN) in Indonesia, the Poultry Farmers Association (PINSAR) representing the major

group of farmers in Indonesia, the traditional private slaughter-point associations, and two government agencies (the agricultural agency and a regional office). We also interviewed other actors such as consultants and representatives from meat-specialty stores, the banking sector and academia.

The data were processed in five steps. First, the value chain map was drawn and completed with the number of actors. Subsequently, the map was classified based on the governance typology. Third, we calculated the economic performance of the governance structure using quantitative estimates. Fourth, we linked details on the value chain governance structure using TCE and the assessment of biosecurity practices. Lastly, we linked the governance structure with the economic consequences of HPAI in the absence of government intervention.

Quantitative estimates of the market power of different chain actors were based on enterprise budgets for each chain actor group by estimating costs of input, returns, and added value.

1. The output of Western Java poultry production was estimated based on the situation in 2013 using secondary data from the Agricultural Census (24). Total farm output was based on the number of broilers in three provinces: West Java, Banten, and DKI Jakarta.
2. The total farm output of Western Java was divided over the traditional and the modern channels. Since no exact information on the distribution of output over the chains was available, we made an estimation based on the focus group discussions and interviews. We assumed that farms in sectors 1 (industrial and integrated farms) and 2 (commercial poultry production with high biosecurity farms) served the modern channel and that farms in sectors 3 (commercial poultry production with low biosecurity farms) and 4 (village or backyard poultry farms) served the traditional channel. The output of these four farm types was distributed over the slaughterhouses and collecting points in their respective value chains.
3. For each actor group in the identified value chains, we calculated output in kilograms of poultry products based on the available knowledge on production size. Since weight was used as the unit of output, farm output was measured in terms of weight of delivered poultry, while slaughterhouse output depended on the carcass weight
4. For each actor, we calculated fixed costs, variable costs, and added value based on the situation in 2013, using secondary data from the Agricultural Census (24).
5. Revenues were calculated as the output of products multiplied by the product market price (average yearly price in 2013).
6. Finally, for each actor group, we calculated the profitability per chain stage (based on a cycle of production activity for farmers, and on a day of selling and production activities for collecting farms and slaughterhouses) by subtracting the costs from the returns. A cycle of production activity for a farmer refers to the growth cycle of poultry from day 1 until harvest.
7. All calculations were made in Indonesian Rupiah and then converted into Euro using the December 2013 exchange rate.
8. The results are presented as a comparison of total profit margin relative to the total turnover in a given chain. The total turnover was defined as the total sales revenue.

RESULTS

Mapping the Poultry Value Chain

The analysis revealed two main marketing channels for poultry in West Java, which are illustrated in **Figure 1**. These channels were classified as the modern and traditional channels only serving the domestic market. The two marketing channels provide poultry meats with different characteristics. The modern channel produces cooled and frozen poultry meat, while the traditional channel produces freshly cut poultry meat without refrigeration or freezing. Therefore, these channels attracted different consumers with different preferences for poultry meat. Within these two channels, four specific chains could be distinguished: the integrator chain and the semi-automated slaughterhouse chain in the modern channel, and the controlled slaughter-point chain and the private slaughter-point chain in the traditional channel. **Figure 1** illustrates the production and financial flows of the four different chains, and identifies the different links within and between the different value chains. The production flows are represented by the downward arrows, while the financial flows are represented by the dashed upward arrows. Stakeholders were characterized as internal or external actors, based on their involvement in the physical transport in the production flows. All stakeholders had both a direct and an indirect influence on the poultry transactions.

Most actors in the Western Java poultry chain were internal chain actors who are physically involved in the meat production and distribution, such as farms, collecting farms, transporters, slaughterhouses, slaughter-points, food processors and retails. These actors differed in number (**Figure 1**) and in their production characteristics. They were involved in transporting live birds and carcasses, using different transportation modes to end consumers. Live birds were produced at farms, and the mode of production depended on the farming system (sectors 1–4). The live birds from sector 1–2 that go to the modern channel were transported directly to the slaughterhouses, while the live birds from sector 2–4 that go to the traditional channel were transported through collecting farms. We noticed a relationship between sector 2 farms from the modern channel and the collecting farms from the traditional channel. Transport tools were owned by both slaughterhouses and collecting farms. Collecting farms are poultry shelters where live birds are brought together and sold. There are two types of collecting farms: controlled collecting farms and private collecting farms. The controlled collecting farms operate in a centralized government area, set up by the government to control the spread of HPAI. The government relocated many private collecting farms to a location owned by the government in order to control live bird movements. By contrast, private collecting farms operate in private locations or through home slaughtering. The average weight of live birds was 2.15 kg for sector 1, 1.5 kg for sector 2 and 1.3 kg for sectors 3 and 4. The average carcass weight by sector was 1.46 kg for sector 1, 1.13 kg for sector 2 and 0.98 kg for sectors 3 and 4.

Live birds were collected and processed in a slaughterhouse (automated or semi-automated) or slaughter-point (manual process), after which they were sold on the market. Live birds from private collecting farms that were to be slaughtered in private slaughter-points were transported by motorcycle. The transporters were informal actors, working part-time

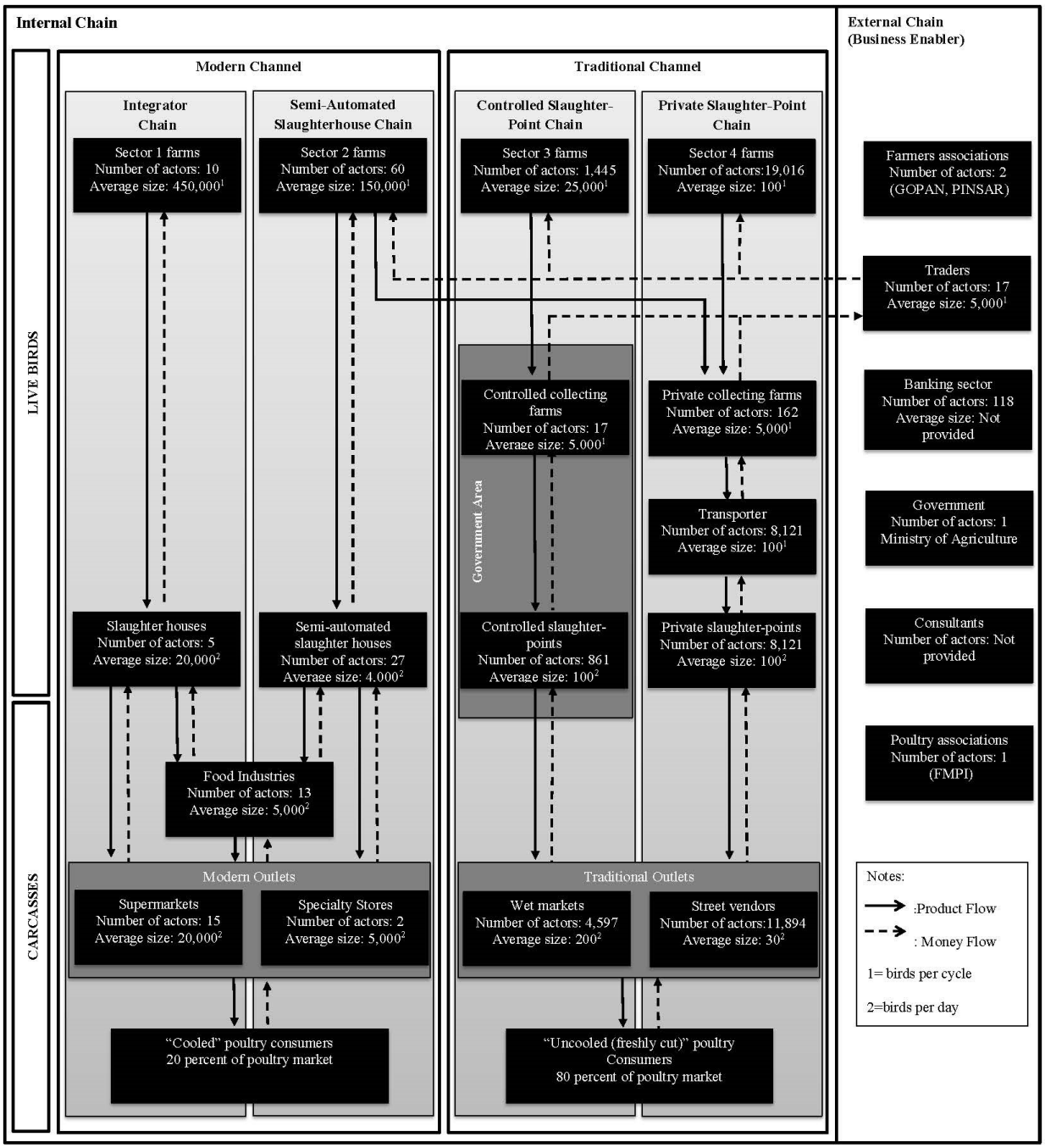

FIGURE 1 | Mapping and approximate number and size (in birds per chain stage) of the actors poultry value chain in Western Java.

and receiving fees from the private slaughter points for their services. There were four types of slaughterhouse systems: the integrator slaughterhouse, the semi-automated slaughterhouse, the controlled slaughter-points, and the private slaughter-points. The integrator slaughterhouses consists of slaughter plants with modern equipment and holding HACCP, ISO, and state (NKV) certificates. The slaughter process at semi-automated slaughterhouses involves automated general stunning (water bath) and plucking, and transportation in shackles, but with all other work in the plant conducted manually. At controlled slaughter-points an actor that bought poultry from the controlled collecting farms, slaughters it in a centralized government area.

Private slaughter-points are private houses in front of which workers slaughter poultry.

The total output was distributed in accordance to the focus group discussion results. The total output from sector 1 was distributed to the integrator chain. We assumed that the excess supply from sector 2 was distributed over the two chains in the traditional channel. Therefore, the higher quality output from sector 2 was distributed to the automated slaughterhouse (50%), while the lower quality was distributed to the controlled slaughter-point chain (5%), and the private slaughter-point chain (45%). Next, the output from sector 3 was distributed to the controlled slaughter-point chain (10%), and the private slaughter-point chain (90%). The total output from sector 4 was distributed to the private slaughter-point chain.

From the slaughterhouses, poultry meat was transported and sold to food processors, modern outlets such as supermarkets, and meat specialty stores. These outlets applied a cold chain and adhered to specific quality standards. Poultry meat from slaughter-points, however, was transported and sold through traditional channel outlets, such as wet markets and street vendors. These outlets sold fresh poultry meat using a temporary structure or mobile stall.

The transaction product flows in the internal chain differed across the modern and traditional chains. In the modern channel, the transactions were coordinated with rules and standards, while the traditional channel engaged in on-the-spot transactions, with low entry barriers but asymmetries in information among actors.

We identified a number of external actors that played a role in the value chain as business enablers, but were not necessarily physically involved in the production or distribution of poultry meat. One important example are traders at live bird markets. Traders are the individual actors between farmers and collectors. They play a critical intermediary role in terms of providing informal financial support in liaising transactions between farmers and collectors, and secondly they act as brokers matching farmers and collectors. Traders provide farmers with cash payments, and receive payments from collecting farms. This role started after the banking sector left the small and medium scale poultry business without support during the economic crisis of 1997. Transactions were based on the daily spot market, and there were no formal contracts or informal relations between traders and other actors. The banking sector provides business services such as the holding of financial assets and financial services for large companies, but far fewer services for farmers. There was no direct involvement from the banking sector to support investments to control HPAI. A number of organizations worked together with the government to address HPAI. PINSAR and GOPAN are the poultry farmer associations that advocate and support farmers, while ARPHUIN is the slaughterhouse union. FMPI is a poultry federation that facilitates communication and advocates for the poultry business on behalf of all poultry actors. The government plays a role in the food safety system to control the transmission of HPAI in the poultry sector production and market. Independent consultancy companies also played a role in the system through the provision of expert advice on the poultry business or on food safety in the modern channel, for example regarding ISO standards and HACCP certification.

TABLE 2 | Types of value chain governance in the poultry meat value chain of West Java.

Chain governance structure determinants (Diversity of transactions Criteria)	Modern channel		Traditional channel	
	Integrator chain	Semi-automated slaughterhouse chain	Controlled slaughter-point chain	Private slaughter-point chain
	Hierarchy	*Modular*	*Market*	*Market*
• *Degree of coordination*	High	Low	Low	Low
• *Capabilities in the supply base*	Low	High	High	High
• *Ability to codify transactions*	Low	High	High	High
• *Complexity of transactions*	High	High	Low	Low

Governance Structures in the Poultry Value Chain

We found a wide range of governance structures in the different poultry value chains. Based on the typology of Gereffi et al. (19), we observed the presence of a hierarchy type governance in the integrator chain, modular governance in the semi-automated slaughterhouse chains, and market governance in the controlled slaughter-point and private slaughter-point chains. The other two typologies, the relational and captive governance structure, were not identified in these chains (**Table 2**).

In the hierarchical form of governance in the integrator chain, slaughterhouses acted as the lead firms with explicit coordination of the other actors in the chain. This chain was vertically integrated, employing full managerial control to produce products in-house. The level of coordination between actors was high because of the complexity in the requirements for meat quality, including standards for cold and frozen products, size/weight, biosecurity, halal certification, NKV certification, HACCP certification, and ISO certification. Only NKV and HACCP certification standards induced the slaughterhouse as the leader of the chain to control HPAI. These certificates are required for doing business in this chain. Prices and volumes were arranged via material requirement planning to ensure timely supply.

The modular form of governance was found in the semi-automated slaughterhouse chain, where suppliers had a responsibility to make products or provide services to meet customer expectations. For instance, farmers needed to meet buyer requirements with regard to size, weight and on-farm biosecurity (e.g., isolation, traffic control and sanitation), and the semi-automated slaughterhouses had to provide a product specified by the retailers. In this chain, no private or public standards induced the slaughterhouse as the leader of the chain to control HPAI. A form of contract was used, but the buyer-supplier interactions were limited to the delivery specifications and prices and not via specific, long-term coordination. The traditional channels were characterized by market governance. In these two value chains, transactions were relatively simple, with no formal cooperation between actors. These channels had a low mutual

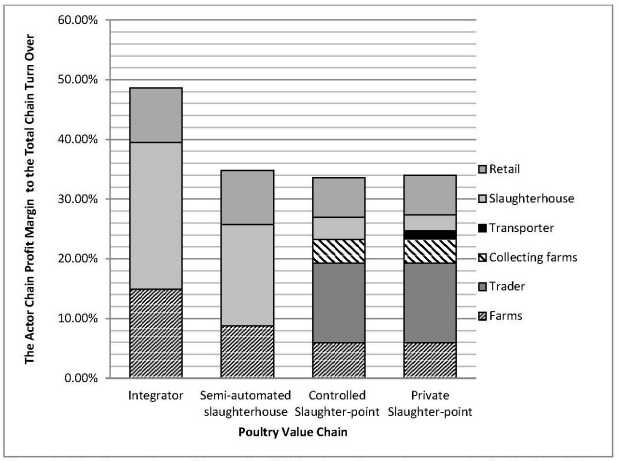

FIGURE 2 | The Comparison of Actors Profit Margin to the total turnover in the Poultry Value Chain. The graphic bars represent the joint profit margin contributed bv each actor groups to total turnover (total sales revenues) in different chain governance. Each block in the graphic bars represents each actor group profit margin to the total turnover.

dependence related to reputation, or family and ethnic ties between actors in vertical chain stages. The buyers provided suppliers with limited or no information about product specifications. We found diseased poultry was sold in these chains, therefore we labeled them as a "sick" poultry market. Traders had a relatively larger role coordinating the chain as external actors.

The Economic Performance of the Poultry Value Chain in West Java

As illustrated in **Figure 2**, we computed economic performance at chain level as the share of the total profit margin relative to the total turnover in a given chain. We found that the integrator chain had the highest economic performance, because the share of the total profit margin relative to the total turnover was the highest (**Figure 2**). The other three chains had similar but lower profit margins.

If we look at the distribution of profit within and across the different groups, a number of interesting results emerge. In the modern channels, slaughterhouses had a higher share of the profit margin than farmers and retailers. By contrast, in the more traditional channels, the total profit margin was distributed over more actors, with the largest share captured by the traders. The comparison of actor profit margins within the different chains may illustrate the power of a specific chain actor. In this case, the slaughterhouse seemed to have the highest power in modern channels, while in traditional channels, the highest power was held by traders. Consequently, those who had market power were acting as the chain leader and had the largest influence on chain governance. Indeed, the presence of only a handful of traders compared to the significantly larger number of other actors (**Figure 1**), suggests a form of oligopolistic power held by traders in the traditional channel. The ability of slaughterhouses and traders to drive the value chain is the key determinant to impose biosecurity standards and control HPAI in all forms of chain governance.

TABLE 3 | Biosecurity practices and governance forms in the poultry value chains of West Java.

Chain Governance Structure determinants (TCE Criteria)	Modern Channel		Traditional Channel	
	Integrator Chain	Semi-automated Slaughterhouse Chain	Controlled Slaughter-point Chain	Private Slaughter-point Chain
	Hierarchy	*Modular*	*Market*	*Market*
• *Level of Asset Specificity*	High	Medium	Low	Low
• *Level of Uncertainty*	Low	High	High	High
• *Transaction frequency*	Low	High	High	High
Biosecurity Practices	*High*	*Medium to low*	*Low*	*Low*

Biosecurity

We looked at the role of chain governance in the application of biosecurity practices for the four different value chains we identified. We assumed that differences in chain governance influence the risk of HPAI transmission (13). In this context, we took a transaction cost economics approach to test our hypothesis as to whether more coordinated chains lead to more investments in biosecurity practices. We differentiated two aspects of biosecurity: (1) the risk of disease introduction, and (2) the risk of disease transmission. The risk of HPAI introduction is the likelihood that the virus enters the value chain, for instance from another value chain. The risk of HPAI transmission is the likelihood of HPAI being transmitted within the value chain, for instance from one stage to the next after introduction of the virus.

Table 3 summarizes the biosecurity practices for each value chain type. While three transaction characteristics were observed, the level of asset specificity was most strongly related to the application of biosecurity practices. The other characteristics of transactions, uncertainty and frequency, were not considered by the actors as drivers for the application of biosecurity measures. Inciting suppliers to adopt biosecurity practices could be a way to mitigate uncertainty in the supply chain. Strong hierarchies and tight coordination amongst actors within the integrator chain facilitated a variety of specific investments, including those on biosecurity. These included maintaining biosecurity through a compartment system at the farm level (among section 1 farms), while slaughterhouses had stringent quality processes, which were HACCP, ISO and NKV certified. In a well-coordinated value chain, such as for the hierarchical form of governance, it was easier to implement and maintain biosecurity practices and, therefore, well-coordinated chains were better protected against HPAI introduction and transmission.

Asset specific investments in the semi-automated slaughterhouse chain (modular governance) were lower than in the integrator chain, because a form of contract (limited to price and weight specifications with general disease status) was used to

support transactions within this chain. While contracts between actors included product specifications with regard to disease status, there were no efforts to support the supplier to increase biosecurity in order to fulfill these requirements. Therefore, biosecurity measures were limited in this chain and depended on the efforts of each individual actor to fulfill the contract requirements. Because of the low level of asset specificity in the semi-automated chain, chain actors were able to trade more freely with other partners, increasing the scope of the transaction but with less coordination. Consequently, investments to promote biosecurity were lower. In this chain, sector 2 farms even traded live birds with collecting farms in traditional channels where biosecurity practices were much lower still. As the risk of HPAI introduction in the controlled and private slaughter-point chains was higher than in the semi-automated slaughterhouse chain, sector 2 farms were at relatively high risk of introducing HPAI in their value chain (**Figure 1**). Improving coordination in the semi-automated slaughterhouse chain and cutting off trade with the controlled and private slaughter-point chains would most likely have a large effect on overall HPAI incidence in this chain.

There were no relation-specific investments in traditional channels. Transactions in these chains were based on price and convenience, in the absence of specific biosecurity requirements or coordination. Sick poultry was traded in these channels, and a sick poultry market was established that was also used by the semi-automated chain upon HPAI occurrence. The intensity of physical exchange and thus the risk of HPAI transmission was high. In the controlled slaughter-point chain, limited levels of biosecurity were applied in the sector 2 farms, that also delivered to the collecting farms. The majority of the live birds that came from sector 3 farms were mixed with those of sector 4 farms which applied only a minimal level of biosecurity. No biosecurity measures were applied in the collecting farms, during transport, or at the private slaughter-points.

Other researchers have shown that the type of governance affects actor perceptions of the importance of biosecurity (14, 23). This difference in perception influences the implementation of biosecurity practices and hence the risk of HPAI introduction and transmission in the different poultry value chains. We assessed this influence based on the four factors that affect the risk of HPAI introduction and transmission in relation to the value chain map, governance structure and the implementation of biosecurity. As shown in **Table 4**, each of the four factors that influenced the risk of HPAI introduction and transmission had a stronger effect in the less coordinated chains. This means that the risk of introduction and/or transmission of HPAI was much higher in the traditional channels as compared to the modern channels. Moreover, the links and contacts between the semi-automated chain and traditional channels created an additional layer of risk of disease transmission. Therefore, the integrator chain provided better protection against HPAI outbreaks as compared to chains with other forms of chain governance.

A TCE perspective highlights that many routes for disease transmission in the value chain were mediated at least in part by investments in biosecurity that arise from the types of governance that exist in the value chain. We found an important risk of backward transmission, e.g., from the markets to the farms or from the slaughterhouses to the farms. Crates and other materials used to transport poultry could act as vectors in the transmission of HPAI. Slaughterhouses were indeed reported to be associated with HPAI outbreaks (25). Poor biosecurity practices at the collecting farms, slaughterhouses, and slaughter-points could lead to infection of farms through interactions between humans, vehicles and crates, especially during the process of returning poultry crates from the market or the slaughterhouses to farms. In order to decrease the risk of introduction or infection in the less coordinated chains, the chain leaders (traders) would need to invest in more formal relationships that include biosecurity requirements, since traders were the only actors with the financial and management capabilities to invest in new production assets. This means that traders should upgrade their role from informal financers of the transaction into more formalized commercial agents, such as financial institutions or collecting farms. This could reduce the number of "infection cycles" in the complex and poorly-coordinated poultry chains. However, traders have no incentive to do so, as improved biosecurity practices do not affect their profits. Indeed, removing the "sick poultry market" would rather reduce trader profitability. This is in contrast with the chain leaders in the modern channels (slaughterhouses) who have incentives (economic performance to protect) to maintain improved biosecurity practices in their chains.

The Economic Consequences of HPAI in Different Poultry Value Chains

The economic consequences of HPAI were influenced by the biosecurity practices in the value chains (23, 25, 26). HPAI incidents

TABLE 4 | Risk factors of HPAI introduction and transmission in different poultry value chains in West Java.

Enabling condition of HPAI introduction and transmission in the chain governance	Modern Channel		Traditional Channel	
	Integrator chain	Semi-automated slaughterhouse chain	Controlled slaughter-point chain	Private slaughter-point chain
	Hierarchy	*Modular*	*Market*	*Market*
1. Number of actors involved	+	+ +	+ + +	+ + + +
2. The frequency of contact	+	+ +	+ + +	+ + + +
3. Number of links in chain stages	+	++	+ +	+ + +
4. Contact structure	+	+ + +	+ + +	+ + + +
Total risk of HPAI	**+**	**+ +**	**+ + +**	**+ + + +**

Note, + = the least likelihood of risk, + + + + = the highest likelihood of risk

TABLE 5 | Consequences of HPAI without government intervention.

Consequences types (Losses)	Modern Channel		Traditional Channel	
	Integrator Chain	Semi-automated Slaughterhouse Chain	Controlled Slaughter-point Chain	Private Slaughter-point Chain
	(Hierarchy)	*(Modular)*	*(Market)*	*(Market)*
Production	+ + + +	+ + +	+ +	+
Farm Price Effect	+ + + +	+ + +	+ +	+
Retail Price Effect	+ + + +	+ + +	+	+
Overall	+ + + +	+ + +	+ +	+

Note, + = the least likelihood of consequences, + + + + = the highest likelihood of consequences

increased the mortality rate of poultry. Hence, the number of live birds that could be sold was reduced. In theory, a lower supply of poultry, will lead to increased prices at the farm gate in all value chains, and eventually the retail price will increase as well. We assessed these consequences based on the information gathered for the production related to disease incidents, including inputs, outputs, and prices per stage in different chains. Our research, however, indicated different consequences of HPAI incidents in the different value chains. The consequences of HPAI incidents in different identified poultry value chains in Western Java are illustrated in **Table 5**.

In the most coordinated chain (integrator chain) HPAI incidents had the most severe consequences (**Table 5**). Because of the biosecurity practices in place, farms were forced to remove sub-clinically infected poultry from their flocks. The removal caused shortages in the supply of live birds (high quality poultry) to the slaughterhouses. Consequently, given the larger volumes traded by integrators, such shortages affected the price at the farm gate and ceased production at the slaughterhouse. The subsequent shortage in meat supply would increase prices at the retailer level. Thus, the reduction in activity would reduce profitability within this chain.

In less coordinated chains, the consequences of HPAI incidents were less severe (**Table 5**). For those actors ordinarily selling to formal markets, incidents of HPAI allowed actors to switch sales to the traditional channel (14), making these chains more resilient to fluctuations in the supply of poultry, but also more prone to new HPAI occurrences. Indeed, the private slaughterhouse chain assisted farmers in the semi-automated slaughter chain to trade their sub-clinically infected poultry. Therefore, during an HPAI outbreak, farmers under modular and market forms of governance (sector 2, 3 and 4 farms) were able to sell their poultry to the sick poultry market and thus mitigate the economic consequences of HPAI at a nodal level. However, the ability to trade across channels depended on the size of the outbreak. When HPAI outbreaks were large, farms in the semi-automated slaughterhouse chain were unable to supply enough poultry to the semi-automated slaughterhouse. In these cases the semi-automated slaughterhouses saw a decrease in production, affecting the farm price of poultry.

In general, the overall consequences of HPAI in situations where market governance prevails were lower than in situations of hierarchy and modular governance. The existence of a sick poultry market in this chain partially mitigated the production consequences of HPAI, leading to smaller effects on farm and retailer prices. Because consumers Any doubtin traditional channels were less informed about the quality of the product, retailers could sell sick poultry, having only to accept a slightly lower price.

DISCUSSION

In this study, we carried out an extensive value chain analysis, paying much attention to the governance structures in the Western Java poultry system. Our results indicate that the economic consequences of an HPAI outbreak vary for different governance structures. In particular, chains that were more tightly coordinated had more incentive to implement HPAI control measures compared to traditional channels. Therefore, the risk of HPAI introduction and transmission was lower.

Like all value chain studies, our approach was limited by its sampling frame. We implemented a convenience sampling framework for the different actors, given the complexity of value chains and the difficulties in obtaining representativeness among certain types of actors, particularly traders, wholesalers, and processors. Rich and Wanyoike (11) used a similar approach to extrapolate the broader value chain impact of Rift Valley Fever in Kenya. Although a larger sample would allow for a more detailed quantitative validation, our goal was to offer a qualitative view of the governance structure of the poultry sector. Moreover, resource constraints limit the ability of practitioners to carry out extensive informant-based data collection. Therefore, a relatively limited number of semi-structured interviews with key informants is justified for this study.

A number of issues with regard to the effectiveness of HPAI control measures and how they are related to governance can be identified. First, the effectiveness of HPAI control measures depends on the removal of the sick poultry market from the poultry chain. Without this, efforts to control HPAI will not be effective, since the existence of this market has largely removed the economic motivation of farmers and other actors to improve biosecurity. Traders will need to included, but it is unclear whether they will have the economic incentives to cooperate. Without this intervention, motivating actors, upstream and downstream in the chain will be difficult. Second, because of their higher risk of disease introduction and transmission as well as the limited economic incentives to prevent and control outbreaks, government interventions should focus on the less coordinated chains. Nonetheless, more moderately coordinated chains (e.g., the semi-automated chain) should receive particular attention, as they sell to both formal and informal markets, presenting a greater transmission risk. Third, the value chain map shows that traders play an important role as external actors in HPAI transmission. Analyzing chain governance shows that traders have an important decision-making role regarding the distribution of sick poultry to the market. Many control measures did not involve the participation of traders; therefore sick poultry markets have remained viable. Government intervention should aim at upgrading the role of traders from informal to formal commercial agents, such as financial institutions or collecting farms.

Existing coordination mechanisms have resulted in a lack of effective interventions within the traditional poultry sales channels, and improving coordination could lead to better HPAI control. Higher levels of coordination are correlated with

improved application of biosecurity measures, thus strengthening coordination will likely reduce the risk of HPAI introduction and transmission in the poultry chains (13). Contracts can be implemented, as one form of a coordination mechanism, to improve meat quality and actor revenues (14). In turn, contract farming would create incentives to increase biosecurity and remove the sick poultry market.

On the other hand, increased coordination comes with higher transactions costs. It is not clear whether chain actors possess either the supply-side or demand-side incentives to address coordination without some form of government intervention or regulation. In order to create effective incentives, an intervention policy tailored to different value chains will be required (8, 14). For the situation in Western Java, a pull-and-push strategy could be applied. This approach uses economic performance as the main driver to stimulate chain actors to invest in improved coordination mechanisms and to produce healthier, higher quality poultry. The push strategy attempts to give greater incentives to chain actors in poultry production. Such incentives can take the form of bottom-up entrepreneurial support to encourage change (financial, organizational or technical incentives, or market access, property rights, and contracts) or a more direct, top-down regulatory approach. Neither approach is mutually exclusive, and indeed a combination of incentives and penalties is crucial where transactions costs and information asymmetries are high (27). The pull strategy is an incentive mechanism that is aimed at consumers. The pull strategy would try to convince consumers to change their preference towards higher quality poultry. Changes in consumer buying preferences would drive actors in poultry chains to meet consumer demand by improving the coordination of transactions (asset specific investments). Both intervention strategies are costly, therefore proving that the regulatory cost of improving standards and controls is a major consequence of HPAI.

CONCLUSIONS

An extensive value chain analysis showed that four different poultry value chains can be distinguished in Western Java: the integrator chain, the semi-automated slaughterhouse chain, the controlled slaughter-point chain and the private slaughter-point chain. These four chains differ in structure, number and size of actors, economic performance, and in governance mechanisms. Moreover, the effects of HPAI vary in the different value chains as well as the effectiveness of HPAI control measures.

A close relationship was found between the poultry chain structure, coordination mechanisms, and the risk of HPAI introduction and transmission. First, the relationship between poultry chain structure and chain governance influences the effectiveness of current HPAI control measures. Second, the diversity in governance implies that there is no "one-size-fits-all" strategy for HPAI control measures that can be applied across different poultry value chains. Third, there are fewer economic incentives in less-coordinated chains (traditional channels) to participate in HPAI control programs. This means that in order to improve the HPAI situation in Western Java, it would be advantageous if government intervention improved incentives for better coordination of the different value chains. Improving the institutional infrastructure is a crucial condition for HPAI control to be effective.

AUTHOR CONTRIBUTIONS

DI designed the study, collected and analyzed the data, and drafted the manuscript. The remaining authors provided input on the design of the study, helped interpreting study results, and critically revised the manuscript. All authors read and approved the final manuscript.

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Geographical and Historical Patterns in the Emergences of Novel Highly Pathogenic Avian Influenza (HPAI) H5 and H7 Viruses in Poultry

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Over the years, the emergence of novel H5 and H7 highly pathogenic avian influenza viruses (HPAI) has been taking place through two main mechanisms: first, the conversion of a low pathogenic into a highly pathogenic virus, and second, the reassortment between different genetic segments of low and highly pathogenic viruses already in circulation. We investigated and summarized the literature on emerging HPAI H5 and H7 viruses with the aim of building a spatio-temporal database of all these recorded conversions and reassortments events. We subsequently mapped the spatio-temporal distribution of known emergence events, as well as the species and production systems that they were associated with, the aim being to establish their main characteristics. From 1959 onwards, we identified a total of 39 independent H7 and H5 LPAI to HPAI conversion events. All but two of these events were reported in commercial poultry production systems, and a majority of these events took place in high-income countries. In contrast, a total of 127 reassortments have been reported from 1983 to 2015, which predominantly took place in countries with poultry production systems transitioning from backyard to intensive production systems. Those systems are characterized by several co-circulating viruses, multiple host species, regular contact points in live bird markets, limited biosecurity within value chains, and frequent vaccination campaigns that impose selection pressures for emergence of novel reassortants. We conclude that novel HPAI emergences by these two mechanisms occur in different ecological niches, with different viral, environmental and host associated factors, which has implications in early detection and management and mitigation of the risk of emergence of novel HPAI viruses.

Keywords: novel HPAI emergences, low pathogenic to highly pathogenic conversion, reassortment, spatial and temporal pattern, phylogeography

INTRODUCTION

Highly Pathogenic Avian Influenza (HPAI) viruses of the H5 and H7 subtypes represent a global human health concern (1) in addition to causing severe economic losses in the poultry industry (2). These viruses have an eight-segmented genome, and undergo frequent genetic reassortment and mutations leading to creation of genetic diversity and emergence of novel viruses.

The natural reservoir of avian influenza (AI) diversity is the wild bird ecosystem, where all subtypes circulate in the low pathogenic (LP) form with in various combinations of one of the 16 haemaggultinin (H1–H16) and one of the nine neuraminidase (N1–N9) surface protein genes (3). Frequent transmission of different AI viruses and their genetic segments between wild bird host species, especially in the orders Anseriforms and Charadriiformes favours maintenance of avian influenza genetic diversity. In addition, contact rates can increase during migration periods, such as in stopover sites, where the host diversity is significant with several species, age groups, of different immune status congregating together. This considerable diversity in host range selects sets of virus subtypes in the low pathogenic form that are capable of maintaining transmission cycles through different hosts (4). In this system, there is predominantly environmental transmission between hosts, with evidence of lower evolutionary rates maintaining evolutionary stasis (5). Thus, virus evolution is characterized by low virulence, and high host survival rates; two conditions that are compatible with long distance migration. In summary, epizootics caused by HPAI in wild bird populations are seldom, and were mostly documented for virus strains that had previously been associated with poultry farming (6).

Poultry farms and their associated value-chain networks form the secondary system for AI transmission. On a host level, these poultry systems are characterized by single or limited host species (primarily galliform poultry and waterfowl), of uniform age and considerably lower genetic diversity, reared in high-density flocks, though those factors vary greatly according to the intensification level of the poultry production system considered (7). In high-income countries (HICs), over 95% of chickens are raised intensively in commercial and highly specialized poultry production systems for eggs and meat production (8). In contrast, in low-income countries (LICs), the large majority of poultry is still raised in backyard extensive poultry production systems, for subsistence and as a way to generate income in rural settings. A full range of intermediate situations exists in between these two extremes, which follow a gradient of income. Middle-income countries (MICs) typically face an intermediary situation where both extensive and intensive poultry production systems co-exist (8–10), and where value-chains involve a number of intermediate workers and live bird markets. These poultry agro-ecosystems can be broadly divided between regions that are mostly free from HPAI and where HPAI outbreaks are sporadic, detected and contained early; and regions where HPAI is endemic or showing frequent reoccurrences, and where there are challenges associated with detection and response. The poultry agro-ecosystem is characterized by anthropogenic transmission risks linked to the farming system and value-chains, a lower diversity of circulating types and sub-types, spillover between host species and occasional emergence of novel HPAI viruses.

The modes of evolution of AI viruses within these two systems vary according to the evolutionary pressures accompanying each system. In wild birds, novel AI viruses evolve using two main mechanisms. First, continuous accumulation of point mutations, deletions and substitutions due to lack of proof reading in the RNA polymerase creates antigenic drift (11). Second, exchange of genetic segments between two co-infecting viruses within a host cell leads to genetic reassortment and yields novel subtypes by antigenic shift. These modes of evolution predominantly result in LPAIV subtypes causing subclinical infections (12). An additional fact is that in countries where long term surveillance is conducted in the wild bird system (over 43 years), no novel emerging HPAIV subtypes have ever been isolated (13).

Within the poultry systems, genetic mutations are constantly occurring, and the emergence of novel HPAIVs have been reported on a regular basis in relation to the following main mechanisms. The presence of a multibasic cleavage site (MBCS) within the HA is one of the properties used by World Organization for Animal Health (OIE) to classify AI viruses as highly pathogenic (14). There are several mechanisms by which this can occur; first, new HPAIVs can emerge from the acquisition of certain stochastic mutations of nucleotide/amino acid substitution leading to the insertion of basic amino acids yielding (MBCS) in the HA gene of an existing LPAI virus (15, 16). The MBCS can also be introduced into the HA gene in an LPAI by recombination with host or viral RNA (17), which only occurred on a few occasions in the H7 subtype (18). Third, a novel HPAIV can emerge from the reassortment between already circulating LPAIV and HPAIV influenza viruses by exchange of genetic segments (12).

In this paper, we aim to describe the conditions of emergence of novel HPAIV in the poultry system. Two main methods of novel HPAIV emergence will be reviewed: the acquisition of the MBCS in an LPAI virusus leading to conversion to HPAI- called as "conversions", and the exchange of genetic segments between viruses leading to generation of a novel HPAI, called "reassortments". A data set of these conversion and reassortment events will be compiled in order to describe their geographical distribution and spatio-temporal trends in relation to poultry production systems. Finally, we will use phylogeography to assess the evolutionary and geographical relationships between these novel HPAI emergences.

METHODS

Compilation of Conversion and Reassortment Datasets:

Case Definitions

HPAI Conversions: The first reports of novel H5 or H7 HPAI viruses which are documented as resulting from the conversion of a LP to a HP strain subsequent to introduction from wild birds and circulation and gain of pathogenicity in poultry were classified as HPAI Conversions. The gain in pathogenicity should have resulted from the insertion of a MBCS in the HA of a LP virus. Only the primary/first report of emergence of an HPAI in poultry was considered, and the secondary spread of the same subtype during an epidemic was excluded. The dataset of HPAI conversions was compiled from 1959 onwards.

HPAI Reassortments: The first reports of novel HPAI viruses generated by inter and intra subtype exchanges of genetic segments were classified as "HPAI-reassortants". Reports HPAI H5 and H7 novel reassortment events were compiled using methods described below. Novel reassortant viruses isolated from primary outbreaks, surveillance of live bird markets (LBMs), wild-bird die-off events, and from human and other mammalian cases of HPAI caused by H5 and H7 subtypes were included in the dataset. The dataset of HPAI reassortments was compiled from 1996 onwards, as we could not find any reports of reassortments prior to that.

It is to be noted, that the primary report of a novel HPAI emergence may not actually be entirely accurate as the primary report actually pertains to a first detection/isolation, which may reflect a surveillance bias and indeed may not be the actual conversion/reassortment event.

Methodology

Internet searches using Google Scholar and PubMed databases for conversions were performed using keywords, "H5/H7 LPAI to HPAI", "LPAI to HPAI outbreaks", " H5/H7 LPAI to HPAI Conversion ", and "H5/H7 LP to HP emergence". For reassortments, the keywords used were "Novel Reassortment", "HPAI Reassortment", "HPAI AND novel reassortant", "Novel HPAI", "Novel emergence" along with H5/H7 keywords. We also looked at the paper cited, or being cited by the papers found using the search terms in order to find further references that would not have been found in the primary search.

Thereafter, internet search engines (Internet explorer/Google) were also searched used using similar keywords. Time filters were applied on these searches by dividing into three time periods: up to 1995, 1996–2005, and 2006–2015. Country names were also added to these keywords to further find epidemiological details regarding these events. The publications citing these novel emergences were reviewed to classify the report into a conversion or reassortment event based on the documented evidence. In several instances, the references within a publication would reference other novel emergences, and these references were also reviewed for addition of events to the dataset.

The geographic coordinates of the outbreak/isolation were recorded. For some of the earlier events, the geographic coordinates are not exact, and are available only at coarse resolutions. For the reassortments especially with reference to China and Vietnam, several isolates were from LBM surveillance and the exact location could not be ascertained beyond administrative level 2 or 3. The time of outbreak/isolation was also obtained, and for the purpose of homogeneity, we kept the time period, as "year" as exact dates of sampling were not available for most viruses. The subtype (HxNx) involved in the conversion and reassortment event was recorded along with the host species and type of poultry farming system (backyard poultry, commercial poultry and wild birds) of the first isolation of the novel HPAI subtype. Where necessary, the information on outbreaks/isolates was crosschecked in publicly available databases, which included the EMPRES-i database (http://empres-i.fao.org/eipws3g/), the OIE WAHID database (14), and the ProMed-mail (www.promedmail.org) to verify the host species, production system, geographic location and add any other information that may be available.

The sequence isolated from each conversion and reassortment event was identified from the literature along with its accession number. The isolate name and accession numbers of the submitted sequences were obtained from GenBank® (www.ncbi.nlm.nih.gov/genbank/) (19), the Global Initiative on Sharing All Influenza Data (GISAID) EpiFlu Database (http://www.gisaid.org) and the Influenza Research Database (http://www.fludb.org/brc/search_landing.spg?decorator=influenza). In addition, source details of the associated host species, subtype/gene segment involved in the generation of the reassortant isolate were also tabulated as described by the referenced publication. The accession numbers of four sequences for conversion events could not be found in either of the databases, as the respective authors may not have submitted them yet. We were able to obtain the accession number of all reassortant isolates from the public databases. The publication citing the conversion and reassortment was also referenced for each novel virus. In some cases multiple strains were cited in the publication, but only some could be classified as a novel reassortants, therefore careful scrutiny of each publication was carried out to include only new reassortants.

Analysis

The data sets were mapped according to three study periods: Up to 1995, 1996–2005, and 2006–2015. These were chosen as they historically represented times at which important changes occurred in the epidemiology of HPAI: the year 1996 marked the emergence of the HPAI H5N1 A/Goose/Guangdong/1/96 virus, which was distinct from the earlier sporadically occurring HPAI H5 subtypes. During this period, geographic spread within southeast and east Asia of the Gs/Gd lineage was documented. Period three was chosen as the year 2006 marked the period of global expansion of HPAI from Asia into the Indian subcontinent, Europe and Africa.

In order to estimate the evolutionary relationships between the selected H5 and H7 sequences associated with conversions and reassortment events, we used the discrete phylogeographic approach implemented in BEAST 1.8.4 (20). This Bayesian phylogenetic diffusion model allows inferrering ancestral times and spatial locations at the internal and root nodes of time-calibrated phylogenetic trees (21). A distinct analysis was performed for the H5 and H7 alignments, for which we partitioned the coding genes into first + second and third codon positions. In BEAST, we specified a general GTR+Γ + I nucleotide substitution model, an uncorrelated lognormal relaxed molecular clock to account for evolutionary rate variation among lineages (22) and a flexible Bayesian Skyride coalescent tree prior (23). The discrete diffusion model was parametrized as a nonreversible continuous-time Markov chain (CTMC) model (24). We used the program Tracer 1.6 (20) to ensure that effective sample sizes were greater than 200 for all the different parameters estimated by BEAST. Finally, TreeAnnotator 1.8.4 (20) was used to summarize the inferred ancestral locations from the resulting posterior distribution on a maximum clade credibility (MCC) tree.

RESULTS

From 1959 to 2015, a total of thirty-nine LPAI to HPAI H5 ($n = 14$) and H7 ($n = 25$) conversion events were documented (**Figure 1**).

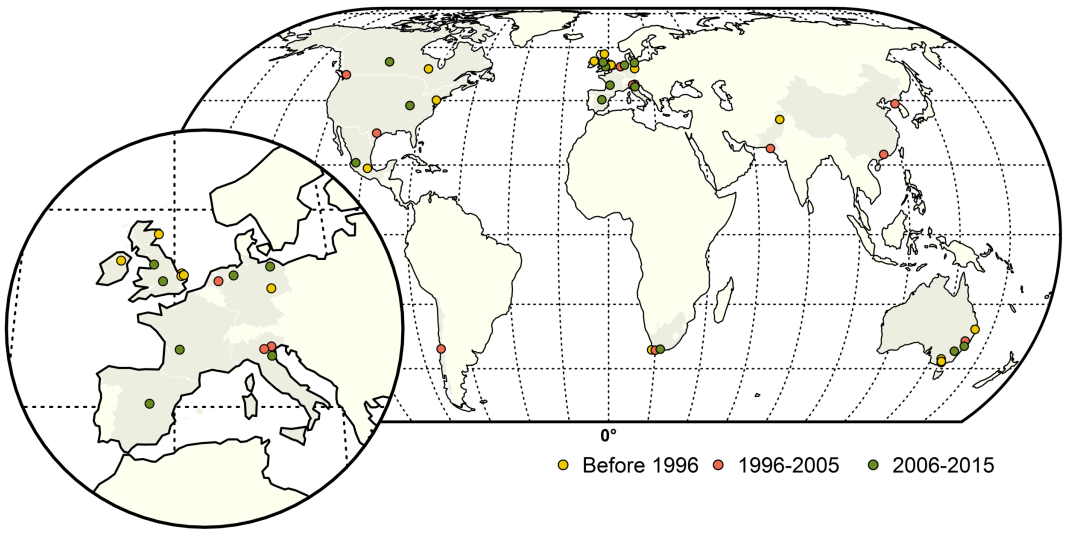

FIGURE 1 | Map of highly pathogenic avian influenza (HPAI) subtype H5 and H7 conversions during three time periods; yellow (before 1996), red (1996–2005) and green (2006–2015). The shapefile data used to produce these maps were made with Natural Earth (http://www.naturalearthdata.com/). The graticule is composed of a 20-degree increments and the coordinate system is Eckert IV (EPSG: 54012).

A total of 127 HPAI reassortments were documented since 1959 (**Figure 2**; Table S1 in SI) and only two of the reassortments were reported from H7 subtype, while the remainder was all reported in H5 subtype. Spatially, a large majority of the conversions occurred in Europe (n = 14), followed by the Americas (n = 9), Australia (n = 7), Africa (n = 3) and Asia (n = 4). The details of all the conversion events are provided in **Table 1**. In contrast, the reassortments were concentrated in Asia (117), with only a few in Africa (n = 5), Americas (n = 3) and Europe (n = 2). There were no reassortments reported from Australia. Within Asia, the highest number of reassortments were documented in China (n = 56), followed by Vietnam (n = 35). Hong Kong (n = 11) Taiwan (n = 4), Korea and Bangladesh (n = 3), Indonesia (n = 2), DPR Korea and Kazakhstan reporting only one reassortment. In

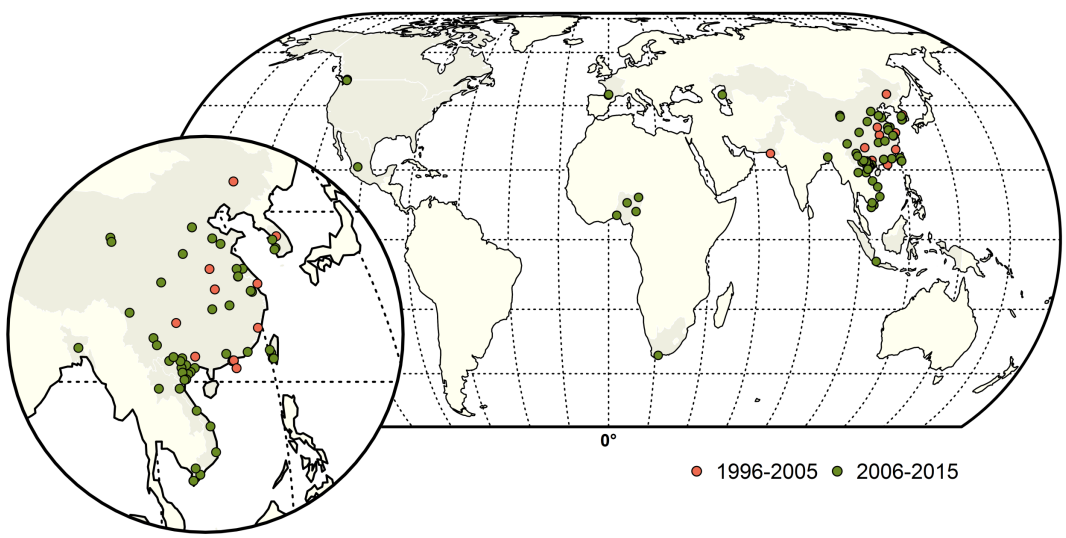

FIGURE 2 | Map of Highly pathogenic avian influenza (HPAI) subtype H5 and H7 reassortments during two time periods; red (1996–2005) and green (2006–2015). The shapefile data used to produce these maps were made with Natural Earth (http://www.naturalearthdata.com/). The graticule is composed of a 20-degree increments and the coordinate system is Eckert IV (EPSG: 54012).

TABLE 1 | List of Highly Pathogenic Avian Influenza Conversion (subtype H5 and H7).

Country	Year	Subtype	Location	Species	Wildbird Link	Interface	AccessionNo	Reference	Strain Name Strain No.
Time Period: before 1996									
UK	1959	H5N1	Scotland	Commercial, Chickens	Y	Interface	X07826	(25)	(A/chicken/Scotland/1959(H5N1)
South Africa	1961	H5N3	Capetown	Wild terns	Y	Interface	U20460	(26)	(A/tern/South Africa/1961(H5N3)
UK	1963	H7N3	England	Commercial, Turkeys	Y	Interface	U20462	(27)	(A/turkey/England/1963(H7N3)
Canada	1966	H5N9	Ontario	Commercial, Turkeys	N	Poultry	CY107859	(28)	(A/turkey/Ontario/7732/1966(H5N9)
Australia	1976	H7N7	Victoria	Commercial, Chickens	N	Poultry	CY061602	(29)	(A/duck/Victoria/1976(H7N7)
UK	1979	H7N7	England	Commercial, Turkeys	Y	Interface	M30122	(30)	A/turkey/England/199/79 (H7N7)
Germany	1979	H7N7	Leipzig	Commercial, Chickens	Y	Interface	U20459	(31)	A/chicken/Leipzig/79 (H7N7)
USA	1983	H5N2	Pennsylvania	Commercial, Chickens	N	Poultry	CY107848	(32)	(A/chicken/ Pennsylvania/1370/1983(H5N2)
Ireland	1983	H5N8	Ireland	Commercial, Turkeys	Y	Interface	CY015089	(33.	(A/turkey/Ireland/1378/1983(H5N8)
Australia	1985	H7N7	Victoria	Commercial, Chickens	Y	Interface	CY025069	(34)	(A/chicken/Victoria/1/1985(H7N7)
UK	1991	H5N1	East Anglia	Commercial, Turkeys	Y	Poultry	EU636692	(35)	(A/turkey/England/50-92/91(H5N1)
Australia	1992	H7N3	Victoria	Commercial, Chicken, Ducks	N	Poultry	CY025077	(36)	(A/chicken/Victoria/224/1992(H7N3)
Australia	1994	H7N3	Queensland	Commercial, Chickens	Y	Interface	CY022685	Rev. sci. tech. Off. int. (37)	(A/chicken/Queensland/1994 (H7N3)
Mexico	1994	H5N2	Puebla state	Commercial, Chickens	N	Poultry	L46586	(15)	(A/Chicken/Puebla/8623-607/94(H5N2)
Pakistan	1994	H7N3	Islamabad	Commercial, Chickens	N	Poultry	AF202226	Naeem and Hussein. 1995	A/chicken/Pakistan/447/94 (H7N3)
Time Period: 1996–2005									
Country	**Year**	**Subtype**	**Location**	**Species**	**Wildbird Link**	**Interface**	**AccessionNo**	**Reference**	**Strain Name Strain No.**
China	1996	H5N1	Guangdong Sheng	Commercial, Geese	Y	Interface	AF144305	(38)	(A/Goose/Guangdong/1/96(H5N1)
Australia	1997	H7N4	New South Wales	Commercial, Chickens	Y	Interface	CY022701	(39)	(A/chicken/New South Wales/327/1997 (H7N4)
Italy	1997	H5N2	Veneto and Friuli-Venezia Giulia regions	Backyard, Chicken	Y	Interface	GU052403	(40)	(A/chicken/Italy/330/97 (H5N2)
Italy	1999	H7N1	Verona	Commercial, Turkeys	N	Poultry	DQ991320	(41)	A/Chicken/Italy/5093/1999 (H7N1)
Chile	2002	H7N3	San Antonio, V Region	Commercial, Chickens	N	Poultry	AY303632	(17)	(A/chicken/Chile/4957/02(H7N3))
Pakistan	2003	H7N3	H7N3	Commercial, Chickens	N	Poultry	FJ577526	(42)	(A/chicken/Karachi/NARC-23/2003(H7N3)
Netherland	2003	H7N7	Gelderland	Commercial, Chickens	Y	Poultry	AY338458	(43)	(A/chicken/Netherlands/1/03(H7N7)
Canada	2004	H7N3	Fraser Valley, British Columbia	Commercial, Chickens	N	Poultry	AY731820	(44)	(A/chicken/British Columbia/NS-1390-2/04(H7N3)
USA	2004	H5N2	Texas	Commercial, Chickens	N	Poultry	AY849793	(45)	(A/chicken/TX/298313/04(H5N2))
South Africa	2004	H5N2	Western Cape	Commercial, Ostrich	Y	Interface	FJ519983	(46)	(A/ostrich/South Africa/ N227/2004(H5N2))
Dem People's Rep of Korea	2005	H7N7	P'yongyang-si	Commercial, Chickens	N	Poultry		ftp://ftp.fao.org/docrep/fao/010/a0115e/A0115E04.pdf	
Time period: 2006–2015									
Country	**Year**	**Subtype**	**Location**	**Species**	**Wildbird Link**	**Interface**	**AccessionNo**	**Reference**	**Strain Name Strain No.**
Canada	2007	H7N3	Saskatchewan	Commercial, Chickens	Y	Interface	EU500860	(47)	(A/chicken/SK/ HR-00011/2007 (H7N3))
UK	2008	H7N7	England	Commercial, Chickens	N	Poultry	FJ476173	(48)	(A/chicken/England/1158-11406/2008(H7N7))
Spain	2009	H7N7	Castilla-la Mancha	Commercial, Chickens	Y	Interface	GU121458	(49)	(A/chicken/Spain/6279-2/2009(H7N7))
South Africa	2011	H5N2	Western Cape	Commercial, Ostrich	Y	Interface	JX069081	(50)	(A/ostrich/South Africa/ AI2114/2011(H5N2))
Mexico	2012	H7N3	Jalisco	Commercial, Chickens	Y	Poultry	JX397993.1	(51)	(A/chicken/Jalisco/12283/2012)(H7N3)

Continued

TABLE 1 | Continued

Country	Year	Subtype	Location	Species	Wildbird Link	Interface	AccessionNo	Reference	Strain Name Strain No.
Australia	2012	H7N7	New South Wales Po river delta of Emilia Romagna	Commercial, Chickens	Y	Interface		FAO Empres-I	NA
Italy	2013	H7N7	Region	Commercial, Chickens	Y	Interface	KF569186	(52) http://www.dpi.nsw.gov.au/__data/assets/pdf_file/0003/458553/AHS-oct-dec-2012.pdf.	(A/chicken/Italy/13VIR4527-11/2013(H7N7))
Australia	2013	H7N2	New South wales Mecklenburg	Commercial, Chickens	Y	Interface	NA		NA (A/turkey/Germany-MV/AR2472/2014; AR2472/14)
Germany	2014	H5N8	Vorpommern	Commercial, Turkeys	Y	Interface	EPI544756	(53)	
Germany	2015	H7N7	NIEDERSACHSEN	Commercial, Chickens	Y	Interface		(54)	NA
UK	2015	H7N7	Lancashire	Commercial, Chickens	Y	Interface	EPI623939	(54)	A/chicken/England/26352/2015 (H7N7)
France	2015	H5N1	Dordogne	Backyard, Chicken	Y	Interface	KU310447	(55)	(A/chicken/France/150169a/2015(H5N1)
USA	2015	H7N8	Indiana	Commercial, Turkeys	N	Poultry	KU558906	(56)	A/turkey/Indiana/16-001403-1/2016(H7N8)

Africa, four reassortment events were reported from Nigeria and one from South Africa. Europe reported two reassortment events. In the Americas, Mexico, Canada and the USA reported one HPAI reassortment each. The details of all the HPAI reassortments events are provided in Table S1 in SI.

Temporally, the conversion events were reported throughout the three time periods, with 15, 11 and 13 events in the 1959–1995, 1996–2006 and 2006–2015 periods, respectively. In contrast, up to the year 1995, no HPAI reassortments were reported in the literature. In the following decade, 45, and in the period of 2006–2015, a total of 82 reassortments were documented respectively. The spatio-temporal pattern of reassortment reports largely followed the spread of the HPAI H5N1 virus, which reassorted with many other viruses giving rise to several H5Nx viruses, as detailed in the Supplementary Information Data Sheet S1.

From 1959 to 1995, the conversion events were spatially dominant in Europe and Australia. The majority of conversions occurred in the UK (1959 H5N1, 1963 H7N3, 1979 H7N7, 1991 H5N1) and Ireland (1983 H5N8) with both the H7 and the H5 subtypes involved. The continent of Australia was affected four times with only H7 LP to HP conversions during this period (1976 and 1985 H7N7, 1992 and 1994 H7N3). Single conversion events occurred in USA (1983 H5N2), Canada (1966 H5N9), South Africa (1961 H5N3), Germany (1979 H7N7), Mexico (1994 H5N2) and Pakistan (1994 H7N3).

During the next decade from 1996 to 2005, Europe still remained the center of conversions, however, unlike the previous time period, none of the events were recorded in the UK. Instead Italy (1997 H5N2, 1999 H7N1), and the Netherlands (2003 H7N7) were affected by outbreaks caused by LP to HP conversions. Interestingly Australia had only one conversion (1997 H7N4) event. Canada (2004 H7N3) and the USA (2004 H5N2) had one event each, similar to the earlier time period. Conversions were also reported from South Africa (2004 H5N2) and Chile (2002 H7N3).

During this period, Asia reported conversions for the first time in Pakistan (2003 H7N3), DPR Korea (2005 H7N7) and China (1996 H5N1). The 1996 H5N1 virus became the progenitor of the HPAI H5N1 virus that began spreading across continents in the following decade.

From 2006–2015, there was a considerable increase in conversion events in Europe (especially towards the latter part of the decade) with seven conversions reported. Two conversions were reported from the Americas and Australia (2012 H7N7, 2013 H7N2). There were no conversions reported from Asia. During the initial part of the decade, the conversions were limited to the UK (2008 H7N7), Spain (2009 H7N7), Canada (2007 H7N3), South Africa (2011 H5N2), Mexico (2012 H7N3) and Italy (2013 H7N7). From 2014–2015 there was a quick succession of conversion events reported from Europe from Germany (2014 H5N8, 2015 H7N7), the UK (2015 H7N7) and France (2015 H5N1). Additionally, a LP to HP conversion involving H7N8 was reported in the USA in 2015. All conversion events are described in **Table 1**, and more details on these events can be found in Supplementary Information (SI) Data Sheet S2A.

In terms of host species and production systems, conversion events are predominant in intensive production systems with conversion events found in commercial chicken farms (n = 25),

followed by commercial turkey farms ($n = 8$), ostrich farms ($n = 2$) and commercial geese farms ($n = 1$). Backyard farms only reported HPAI conversions on two instances: in 1997, in Italy with an HPAI H5N2 virus, and in France with an HPAI H5N1 virus. The only instance of a conversion being documented in wild birds was the large die-off of wild terns caused by HPAI H5N3 in 1961, off the coast of South Africa (26). Evidence of a direct interface with wild birds was reported in 19 of the 41 conversion events. This includes proximity to areas inhabited by wild birds, areas of overlap with migratory bird flyways and direct links established with wild bird sequences through phylogenetic analyses.

In comparison, the majority of the reassortants were isolated from chicken and ducks during live bird market (LBM) surveillance conducted in China and Hong Kong ($n = 42$). Other than that, 6 reassortants were isolated from geese samples and a single isolate came from a partridge sampled in LBMs. In Bangladesh, three reassortant isolates were isolated from chickens in LBM and poultry farms. In Vietnam, most reassortants were isolated from poultry farms from chickens ($n = 15$), ducks ($n = 17$), and a single isolate from ostrich and quail each from surveillance following outbreaks. Reassortant viruses were also isolated from commercial chicken farms in Mexico, Canada, China, Hong Kong, DPR Korea, Nigeria, Lao PDR and Taiwan. In Taiwan, three reassortants were also isolated from commercial goose farms. In France, two reassortant viruses were isolated from fattening duck farms.

Reassortants ($n = 3$) were also reported in commercial ostrich farms from South Africa, Nigeria and Vietnam. Six reassortants HPAI viruses were isolated from human cases, of which three were from China and one each from Hong Kong and Vietnam. Reassortant isolates were obtained from wild birds following reports of die-offs in 9 instances. Reassortants were also isolated from sparrows ($n = 1$), swine ($n = 1$), cats ($n = 1$) and a captive tiger ($n = 1$). To summarize, the large majority of the 127 HPAI H5 and H7 reassortants were reported from chicken ($n = 51$), ducks ($n = 46$), and geese ($n = 8$), and only few of them were sampled from other bird species.

We were able to obtain the accession numbers of all LPAI to HPAI conversions events, except four sequences (**Table 1**). For the reassortments, all accession numbers for the HA sequences could be obtained (Table S1 SI). The time scaled phylogeographic history of the available HA H5 and H7 subtype novel viruses produced by conversions and reassortments are presented in **Figure 3**, and present the time, region and subtype, that the conversion or reassortment event is associated with.

Within the H5 subtype, one can observe the dominance of conversion events until 1995, and these were largely restricted to Europe and America (**Figure 3**) apart from a single conversion of H5N3 into HPAI causing an outbreak in wild terns off the coast of South Africa. Sometime around late 1980s, the H5N2 subtype was introduced from the Americas (Mexico) into Taiwan (East Asia), which caused a reassortment between a Mexican-like H5N2 virus and a locally circulating H6N1 virus (57) and yielded a virus that was very similar to the vaccine strain used in Mexico. From 1995 onwards, with the emergence of the Gs/Gd HPAI H5N1 subtype, the emergence of novel reassortments started to be reported from eastern Asia (China and Hong Kong) and reassortments were only described as being of the H5N1 subtype until 2008–09. Introduction of HPAI of the H5 subtype to Southeast Asia took place around year 2000, which was followed by the regular isolation of multiple reassortant genotypes and subtypes. Multiple introductions followed by reassortments are also reported from East Asia (China) into Southeast Asia, indicating a continuous gene flow from eastern China into primarily Vietnam. The spread of H5 HA from China into Africa around 2005 is also noticeable, leading to the emergence and evolution of the European-Middle Eastern-African (EMA) (58) lineage that clusters in Africa. The HA of the South Asian H5N1 viruses also groups together (**Figure 3**, annotated in blue) indicating a single introduction from Southeast Asia followed by reassortments due to enzootic circulation of subtype H9N2. From 2009 onwards, the H5Nx reassortants start emerging, initially restricted to China, followed by expansion into Southeast Asia, and then global spread into Europe. Simultaneously, reassortment between the Asian H5N1 with the North American viruses yielded to the reassortant HPAI H5N1 and H5N2 in 2014 (**Figure 3**).

The H7 HA phylogeography does not provide any evidence for conversion or reassortment events involving H7 in Africa (**Figure 3**). The H7 subtypes in the Americas group together as is also the case in Australia. The HPAIV conversions in Australia are part of a discrete monophyletic lineage that diverged from the Eurasian lineage (**Figure 3**). The first conversion event took place in Australia around 1976 and thereafter, all conversion form a single cluster with no introduction from other regions. Similarly, the H7 subtypes in Europe group together, and are only associated with conversion from LPAI to HPAI. The H7 HA was introduced into South Asia (Pakistan) around 1994, where it converted into a HP subtype.

DISCUSSION

The spatial and phylogenetic descriptive studies indicate that conversion and reassortment events, which are the two main evolutionary mechanisms by which a novel HPAI could emerge in the poultry systems, appear to show distinct geographical patterns.

The number of independent LPAI to HPAI conversion events is fairly low, even if some of these conversions lead to epidemics with very important consequences. The large majority of the detected conversions events took place in high-income countries, in poultry farms located within high poultry density areas. Out of the 41 conversion events documented, only 2 occurred in backyard rural flocks, and even those took place in regions of intensive poultry production (Italy 1997 and France 2015).

The surveillance and detection capacity can hardly be considered to be homogenous in space and time, and this may introduce a significant observational bias. So, a first hypothesis to explain the predominance of HPAI conversion in intensive poultry settings could be that this simply reflects the country's surveillance capacity, but that other HPAI viruses could emerge in less intensive settings, and go extinct before being detected. Most HIC countries where HPAI conversions were described have regular and standardized surveillance and detection plans for sub-clinical LPAI and HPAI in poultry, and sometime in wild birds, too. In MICs, where poultry production systems are still transitioning toward more intensive production systems, only the large scale mortalities caused by HPAI

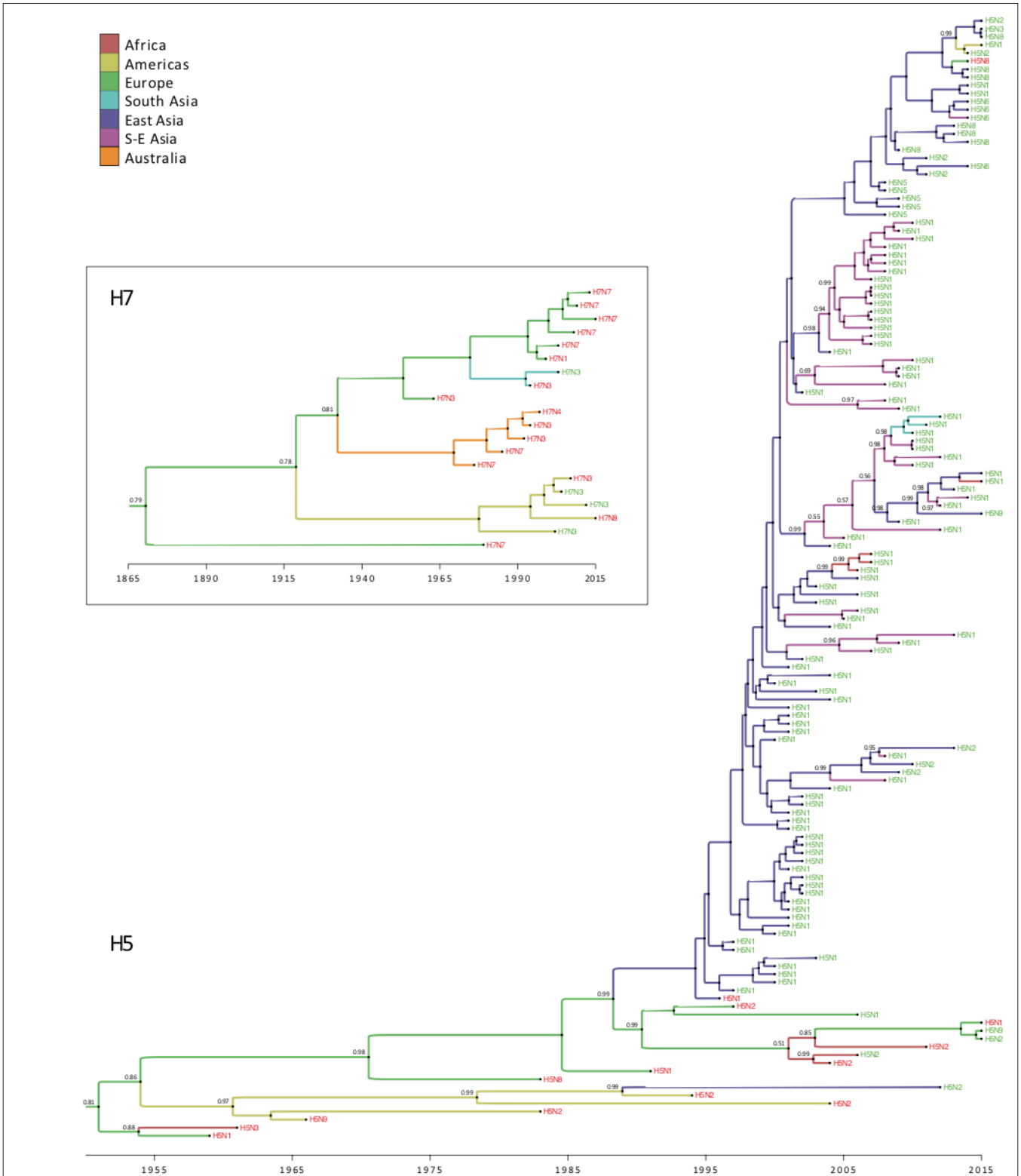

FIGURE 3 | Time-scaled phylogeographic history of H5 and H7 sequences associated with HPAI conversions and reassortments. Branch colors represent the most probable location of the parental node of each branch. Tip labels indicate the subtype and are colored according to the associated event, i.e., conversion (in red) or reassortment (in green). When they are lower than 0.95, posterior probabilities of the most probable ancestral state (i.e. geographic location) are reported next to internal nodes.

in poultry would probably be noticed, often at times when the epidemic would have spread far and wide (e.g., Mexico, Pakistan, China). HPAI conversion in countries practicing vaccination as a means of control may be even more complicated to detect, as masking of clinical signs may confound the passive surveillance. The willingness to report an outbreak is also a factor that needs to be considered and commercial farms are more proactive when it comes to reporting (59), as compared to backyard farms. This willingness to report is more likely to be higher among farmers of HICs than in MICs too.

A second hypothesis is that HPAI conversions can only take place in intensive poultry rearing conditions with high contact rates, where certain mechanisms and conditions encountered in those systems aid conversions. Once a virus starts circulating in poultry, several variants are produced on account of antigenic drift and only the fittest variants persist. If host density and contact rates are low, the most virulent viruses may face a shortage of susceptible hosts and the chain of transmission may be broken. When the density and contact rates of immunologically naïve hosts increases, as it is encountered in a flock of intensively managed and unvaccinated broilers, for example (densities are typically >10 birds/m^2 in a barn), the cost of gaining virulence decreases (the pathogen can kill its host quickly and still face a sufficiently large number of in-contact susceptible hosts to pursue its transmission in the population) and being highly pathogenic doesn't limit transmission any longer (60, 61). Retrospective studies of LPAI and HPAI viruses from the same epidemic have shown that some of the key mutations that were present in the HPAI variants were also identified in the LPAI precursors, albeit at lower frequencies; and as the LP virus transmitted through the poultry population, it accumulated further mutations driven by a multitude of ecological drivers such as host, population and environmental changes, resulting in the conversion to a HPAI variant (62).

Another possible mechanism linking intensive poultry production systems to the evolution of virulence is the all-in/all-out practice, whereby entire cohorts of birds are managed simultaneously, and where the birds surviving an HPAI outbreak would be culled, which prevents the selection of natural resistance in the poultry host population. In contrast, in backyard poultry settings, birds that may have survived a local outbreak would possibly be used to restock with the possibility to select natural resistance genes, and mathematical models indicate that this may influence the evolution of virulence and host resistance (63).

The species composition also plays a role; there are differences in immune response to a LPAI and a HPAI infection within different species of poultry (64). In ducks, there is positive selection for genes that help to down regulate the immune response leading to tolerance for LPAI and HPAI infection. In chickens, immune response to LPAI is common (e.g., H9N2) but there is very little protective immune response to HPAI, and which may explain their increased susceptibility. Experimental studies in chicken show that the mutations like the C-terminal truncations at the non-structural 1 (NS1) protein occurring during the LP to HP conversion may help in increasing viral pathogenicity (65). Higher virulence can also be acquired with the insertion of polybasic amino acids in the HA cleavage site (HACS) motif (66). Another is the neuraminidase (NA) stalk region deletion that favour adaptation to terrestrial poultry (67), as well as increased respiratory shedding and virulence (68). However, the knowledge of selection pressures that lead to these mutations and subsequent switch can only be hypothesised and therefore requires further elucidation.

The role of vaccination in driving conversion also needs to be explored. Vaccination used for HPAI control following outbreaks in some of the countries may also favour the evolution of escape mutants that may convert into HP variants. Studies in Italy have shown the presence of certain mutations that were acquired after introduction of heterologous vaccination following the 1999 HPAI H7N1 epidemic (69) and the LPAI H7N3 outbreaks (70). Also in Mexico, antigenic drifts was observed in the HA gene of LP H5N2 AIV isolates over a period of time in vaccinated birds (11). In Egypt, a variant group of HPAI H5N1 viruses with increase in HA substitutions was isolated from vaccinated poultry following the implementation of vaccination in 2006 (71). These vaccination pressures may help in driving changes in the antigenicity that confer an evolutionary advantage and allowing re-infection of hosts, actually increasing respiratory shedding, onward transmission and a chance to change phenotype into HPAI.

In addition, the virus, host, and environment interactions driving conversions in Australia may be unique from other parts of the world. H7 viruses have not evolved antigenically over the last 30 years (72). Yet, there is remarkable diversity of the NA types (H7N2, H7N3, H7N4, H7N6, and H7N7) suggesting frequent reassortment with the wild bird system (73, 74). Australia and New Zealand lie at the southernmost edge of most of the migratory pathways with a climatic pattern quite different from that of the northern hemisphere with alternating wet and dry seasons that impacts the availability of food and breeding requirements for migrating Anseriformes (75), ducks, geese and swans, which represent one of the main reservoir of AI viruses. The Wallace Line, a well-known biogeography limit separating Australasia from Southeast Asia, may represent a barrier to the spread of AI into the Australian continent, as there is limited migration of Anseriformes birds across this line (76). Many species of shorebirds indeed do migrate across the Wallace Line to Australia after numerous stopovers in Asian countries with ongoing HPAI epizootics (77). However, the role of shorebirds in transmitting AI viruses to poultry over short and long distances is less well documented than from Anseriformes, and the LPAI viruses circulating in Australia may have limited genetic relatedness with those circulating in Asia.

The geographical distribution of HPAI reassortments is completely different, and points to the epidemiological conditions encountered in areas where HPAIV circulates endemically, the presence of a pre-existing HPAI being a necessary condition for the emergence of novel HPAI reassortants. The observational and surveillance intensity bias over space and time is likely to be much stronger for the dataset of HPAI reassortants than for the HPAI conversions. In a situation of HPAI endemicity where HPAI outbreaks are not systematically sampled and sequenced, active surveillance followed by sequencing is the main way of detecting a reassortant HPAI.

The epidemiological conditions promoting endemicity were documented in prior studies (78, 79). High duck density, with free-grazing in rice paddy fields are widespread in east Asia, and

have been known to be associated with spread and persistence of HPAI (80). The production and marketing value chains associated with poultry production systems in middle income countries are characterized by many facilitators such as middlemen or aggregators who visit different farms, including some with low biosecurity, collect and redistribute multiple species of poultry and waterfowl, taking them to LBMs where there are ongoing possibilities of direct and indirect transmission (81). The LBMs of Asia have a high environmental load of AI viruses, low biosecurity and regulatory processes for minimizing contact points that foster virus survival and re-circulation and are key points of virus exchange and onward virus transmission (82).

Many countries in Asia and elsewhere have similar conditions, such as Bangladesh, Egypt, Indonesia, but yet had much fewer reassortants than China or Vietnam. In Bangladesh the detection rate of AIVs in the LBMs is quite high (24%) (83), the country has a sizeable population of ducks (though less intensively raised than China) and abundant waterfowl in the delta region between its two large rivers, which are also visited by migratory waterfowl from Europe and Central parts of Asia (84). Mixed rearing is common, with poultry and ducks raised together in a semi-scavenging system that allows contact with wild waterfowl (85). In Egypt, even though there has been ongoing circulation of HPAI H5N1 virus since 2006, the evolutionary trajectory has largely been of antigenic drift resulting in phenotypic variation, and few reassortants were reported. In Indonesia, apart from the reassortments that occurred in 2005–06, most of the evolution has been limited to point mutations (86) even though the country has numerous live-poultry markets with mixed species.

China and Vietnam implemented several national and regional level surveillance and epidemiological programs in the last decade, hence these were regions of increased sampling, and the higher frequency of reassortants found in these countries compared to other countries where similar risk factors prevail is likely influenced by this sampling bias.

However, this may not necessarily be the only explanation. We referenced the number of samples submitted to GenBank during each of the three time periods from the majority of countries reporting HPAI emergences (China, VietNam, Indonesia, Bangladesh, Egypt, and USA) (SI-Table S2), and found that the number of sequences submitted during the third time-period (2006–2015) is fairly homogenous relative to the chicken stock between all these countries (7) which suggests that the difference in reassortments cannot be fully explained by differences in sampling intensity.

Some epidemiological factors found in China, Hong Kong and Vietnam may differ from Bangladesh, Egypt and Indonesia. In China, Hong Kong and Vietnam, duck meat consumption is more popular, which translates into more intensive duck production systems and trade-related activities, which may also account for the diversity of subtypes of AIVs circulating within the LBMs. Particular subtypes/species combinations in the chicken/duck ecosystems are more prone to reassortment. A higher presence of particular AI subtypes in Anseriformes was described to be associated with higher reassortment rates as compared to Galliformes. Lu et al., (87) found that the H6 subtype that was more abundant in domestic ducks was associated with a higher rate of reassortment. They also found a positive correlation between subtypes with high reassortment rates and Anseriformes, i.e., waterfowl (87). In contrast, lower reassortment rates were observed in Galliformes.

China is also the world's largest producer of swine and the presence of pigs at LBMs creates a higher risk of infection of LBM workers to poultry AIV and swine H1N1 viruses, additionally increasing the risk of emergence of novel HPAI (88). Bangladesh, Egypt and Indonesia are predominantly Muslim nations, and swine production is minimal. In China, other types of agricultural production, such as swine production and aquaculture have also been intensifying rapidly. The production practices unregulated with one industry's waste being used as another industries input. For example, wastes from the swine and poultry industries are most often subjected to land disposal, which can lead to contamination of inland ground and surface water (89). Increasingly, poultry waste is also being used for land based aquaculture feeding in many countries (Little et al., 1996; Little and Edwards, 2003). Presence of remnant feed in poultry and pig wastes attracts waterfowl and wildbirds, which may also promote virus transmission if these waste disposal sites are located along migratory routes [such as in Guangdong, Southern China; (90)].

There is also an extensive wild bird interface in China that may explain the higher reassortment rates observed in east Asia, where large domestic ducks and geese populations are reared in mixed, free range settings that allows close contact of migratory and local waterfowls with ducks and geese, allowing for genetic exchange between viruses (91, 92). Several of the novel reassortants have had multiple gene segments from AI viruses of wild-bird origins (93).

Finally, in China, the rapid increase in HPAI reassortments and the increase in antigenic diversity also coincided with the time when mass-vaccination for HPAI control was implemented. Increase in antigenic drift and diversity promotes rapid antigenic evolution, which further complicates control by vaccination (94). It has not been conclusively proven that vaccination correlates with genetic reassortment, and even though vaccination protects poultry flocks from overt shedding of virus and clinical signs, subclinical infection and silent circulation in poultry does occur (95) and even leads to generation of reassortants as seen by the isolation of vaccine escape variants of HPAI H5N2 in China (96). There are also challenges associated with unregulated vaccine use in several countries that may lead to use of improperly inactivated or attenuated vaccines. In Taiwan, reassortant HPAI H5N2 viruses were isolated from outbreaks, which had HA and NA genes similar to the Mexican vaccine H5N2 strain, and other genes from enzootic AI H6N1 viruses (57).

The recent increase in the number of reassortments in east Asia, accompanied by rapid evolution and global spread of HPAI viruses deserves further investigations. In addition, the H5 HA that was almost exclusively associated with a monophyletic NA over the past decade had recently acquired the ability to combine with several NA subtypes (97) leading to generation of several H5Nx novel reassortants. The reason for this dramatic change in geographic and host range has been described in molecular terms (98). However, the underlying reasons or evolutionary pressures having favored these molecular changes are unknown. It has been hypothesized

that recent changes in the spatial epidemiology of these reassortants HPAI H5Nx may have been associated with land use changes in northeastern China, having affected the spatial patterns of the wild – domestic interface (99).

Further studies on these aspects of differing duck densities and the associated rice-paddy-duck wild bird interface, leading to the risk of generation of genetic diversity will help to gain interesting insights into the generation of HPAI reassortants.

In conclusion, conversions and reassortments have distinct geographical and temporal patterns. The large majority of conversion events took place in intensively raised poultry conditions, but the precise role of this factor as compared to an observational bias would deserve a further quantitative investigation. The distribution of reassortments, that dominate in China and Vietnam, may be influenced by a surveillance and sequencing bias too, but one cannot exclude that other underlying causal factors favoring virus persistence, endemicity and co-circulation of several strains, in these transitioning poultry production systems may have been important too. There are still gaps in our knowledge of the drivers behind the differences in emergences of novel variants, either by conversion or reassortment. With the advent of deep sequencing methods, rapid progress is being made in the identification of molecular changes and mutations associated with evolution. Attempts should be made to monitor and link these changes to agro-ecological drivers of HPAI emergences, with an aim to adapt surveillance and control accordingly. Risk mitigation approaches to prevent conversions should consider the changing climate, land use, and poultry production scenarios, in order to adjust and target surveillance in changing wild-domestic bird interface for early detection of LP to HP conversion linked molecular signatures. Vaccination for prevention and control, should be accompanied with regular monitoring of post vaccinal sero-conversion, field virus surveillance and updating of vaccine strains. Most importantly, areas of intensifying poultry production need to be regulated to make sure that biosecurity and biosafety practices at all levels of the value chain reduce risk and are able to cope with the demands of intensification, and to avoid ongoing virus evolution.

AUTHOR CONTRIBUTIONS

MG, MSD and JA designed the study and reviewed methods, results and the manuscript. MSD, JA, SD, TVB, PL, collected data, analysed the data and wrote the manuscript. SM, DMC, SVD, GD, PL and MG interpreted the results, wrote and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fvets.2018.00084/full#supplementary-material

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Establishing Monitored Premises Status for Continuity of Business Permits During an HPAI Outbreak

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Recent experiences with avian influenza outbreaks in poultry in the United States have tested biosecurity protocols and outbreak management strategies. During an outbreak, regulatory officials managing the emergency response need to make timely decisions in order to achieve disease control and eradication goals while simultaneously decreasing the unintended consequences of the response. To move susceptible animals or animal products out of a disease Control Area via a secure food supply continuity of business (COB) permit without the risk of expanding a disease outbreak, premises must be designated as Monitored Premises (MP) by regulatory officials. The experience of and lessons learned from the 2014 to 2015 highly pathogenic avian influenza (HPAI) outbreak have resulted in defined criteria necessary to establish MP status during an HPAI outbreak and highlighted the need for a clear method to determine that those criteria have been met. Establishing MP status is different from an epidemiologic investigation, though they both require analyses of how avian influenza virus may enter poultry premises and can take significant staff time. MP status of premises seeking to move animals or animal products must be continuously re-evaluated as Infected Premises status, and resulting epidemiologic contacts, can rapidly change during an outbreak. We present here a questionnaire to establish MP status, designed to be initially completed by industry representatives in an attempt to streamline processes and conserve resources. During an outbreak, the MP status questionnaire is an essential risk-based management tool used to establish premises status, as part of operationalizing permitted movement to support COB.

Keywords: HPAI, disease outbreaks, monitored premises, continuity of business, permit, permitted movement, questionnaire

INTRODUCTION

The process for moving animals and animal products in the United States (US) can be challenging to implement when quarantine and movement control activities are in place to contain and eradicate a foreign animal disease (FAD) such as highly pathogenic avian influenza (HPAI). However, facilitating the movement of non-infected animals and non-contaminated animal products into, within, and out of a disease Control Area during a disease outbreak,

while minimizing risk of disease introduction and/or spread, is critical in order to maintain continuity of business (COB) for animal agricultural industries (1, 2). For poultry, COB is achieved when the movement of non-infected birds and non-contaminated poultry products are allowed during an HPAI outbreak, thus helping prevent many potentially devastating unintended economic consequences of the outbreak and securing the US food supply. Additionally and importantly, though outside the scope of this manuscript, maintaining COB helps address animal welfare issues that can arise due to restricted movements.

FEDERAL GUIDELINES AND DEFINITIONS

"The United States Department of Agriculture Animal and Plant Health Inspection Service (USDA APHIS) HPAI Response Plan: The Red Book" is the federal document detailing the FAD Preparedness and Response Plan to HPAI in the United States. The plan stipulates that when HPAI is detected in the US, appropriate regulatory officials issue a quarantine, hold order, or standstill notice for the Infected Premises and establish the boundaries of a Control Area (3). Regulatory officials also work to determine appropriate premises designations (i.e., Infected, Contact, Suspect, At-Risk, and Monitored) for other poultry operations within that Control Area (**Figure 1**). The area and premises designations are used for quarantine and movement control efforts, which extend beyond the Infected Premises and are implemented as rapidly as possible [see also Figure 5-4 in The Red Book for a graphic representation of premises designations in relation to permitting and movement control (3)]. Quarantine and movement restrictions are important tools in controlling and eradicating any FAD outbreak; however, there remain multiple types of movements that occur during an FAD outbreak that are critical to the vitality of the animal agriculture business and which can be done with minimal risk of spreading disease (e.g., movements of feed, liquid pasteurized egg products, processed meat, or newly hatched birds). So as not to create an unacceptable risk of disease spread, the current US approach to HPAI emergency response involves regulatory officials issuing permits for some movements to, from, and within Control Areas [e.g., from movements of susceptible animals and animal products to movements of fomites and materials (4)]. Commodity-specific proactive risk assessments help inform the permit decision-making processes with regard to which types of movements from apparently healthy animals (flocks or herds) may pose acceptable risk.

According to federal guidelines, there are two primary types of permits: (1) Specific, and (2) COB (4). Specific permits are used for movements from Infected, Contact, and Suspect Premises—which are under quarantine during an FAD outbreak. COB permits may be issued for movements from At-Risk Premises or Monitored Premises (MP) and are vital for the production of animals and animal products (4). COB permits are meant to facilitate the continuation of business operations for those premises not infected by the disease agent but still affected by their location within a Control Area and the associated movement restrictions therein. Secure Food Supply (SFS) permits, a type of COB permit, allow movements of animals and animal products into the supply chain for further feeding, growing, processing, or to market (4). At-Risk Premises may seek a COB SFS permit to move susceptible animals or animal products *within* the Control Area. Monitored Premises (MP) meet a set of defined criteria and may seek to move susceptible animals or animal products both within and out of the Control Area by COB SFS permit. Movements into the Control Area under COB SFS permits are less common (4). An MP objectively demonstrates that it is not an Infected Premises, Contact Premises, or Suspect Premises.

Our focus here is on COB SFS permitted movements from MP [for further information about other movement or permit types see the FAD Preparedness and Response Manual, Permitted Movement (4)]. It is important to note that outbreak-specific circumstances cannot be predicted in advance and therefore movement permitting decisions may ultimately depend on relevant risk and epidemiologic determinations made by regulatory officials for any given outbreak.

BACKGROUND

The goals of HPAI response in the US include eradicating HPAI (using strategies that stabilize animal agriculture, the food supply, and the economy while protecting public health and the environment) and providing science- and risk-based approaches and systems to facilitate COB (3). Achieving these goals will allow US industries to resume normal production as quickly as possible and the US to regain disease-free status, ideally without the response effort causing more disruption and damage than the disease outbreak itself (3) (e.g., since avian infection with AI viruses is notifiable to the World Organisation for Animal Health [or OIE] there may be significant international trade consequences until disease-free status can be regained). With these goals in mind, the concepts and definitions for premises designations have evolved with FAD preparedness and response in the US. The term "Monitored Premises" first appeared in the USDA HPAI Response Plan (i.e., The Red Book) in 2011. Only At-Risk Premises are eligible to become MP (i.e., currently Infected, Contact, and Suspect Premises cannot become Monitored Premises) (3). According to the 2017 Red Book, MP meet a set of defined criteria in seeking to move susceptible birds or poultry products out of the HPAI Control Area by permit; these criteria are based on the level of risk of the movement and are set out by the Secure Poultry Supply Plan.[1] For the Secure Poultry Supply Plan, the

Abbreviations: COB, Continuity of Business; FAD, Foreign Animal Disease; HPAI, Highly Pathogenic Avian Influenza; MN, Minnesota; MP, Monitored Premises; MPSQ, Monitored Premises Status Questionnaire; SFS, Secure Food Supply; UMN, University of Minnesota; USDA APHIS, United States Department of Agriculture, Animal and Plant Health Inspection Service.

[1]The Secure Poultry Supply Plan (SPS) is a translation of the science in the Secure Egg (SES), Turkey (STS), and Broiler (SBS) Supply plans into a harmonized permitting approach that can be readily accessed (Grab n' Go) in the event of a disease outbreak such as HPAI (https://securepoultrysupply.umn.edu/).

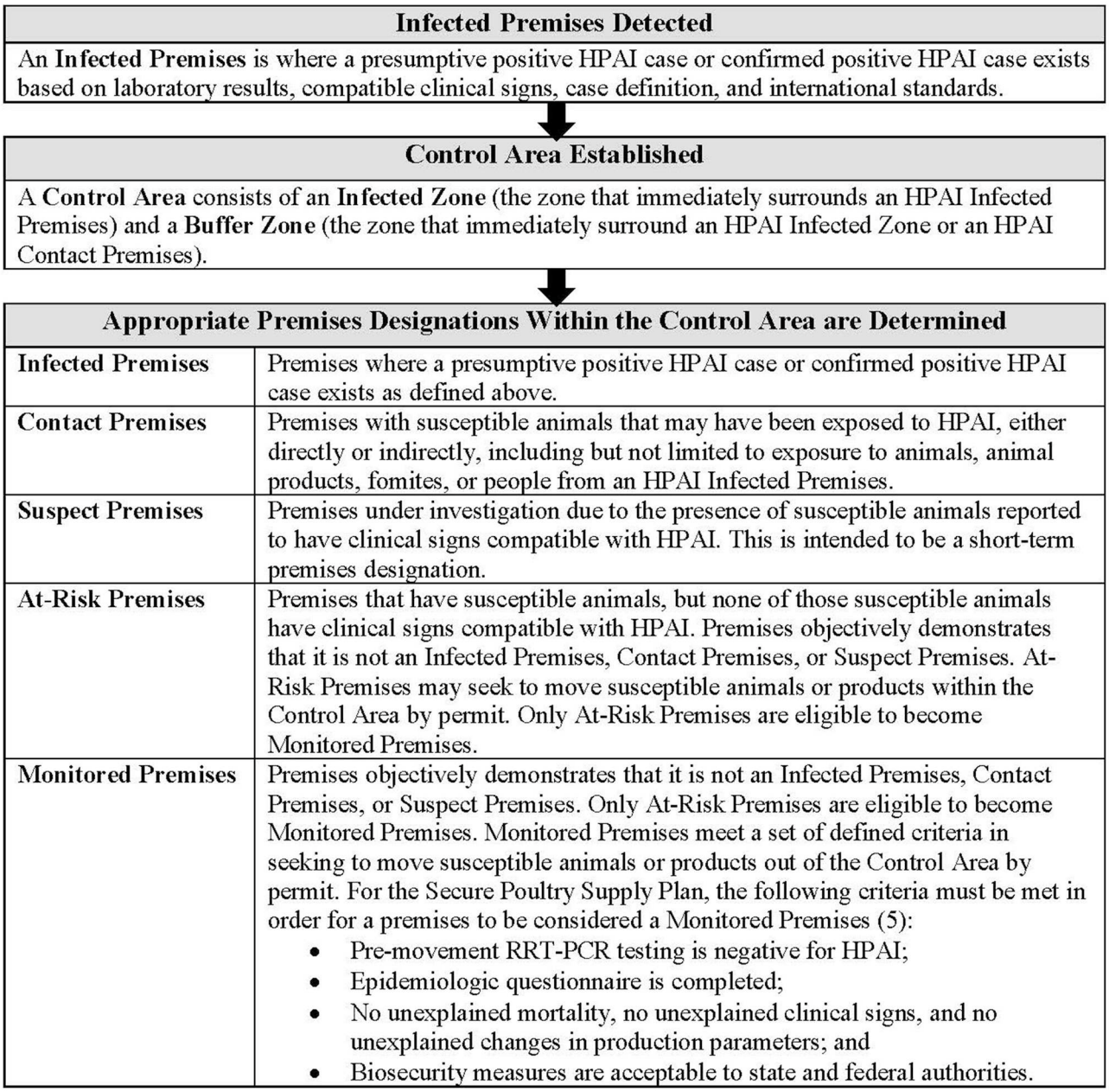

FIGURE 1 | USDA APHIS and Secure Poultry Supply Plan definitions of and steps leading to HPAI area, zone, and premises designations. Additional FAD zone, area, and premises designation definitions not applicable to this manuscript are available in The Red Book (3). Within the unified Incident Command*, the Incident Commander works with the Operations Section and Situation Unit (in the Planning Section) to determine zone, area, and premises designations. These designations are evaluated and reevaluated as needed throughout an outbreak based on the epidemiological situation; specific guidelines as to how to and who will conduct such evaluations to determine appropriate premises designations do not exist. The MPSQ provides a method to establish Monitored Premises status. *In the US, HPAI response is based on the principles found in the National Response Framework (NRF) and National Incident Management System (NIMS); response efforts should be implemented through a Unified Incident Command (i.e., in a manner consistent with the Incident Command System [ICS]) (see the HPAI Red Book for more detailed information and references for the Unified Incident Command organizational structure).

following criteria must be met in order for a premises to be considered an MP [the combined USDA APHIS and Secure Poultry Supply Plan definition of MP is included in **Figure 1**; (3, 5)]:

- Pre-movement RRT-PCR testing is negative for HPAI;
- Epidemiologic questionnaire is completed;
- No unexplained mortality, no unexplained clinical signs, and no unexplained changes in production parameters; and

- Biosecurity measures are acceptable to state and federal authorities.

The criteria specified in the Secure Poultry Supply Plan MP definition were harmonized across poultry industries based, in part, on observations during the 2014–2015 HPAI outbreak in the US and on consultations with the Secure Poultry Supply Working Groups representing the egg, turkey, and broiler industries. While these overarching criteria were harmonized, details regarding each component may be industry specific. Numbers and timing of samples collected for RRT-PCR testing may differ by industry based on transmission rates, products being moved, and nature of the production system, for example. Both the Secure Egg Supply and the Secure Turkey Supply plans include epidemiology questionnaires (which are tailored to identify any possible source of HPAI retrospectively and prospectively on egg or turkey operations, respectively), while the Secure Broiler Supply plan does not.

Lessons learned regarding permitted movement during the 2014–2015 HPAI outbreak in Minnesota (MN) were the subject of several cross-sector/cross-commodity meetings of individuals from the poultry industry, academia, and state and federal agencies that began in late 2015 with a goal to improve future permitting. These meetings comprised larger group discussions as well as smaller working group discussions. The first multi-disciplinary meeting was held by the University of MN (UMN) in December 2015, and it highlighted how permitted movement must be a collaborative effort and resulted in the formation a working group charged with creating a revamped permitting process for MN (6). A key question that this permitting working group faced was how to establish MP status (i.e., how to determine that all necessary criteria had been met) and who will do it.

DEVELOPMENT OF A TOOL TO ESTABLISH MONITORED PREMISES STATUS

Prior to the 2014–2015 HPAI outbreak in the US, permit guidance in MN generally was written *ad hoc* (to address biosecurity and testing requirements) for use by regulatory officials to issue movement permits for poultry. Regulatory officials would review basic production parameters and epidemiologic links among poultry farms that requested to move birds or poultry products. During the 2014–2015 HPAI outbreak in MN, the permitting language was long and very product-specific, all permits were hand-signed, and initially trucks were officially sealed and followed to destinations such as processing plants. This permitting process created a significant workload burden on regulatory officials during the outbreak, requiring an average of 288 staff hours per week (equivalent to 7 full-time employees) for 16 weeks, not including federal staff or indirect state staff time. In the end, over 900 permits were approved in MN that encompassed over 3,000 movements (not including feed or slaughter product permits and their associated movements) (7).

While the permitting process in MN ultimately was successful during the 2014–2015 HPAI outbreak, the substantial time and effort required by regulatory officials and industry alike for permitting was sufficient to motivate an improvement of the process. According to the USDA, during the entire 2014–2015 HPAI outbreak (which involved 15 states that had positive commercial or backyard poultry producing premises), there were over 7,500 permits—and over 20,000 individual movements associated with those permits—that were entered into the USDA APHIS Emergency Management Response System 2.0 (EMRS2). EMRS2, a secure information management system, is used by APHIS personnel for all permitting processes, including issuing permits and tracking movements (4). While a large portion (36%) of the total permits during the outbreak were issued in MN (8), given the sheer number of permits involved for the US as a whole, the burden of the permitting process is likely to have been a challenge for other states as well, not just MN.

The cross-sector/cross-commodity meetings held in MN following the 2015 outbreak were an opportunity to garner the expertise of people involved in this very large outbreak on the aspects of response that went well and aspects that needed improvement. Through multiple meetings and conversations, it became clear that one key component of the permitting process was lacking—a clear method for establishing MP status. Thus, developing a procedure and the tools to establish that all necessary criteria for MP status have been met could help improve the permitting process in future disease outbreaks. Determining MP status is different from an epidemiologic investigation, making epidemiologic questionnaires inefficient tools for determining MP status. When determining MP status, it is important to elicit evidence of potential infection via contact specifically with Infected, Suspect, or Contact Premises in a Control Area. In contrast, epidemiologic investigations are more open-ended and examine contact with *all* potential sources (including other poultry operations and wild birds) and may also seek to gather information about a premises that is not central to determining MP status, such as management type. As such, having a targeted, pre-existing "Monitored Premises Status Questionnaire" (MPSQ) could assist regulatory officials and industry representatives when they are seeking to move poultry/poultry products via COB SFS permits. As the MN working group began development of the MN MPSQ, the 2016 AI outbreak in Indiana re-confirmed the need for such a tool. The response coordinators in the 2016 Indiana outbreak benefited from lessons learned and tools developed as a result of the 2014–2015 outbreak; however, no pre-existing method for determining MP status had been developed at that time. The Indiana Incident Command created their own impromptu strategy during the outbreak. Their method involved using direct communications with the poultry industry premises, completing a biosecurity checklist (using the existing biosecurity checklist in the Secure Egg Supply Plan), and conducting daily sampling (personal communication Dr. Mike Kopp, Sept 2017).

It follows that a pre-existing MPSQ should enable more efficient determination of the appropriate premises designation, that is, if a premises meets the defined criteria to be designated as an MP. This determination would ideally be made without having to have as many direct conversations or searching for then modifying existing checklists or questionnaires to address all of the defined criteria. A set of targeted questions was thus compiled

TABLE 1 | Questions included in the Minnesota Monitored Premises Status Questionnaire (MPSQ).

IDENTIFICATION AND LOCATION OF PREMISES
1. What is the national Premises Identification Number (PIN) for the premises?
HPAI
Responses indicate whether or not a premises is an Infected Premises (#2) or a Suspect Premises (#3–5); Yes answers will be referred to Incident Command for follow-up
2. Does premises have a diagnosis of HPAI? (Yes/No)
3. Does premises have any unexplained clinical signs or clinical signs indicating HPAI? (Yes/No)
4. Does premises have any unexplained mortality or mortality indicating HPAI? (Yes/No)
5. Does premises have any unexplained changes in production parameters or production parameters indicating HPAI? (Yes/No)
EPIDEMIOLOGIC LINKS/EXPOSURES TO INFECTED PREMISES
Responses indicate whether or not a premises is a Contact Premises; Yes and Unknown answers will be referred to Incident Command for follow-up
6. Has this premises been exposed to poultry manure from an infected flock (HPAI virus in manure) in the past 14 days? (Yes/No/Unknown)
7. Has this premises been exposed to dead poultry from an infected flock (HPAI virus in carcasses, etc.) in the past 14 days? (Yes/No/Unknown)
8. Has this premises been exposed to live poultry from an infected flock (HPAI virus in bird secretions and excretions) in the past 14 days? (Yes/No/Unknown)
9. Has this premises been exposed to eggs or egg-handling materials from an infected flock (HPAI virus in and on eggs from infected birds) in the past 14 days? (Yes/No/Unknown)
10. Has this premises been exposed to semen or semen-handling materials from an infected flock (HPAI virus in semen) in the past 14 days? (Yes/No/Unknown)
11. Has this premises had unmitigated exposure* to equipment that has been in contact with poultry manure, dead poultry, live poultry, eggs, egg-handling materials, semen, or semen-handling materials from an infected flock in the past 14 days? (Yes/No/Unknown)
12. Has this premises had unmitigated exposure** to people who have been in contact with poultry manure, dead poultry, live poultry, eggs or egg handling materials from an infected flock in the past 14 days? (Yes/No/Unknown)
13. Have the people or the equipment from this premises been involved in the depopulation of infected flocks in the past 14 days? (Yes/No/Unknown)
BIOSECURITY
An answer of No will be referred to Incident Command for follow-up
14. Is an Accredited Veterinarian (or other Biosecurity Coordinator) responsible for the development, implementation, maintenance, and ongoing effectiveness of a premises biosecurity program that conforms to the National Poultry Improvement Plan (NPIP) guidelines? (Yes/No)

Additional required and critical information also is gathered in the full MPSQ (i.e., date and time that status of Infected Premises was last checked in order to ensure up-to-date Contact Premises information; and explanations of any "yes/no/unknown" answers where appropriate).

**Unmitigated exposure to equipment means inadequate sanitation procedures for those items that come into contact with an infected flock or infectious materials such as trucks/trailers used to transport live birds, eggs, or eggshells; load-out equipment; dumpsters; etc. (a longer list of examples is included with the full MPSQ).*

***Unmitigated exposure to people means inadequate biosecurity, sanitation, or downtime procedures for those people who come in contact with an infected flock or infectious materials such as might happen with working at other poultry operations, visiting a poultry processing plant, visiting a manure handling plant, etc. (a longer list of examples is included with the full MPSQ).*

to create an MPSQ that, taken together, industry could use to establish whether a premises had met all the necessary criteria for MP status (**Table 1**). The MPSQ is divided into four sections: Identification & Location of Premises; HPAI; Epidemiologic Links/Exposures to Infected Premises; and Biosecurity. The Identification & Location of Premises section identifies the premises via the national Premises Identification Number. The HPAI section contains questions to help determine whether or not a premises is an Infected Premises or a Suspect Premises (e.g., does the premise have a diagnosis of HPAI or does the premises have any unexplained clinical signs or clinical signs consistent with HPAI). The Epidemiologic Links/Exposures to Infected Premises section contains questions to help determine whether or not a premises is a Contact Premises (e.g., has the premises been exposed to an HPAI infected flock via poultry manure, poultry carcasses, equipment, etc.). The Biosecurity section helps determine if the premises has a biosecurity program in place that likely will be acceptable to regulatory officials.

The questionnaire was designed with the intent that industry representatives, who actually know answers to farm level questions, will initially answer the questions. Gearing the questionnaire toward industry-initiated determination of MP status was an intentional redistribution of permitting-related responsibility based on two lessons learned: (1) determining appropriate premises designation can take more time and poultry commodity expertise than regulatory personnel may have available during an outbreak, and (2) for products that move daily, MP status must be continuously re-evaluated during an outbreak as Infected Premises status (and thus potential Contact Premises) can rapidly change; this amount of work for regulators concerned with outbreak control may not be justified for low-risk products but is desired by producers who want risk for moving product to be as low as possible. Additionally, the MPSQ questions were designed to be cross-sector and appropriate for any poultry/poultry product (e.g., the terms poultry and bird are used throughout rather than specifying sector/bird type). If a premises meets all of the criteria to be designated as an MP, then the request for permitted movement from that premises should be more easily evaluated by regulatory personnel who make the final determination as to whether to designate a premises as an MP, evaluate compliance with product-specific permit guidance criteria, and issue a permit or not. The pre-existing, targeted, cross-sector MPSQ can enable a more efficient evaluation, and re-evaluation if necessary, by both industry and regulators.

Since the development of the MPSQ, multiple HPAI outbreak tabletop exercises have been conducted throughout the US by the SFS team. These exercises have underscored the complexities involved with the permitting process during an FAD response. Indeed, one of the most commonly identified exercise benefits noted by participants has been knowledge gained about COB permitted movement (9). Discussions from these exercises reinforce that the MPSQ is a tool that can streamline the COB SFS permitting process during what can be a chaotic time. Specifically, it provides industry representatives with a "Grab n' Go" list of questions that they can answer before requesting a COB SFS permit; and it allows regulatory officials to assess those answers quickly (i.e., the answer to all of the MPSQ questions, except for the very first and last questions, should be NO otherwise further follow-up is needed by Incident Command) (see **Table 1**). Ultimately, the MPSQ makes the evaluation process to determine MP status operational rather than theoretical.

CONCLUSION

The need for a logistically feasible operational process that could support the efficient and high throughput of COB permits was a driving force for a series of meetings following the 2014–2015 HPAI outbreak in order to improve the permitting process in MN and gather the experiences gained in the largest FAD outbreak in US history. The process undertaken by MN stakeholders has been collaborative, multi-disciplinary, and multi-layered. Ultimately, the lessons learned from the 2014–2015 outbreak and open discussions resulted in the development of an MN MPSQ with the intent that this instrument could be a universal tool for all poultry commodities and eventually all animal agricultural businesses such as beef, dairy, and pork. MP status is central to the assumptions used to determine the risk of commodity movements during an HPAI outbreak. The MPSQ is a tool that helps to further operationalize the process used to determine MP status for the purposes of permitting, and together with other improvements (e.g., Secure Poultry Supply Plan harmonization, electronic forms and web applications, and risk-based permitting approaches), has improved and streamlined the permitting process in MN and could likely be a beneficial tool for other states' response plans as well.

AUTHOR CONTRIBUTIONS

JU wrote the manuscript. RJ provided valuable background information regarding the entire permitting improvement process undertaken in Minnesota. EL provided helpful research assistance as well as additional background information regarding the cross-sector meetings. RJ, CC, and DH conceived the MPSQ. MK provided poultry industry leadership throughout the permitting improvement process and review of the MPSQ. RJ, MC, TG, DH, and CC provided valuable expertise, feedback, and editing on all topics included in this manuscript. All the authors have read and approved the manuscript.

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Developing Farm-Level Post-vaccination Sero-Monitoring Systems for H5N1 Highly Pathogenic Avian Influenza in an Endemically Infected Country

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Whilst the serological responses of poultry following vaccination against highly pathogenic avian influenza H5N1 has been extensively investigated under laboratory conditions, there have been fewer studies conducted in the field. This applies particularly to the endemically infected countries routinely practicing vaccination, where the combination of multiple circulating clades and/or the use of vaccines with different seed strains makes the design and interpretation of field studies especially problematic. To address this for the particular situation of layer hens in the small to medium commercial sector in Indonesia, we developed a sampling regime before and after the vaccination given to point-of-lay pullets, and assessed serological response with a panel of test antigens. This confirmed that high titres were induced in those birds vaccinated with locally produced homologous H5N1 vaccines administered two or more times, but in flocks using imported heterologous H5N2 vaccines median titres were significantly lower, and unlikely to provide protection throughout the production cycle, without additional vaccination. Comparing the HI responses against the panel of antigens enabled the detection of the flock's exposure to different vaccine antigens, and made possible the detection of mislabelled vaccine seed strains. Furthermore, we show that test antigens need not be exactly matched to assess sero-protection in well vaccinated birds. Finally our study suggests that the POL vaccination serves as a useful reference point for following cohorts of layers throughout their production cycle, and thus enabling robust vaccination field effectiveness studies.

Keywords: avian influenza virus subtype H5N1, avian influenza vaccines, haemagglutination inhibition test, highly pathogenic avian influenza, poultry vaccination

INTRODUCTION

The epidemic of H5N1 highly pathogenic avian influenza (HPAI) virus in Indonesia was initially detected in poultry farms in central Java in August 2003 (1). Although the source of the virus has been traced by sequence comparisons back to Hunan province in southern China (2), the mechanism of the introduction has not been definitively determined. Initially it was suspected

to be via migrating birds, but there is evidence that the pathway of introduction of the virus occurred through the transboundary movement of poultry and/or poultry products (3). Following this introduction, the disease spread rapidly, and by 2005 had been detected in poultry flocks in 30 out of Indonesia's 33 provinces (4).

Initially the Indonesian veterinary authorities attempted a stamping out policy, but when the extent of the spread of the disease became clear, this strategy was changed to one of vaccination (4). While vaccination proved successful in controlling the epidemic (5), there are a number of outstanding questions about its sustainability as a control measure (6, 7). Of particular concern is the extent to which antigenically variant strains are induced by vaccination, which in turn may lead to vaccine failures (8). In response to field reports that this might be occurring in Indonesia, Swayne et al. (9) undertook a challenge study using three Indonesian field strains and found that for one of these, *A/chicken/West Java/PWT-WIJ/2006*, all the tested vaccines were ineffective to prevent death in the challenged birds. This was supported by antigenic cartography which showed considerable drift from the then predominant strain used in the locally made vaccines, *A/chicken/Legok/2003*. Nevertheless, there are other reports of continued effectiveness of the vaccines, and this was supported by a field study in West Java demonstrating that vaccination could prevent disease in layer flocks and native chickens (10).

In a pilot survey of vaccination practices we conducted in 2008 in small to medium sized layer and broiler flocks in western Java, we confirmed that vaccination was being routinely used in the layer farms, but not in the majority of the broiler farms, which relied on biosecurity to prevent disease. With respect to the vaccination regimes used on the layer farms, these varied considerably, especially as regards the vaccine used and the number of vaccinations administered. Nevertheless, we did identify a consistent practice of giving pullets a HPAI vaccine at or around the point-of-lay (POL) stage, which was reported to occur between 16 and 20 weeks of age.

Arising from the initial survey a number of questions were posed, and accordingly we undertook a follow-up study with the general objective of providing baseline data to improve the advice on vaccination regimes. Specifically, in the layer flocks, we sought to establish the effectiveness of the POL vaccination to protect the birds during their early laying period. Due to the variety of vaccines being used, we recognized the need to undertake serological assays using antigens identical, or else closely matched to the vaccine strain. This required us to obtain detailed data about the farm management and vaccination practices, and to explicitly frame our study as an integration of field epidemiology and laboratory diagnostics, as well as extending and building on comparable studies being undertaken on vaccine efficacy and effectiveness at the same time within Indonesia (9–11).

Abbreviations: DOC, day-old-chicks; FAO, Food and Agricultural Organization (of the United Nations); HA, haemagglutination assay/haemagglutinin (gene); HI, haemagglutination inhibition (assay/test); HPAI, highly pathogenic avian influenza; LPAI, low pathogenic avian influenza; OFFLU, OIE/FAO influenza (network of expertise); OIE, Office International des Epizooties (World Organization for Animal Health); POL, point-of-lay; RBC, red-blood cells.

MATERIALS AND METHODS

Study Farms

The study was carried out in 15 commercial layer poultry farms, all located in the districts of Sukabumi and Cianjur in the province of West Java, Indonesia (**Figure 1**). These districts have a well-developed poultry industry, being suitably placed to supply the large Jakarta market. Although some of the farms were large, all were considered by the district animal health office staff as Sector 3 under the FAO classification (12). The initial farm sampling visit occurred between December 2009 and January 2010.

The study farms were selected by officials of the local district animal health office ("Dinas Peternakan Kabupaten"), as the unavailability of a listing of commercial poultry farms in the two districts precluded random selection or formal sampling size calculations. However, there was no deliberate selection for any production or health criteria, although implicitly, participating farms tended to have good relations with the local animal health office. Thus, although not a random sample, the flocks were considered by the animal health staff to be representative of the layer farms in the area.

Sampling

For the layer flocks, a structured sampling regime was developed to collect serum before and after the POL vaccination, which based on our pilot survey had been identified to be typically given when the pullets were between 16 and 20 weeks of age (**Table 1**). Farms were contacted to determine when this vaccination was intended to be administered for the next cohort of layers, and then visited ∼1 week before this date (**Figure 2**). From each farm, 11 pullets were randomly selected, this sample size being chosen based on previous experience of estimating flock serological responses. From each bird, 0.5–1.0 ml of blood was collected from the wing (brachial) vein using a needle and syringe. Three to four weeks after vaccination, a second visit was undertaken and the procedure repeated. The birds were not individually marked, and therefore no attempt was made to resample exactly the same birds. However, the birds in the two samplings did belong to the same cohort.

At the time of the first blood sampling, a questionnaire was administered to the farm manager, to obtain data about the flock and the sampled cohort. This questionnaire had two broad sections: the first asking general details of the farm and the management of the pullets and layers, and the second about the HPAI vaccination practice, including the vaccine used for the POL vaccination (**Table 1**).

Vaccines and Panel Antigens

For the 15 sampled farms, we identified seven vaccines being used for the POL vaccination (**Table 2**), and for each of these

FIGURE 1 | The location of the 15 study farms in the districts of Sukabumi and Cianjur in the province of West Java, Indonesia.

TABLE 1 | Summary of the farm survey questionnaire responses with respect to practices relevant to the study.

Farm ID	Layer replacement (and age of purchase if growers/pullets)	Age at commencement of lay (weeks)	POL vaccine administered	Age vaccinations given (weeks)
1	DOC	19	IND_2	4/17
2	DOC	20	CHI_3	5/15
3	DOC	19	MEX_1	6/17
4	DOC	20	CHI_2	4/9/12/17
5	DOC	19	IND_1	2/5/10/17
6	DOC	20	CHI_3	3/8/14/22
7	Growers (12w)	19	CHI_3	nk/14
8	Pullets (14w)	20	IND_2	4/12/16
9	DOC	19	MEX_1	6/17
10	Pullets (14w)	18	IND_1	nk/16
11	DOC and Pullets (14w)	19	CHI_1	DOC: 4/10/18 Pullets: nk/18
12	DOC	19	IND_2	3/9/18
13	DOC	20	CHI_2	4/9/20
14	DOC	19	CHI_3	4/9/20
15	Pullets (14w)	20	MEX_2	4/12/20

DOC, day-old-chicks; nk, not known; POL, point-of-lay.

we determined the registered seed strain by reference to H5N1 vaccine listings (13, 14) or else by direct communications with the vaccine manufacturers. Antigens were then chosen to match these seed strains (**Table 3**), except for the IND_1 vaccine, for which we substituted a near identical isolate, *A/chicken/West Java/SMI-CSLK-EB/2006* (**Table 4**), as this was found to be more stable and thus provided a more consistent titer on culture. In addition to the four homologous antigens, we included in the panel one derived from an isolate representative of the then commonest circulating clade ("2.1.3.1"), *A/chicken/Konawe Selatan/BBVM204(O)/2007* (**Table 3**). Thus in the panel, one antigen was intended to be identical ("homologous") to the seed strain, and the other four non-identical ("heterologous").

Serology

All 330 collected blood samples were transported at room temperature to the Indonesian Research Center for Veterinary Sciences within 12 h. The serum was then extracted from the syringe, and transferred to a 1.8-ml Eppendorf tube, where it was then stored at −20°C. After preliminary testing to confirm the quality of the serum, the samples were then transported to the Australian Animal Health Laboratory (AAHL) where the Haemagglutination Inhibition (HI) tests were performed against the panel of selected antigens (**Table 3**). This testing used the standard AAHL SOP, which broadly follows that outlined in the OIE Terrestrial Manual of Diagnostic Tests (15). In brief, 25 μl of serum was diluted two-fold in PBS, starting from 1:4 and

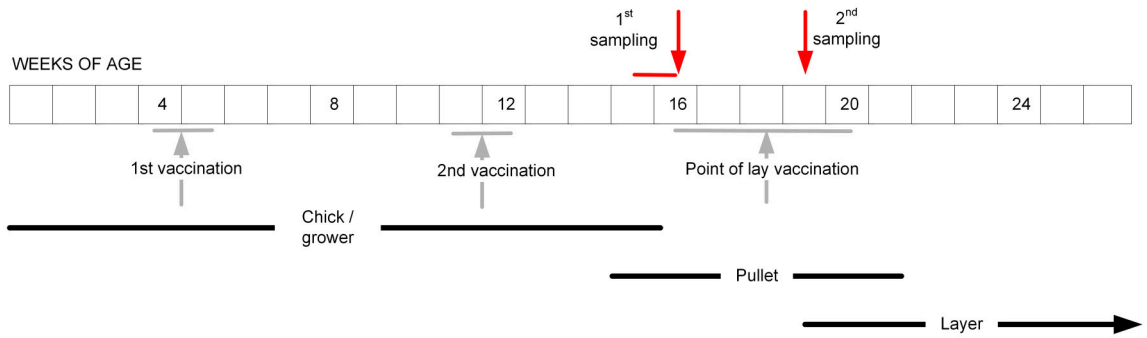

FIGURE 2 | The intended sampling regime for the pullets in the study layer flocks, aiming to occur before and after the administration of the POL vaccine. In practice, the vaccination regimes on the farms turned out to be more variable than indicated (**Table 1**).

TABLE 2 | Anonymised details of the vaccines reported to be used by the farms for the sampled pullets, including the registered seed strain.

Anonymised vaccine name	Country of production	Subtype/pathogenicity	Registered seed strain	Number of farms using
CHI_1	China	H5N2–LPAI	A/turkey/England/N28/1973	1
CHI_2	China	H5N2–LPAI	A/turkey/England/N28/1973	2
CHI_3	China	H5N2–LPAI	A/turkey/England/N28/1973	4
IND_1	Indonesia	H5N1–HPAI	A/chicken/West Java/PWT-WIJ/2006	2
IND_2	Indonesia	H5N1–HPAI	A/chicken/Legok/2003	3
MEX_1	Mexico	H5N2–LPAI	A/chicken/Mexico/232/1994	2
MEX_2	Mexico	H5N2–LPAI	A/chicken/Mexico/232/1994	1

TABLE 3 | Details of the antigens used for the HI test.

HI Antigen	Isolate	Antigen abbreviation	H5N1 Clade	GenBank accession number for the HA gene
Legok/03 H5N1	A/chicken/Legok/2003 (H5N1)	Leg/03	2.1.1	GU052426
CSLK-EB/06 H5N1	A/chicken/West Java/SMI-CSLK-EB/2006 (H5N1)	Cis/06	2.1.3.2	EU124276
Konawe/07 H5N1	A/chicken/Konawe Selatan/BBVM204(O)/2007 (H5N1)	Kon/07	2.1.3.1	Not deposited
England/73 H5N2	A/turkey/England/N28/1973 (H5N2)	Eng/73	n/a	EU636684
Mexico/94 H5N2	A/chicken/Mexico/232/1994 (H5N2)	Mex/94	n/a	AY497096

finishing at 1:2048, in U-bottomed microwell plastic plates and 4 HA units of antigen was added to each well. Following incubation at room temperature for 60 min, 50 μl 0.5% chicken RBC was then added to each well, and the plates were incubated at 4°C for 30–40 min to allow the RBCs to settle. Plates were read and the HI titer was determined as the value of the highest dilution of serum causing complete inhibition of the 4 HA units of virus.

Data Analyses

For each individual HI result, negative titres (<1:4) were re-coded to have a value of "2," and then each individual titer transformed to $\log_2$ titres for further analysis. The individual farm results of the sampled birds HI titres were highly variable, reflecting in part the fact that the sampling at the two visits were not from the same birds. Accordingly, for the purpose of our analysis we treated the HI titer results as a flock test, i.e., where the results are interpreted as estimating a flock-level parameter (16), by taking the median of $\log_2$ titres from the 11 sampled birds as the measure of the flock's serological status before and after the vaccination.

As anticipated, the vaccination caused increases in the median titer in the majority of the farms, but the actual extent of the increase was highly conditional on which test antigen was used. An added complexity was that some of the responses to these test antigens were highly correlated to each other. In order to provide a more complete analysis of the before and after responses, we treated the median responses for each farm to the test antigens as a multivariate response variable. The overall effect of the vaccination response was then tested for a significant increase using a multivariate analysis of variance (MANOVA). As a traditional sum-of-squares MANOVA is dependent upon the assumptions of multivariate normality, which was not shown to be met for our dataset, we conducted a nonparametric permutation MANOVA ("PERMANOVA") to assure against Type I error (17). Aside for testing for an overall significant increase in median farm titres following vaccination, we also

TABLE 4 | Nucleotide percentage identity (green) and amino acid percentage similarity (red) distance matrices for the HA1 genes and proteins of the reported and presumed vaccine seed strains (**Table 1**) and the HI test antigens (**Table 2**).

	Seed strain/HI antigen						
	Leg/03 (%)	**Cis/06 (%)**	**Pwt/06 (%)**	**Kon/07 (%)**	**Eng/73 (%)**	**Mex/94 (%)**	**rgGD/96 (%)**
Leg/03		96.2	96.3	97.4	86.9	79.3	96.1
Cis/06	95.0		99.9	95.7	85.0	77.6	93.0
Pwt/06	95.0	100		95.8	84.9	77.5	92.9
Kon/07	98.4	96.0	96.0		85.6	79.1	93.7
Eng/73	95.7	91.3	91.3	94.7		82.2	89.8
Mex/94	92.9	88.8	88.8	92.2	94.4		80.6
rgGD/96	98.8	93.8	93.8	96.9	96.9	93.8	

Heatmap classes

Nucleotide identity	**Amino acid similarity**
>95%	>97.5%
90–95%	95–97.5%
<90%	<95%

assessed the effect of the provenance of the vaccine on the increase in the titres. Following the detection of a significant provenance effect, we then used orthogonal contrasts to explore whether the Chinese-origin vaccines were significantly different in their responses to the Mexican-origin vaccines, despite both being registered as using H5N2 seed strains (**Table 2**). Finally, to explore the patterns of the HI titer profiles at an individual farm level, we undertook an unsupervised classification of the post-vaccination serological responses for each of the 15 farms, using a hierarchical (agglomerative) cluster analysis, with Ward's linkage used to assess inter-cluster dissimilarity.

All statistical analyses were done within the *R* statistical framework (v. 2.16 or v. 3.0), using either functions from the base or statistics libraries, or else from specialized packages. For the PERMANOVA we used the *Adonis* function of the *vegan* package (version 2.0–10), and for the cluster analysis we implemented the "*hclust*" function of the *cluster* package version 1.15.2.

Bioinformatics Analyses of HI Test Antigens

The clade classification of the H5N1 HI test antigens followed that outlined in the publication by the WHO/OIE/FAO H5N1 Evolution working Group (18). For each of these test antigens, as well as those subsequently presumed to be used as seed strains in the vaccines, a pairwise distance matrix determination was undertaken on alignments of the HA1 gene and its corresponding protein chain (**Table 4**). Nucleotide distances were determined by pairwise identity without assuming a substitution model, and amino acid similarity was calculated using the BLOSUM90 algorithm (19). Alignments and distance matrices were undertaken using *Geneious Pro* version 10.1.3 (www.geneious.com).

Animal Welfare and Ethical Considerations

The sampling of the birds followed the standard practice used for the testing of flocks by the farm managers for their normal sero-monitoring and therefore was treated as a veterinary routine with benefit to the welfare of the birds, as a low titer would result in revaccination and therefore prevention of disease. The questioning of the farm managers was conducted in an ethical framework in which the following conditions applied: (i) the data collected was not of a personal nature; (ii) the data was anonymised before and during publication; (iii) the objectives of the survey were discussed with the farmers beforehand; (iv) taking part in the survey was voluntary and there was no implied pressure to participate; (v) there was no financial or social penalty for not taking part in the survey; (vi) the study was part of disease control research which benefits the farmers; and (vii) the results of the tests on the individual birds–and the wider survey results and their implications for post-vaccination monitoring–were communicated back to the farmers.

RESULTS

General Properties of the Sampled Farms

All the farms were relatively large, with the median number of layers in production being 60,000 birds (range 30,000–138,000). Median egg production per month was 90,000 kg (range 27,000–204,000), with the median egg weight 64 g (range 60–65 g). The predominant breed was "ISA Brown" followed by "Lohmann Brown."

Ten of the farms purchased only DOCs as their replacement strategy, while 4 purchased grower/pullets, and one farm purchased both DOCs and grower/pullets (**Table 1**). For those that purchased pullets, these mostly came from a single supplier, which was different for the five farms.

The median target age for pullets to begin laying was 19 weeks, and the median age for culling was 85 weeks. Premature culling of layers was low with a median of 2% (range 1–10%). Management practices were stable, with 14 of the managers reporting no change over the previous 3 years.

Vaccination Practice for HPAI H5N1

With respect to HPAI H5N1, none of the farms reported outbreaks within the previous 3 years, indicating that the vaccination strategy they had adopted was effective. However, the vaccination regime used on the farms was more variable than indicated in the pilot study, both with respect to its timing and the vaccines used. The predominant practice ($n = 9$ farms) was to give the vaccination at the time of the start of laying, or up to 2 weeks beforehand (**Table 1**). Two farms gave the POL vaccination 1–2 weeks after the start of laying, and 4 farms gave the pre-laying final vaccination more than 4 weeks prior.

For the 11 farms that brought in DOCs (including the single farm that also brought in growers/pullets), 3 gave 4 vaccinations before or shortly after the POL, 4 gave 3 vaccinations and one 4 gave two vaccinations. For the 5 farms that brought in grower/pullets, the HPAI vaccination practice used by the supplier was known for two of these. These both administered a vaccine at 4 and 12 weeks to the growers, and were reported to be the same vaccine as was given by the farm at the POL.

Seven different vaccines were reported to be used by the farms (**Table 2**). These broadly fell within three groups: (1)

Indonesian manufactured vaccines, using Clade 2.1 H5N1 seed-strains that had been isolated from outbreaks on farms in western Java; (2) imported Chinese manufactured vaccines registered as using a H5N2 LPAI seed-strain originally isolated from turkeys in England in 1973; and (3) imported Mexican manufactured vaccines using the *A/chicken/Mexico/232/1994* H5N2 seed-strain. All vaccines were oil emulsion, inactivated vaccines, sold as multi-dose bottles, and sourced from local suppliers. All vaccines were recommended by the manufacturer to be refrigerated when not in use.

All the farms reported to undertake post-vaccination sero-monitoring following the POL vaccination. The exact details regarding this were not asked in the questionnaire, but presumably followed standard practice of sending serum to a private laboratory to determine that the pullets had a titer greater than or equal to 1:16, the threshold which is generally taken to indicate sero-protection for poultry in Indonesia (4, 10, 20).

Flock-Level Serological Responses Before and After POL Vaccination

As assessed by the MANOVA, vaccination resulted in an overall increase in the flock median $\log_2$ titer (from 5.36 to 6.64, a 1.24 fold increase), which was highly significant ($p < 0.01$). This rise was most consistent for the Indonesian vaccines, wherein flock median $\log_2$ titer responses showed a 1.58-fold increase (from 5.22 to 8.27), irrespective of which test antigen was used (**Figure 3**, **Table 5**). The fold increase for the Chinese and Mexican vaccines was less than for the Indonesian vaccines, being 1.11 and 1.35 respectively. However, the Chinese vaccines had a much higher overall pre-vaccination baseline than the vaccines originating from Mexico (6.29 vs. 3.91), which was a highly significant difference ($p < 0.01$). There were however, considerable differences between the responses of the same serum when tested against the different antigens, with the H5N2 test antigens recording pre-POL vaccination titres below that of the international standard for sero-protection against mortality, *viz.* ≥1:32 (15).

The responses to the Chinese vaccines were highly variable (**Figure 3**), with responses intermediate between the Indonesian and the Mexican vaccines. The responses from the farm using the CHI_1 vaccine were similar to those of the farms using the Mexican vaccines, but the farms using the other two Chinese vaccines had responses comparable to the farms using the Indonesian vaccines, as was demonstrated with the hierarchical cluster analysis (**Figure 4**). This was consistent with the results of the orthogonal contrast analysis for provenance, which showed that the overall responses of the Indonesian-origin vaccines did not differ significantly from those originating from China, but these Chinese-origin vaccines were highly significantly different to the Mexican-origin vaccines ($p < 0.01$). Based on the evidence from the two different statistical analyses, it is concluded that two out of the three of the Chinese vaccines (CHI_2 and CHI_3) contained seed strains antigenically closer to H5N1 than the H5N2 for which they were registered, most probably a reverse genetics-generated H5N1 LPAI virus using the *A/goose/Guangdong/1/1996* isolate (rgGD/96) (9).

Adjusting the interpretation of the serological responses for the six farms which used the mislabelled Chinese-origin vaccines, it is possible to assess the capability of the vaccine seed strains to induce immunity in the early layer stage (i.e., after the POL vaccine) allowing for both the effect of the different test antigens and the two accepted thresholds for sero-protection (**Tables 5**, **6**). This shows that all the 5 farms using the locally produced Indonesian vaccines had a median flock titer ≥1:32, which is the minimum recommended threshold to prevent mortality following exposure to HPAI viruses (15). Furthermore, this strong response to these vaccines were seen irrespective of which of the three H5N1 test antigens were used. Similarly there were strong responses to the vaccines surmised to contain rgGD/96, all producing median titres ≥1:32 when assessed using the H5N1 test antigens. However, for some of the Indonesian and Chinese H5N1 containing vaccines, the titres when assessed against the H5N2 test antigens were in the intermediate sero-protective range (i.e., ≥1:16, but <1:32), which undoubtedly reflects the antigenic distance between the subtypes (**Table 4**). Regarding the flocks which were vaccinated with H5N2 containing vaccines, one of these (Farm 3) had a titer below the sero-protective threshold when assessed against three of the test antigens, including the one to which it was homologous (Mex/94). This was not seen in the other flocks using the H5N2 vaccines, and may reflect other factors not accounted for in our analysis (due to insufficient replication) such as the timing and number of vaccinations (**Table 1**).

Our use of a panel of antigens allows an assessment of the practice of the Indonesian testing laboratories of using a single standardized HI test antigen to assess the flock level of sero-protection, at both the lower (1:16) and upper (1:32) thresholds (**Table 7**). When the classification using standardized antigen (Leg/03) is compared with the commonest circulating strain (Kon/07), there was agreement for all flocks at the 1:16 threshold, and all but one for the 1:32 threshold, the latter being for flock 11, which used the CHI_1 vaccine containing a H5N2 antigen. Comparing the Leg/03 test antigen against the homologous test antigen, which would be expected to give the most accurate titer (21) is made complex by not having data for the presumed rgGD/96 containing vaccines, but for the 9 farms for which the homologous antigen data was available, the predictive value of the standardized antigen was similarly very high.

DISCUSSION

The HI test for the assessment of vaccine induced immune responses in poultry has a long history of usage, dating back over 50 years (22). By the time the H5N1 HPAI panzootic strain appeared in Hong Kong, it was a mature test, and thus used to assess the effectiveness of vaccination to prevent onward transmission of the disease (23). However, this use of the HI test describes a situation where the causative strain of the outbreak, the exact seed strain of the vaccine, the timing of the vaccination and prior exposure of the vaccinated birds are all known. A much more complex situation occurs when the disease is endemic, such as in Indonesia, where many of

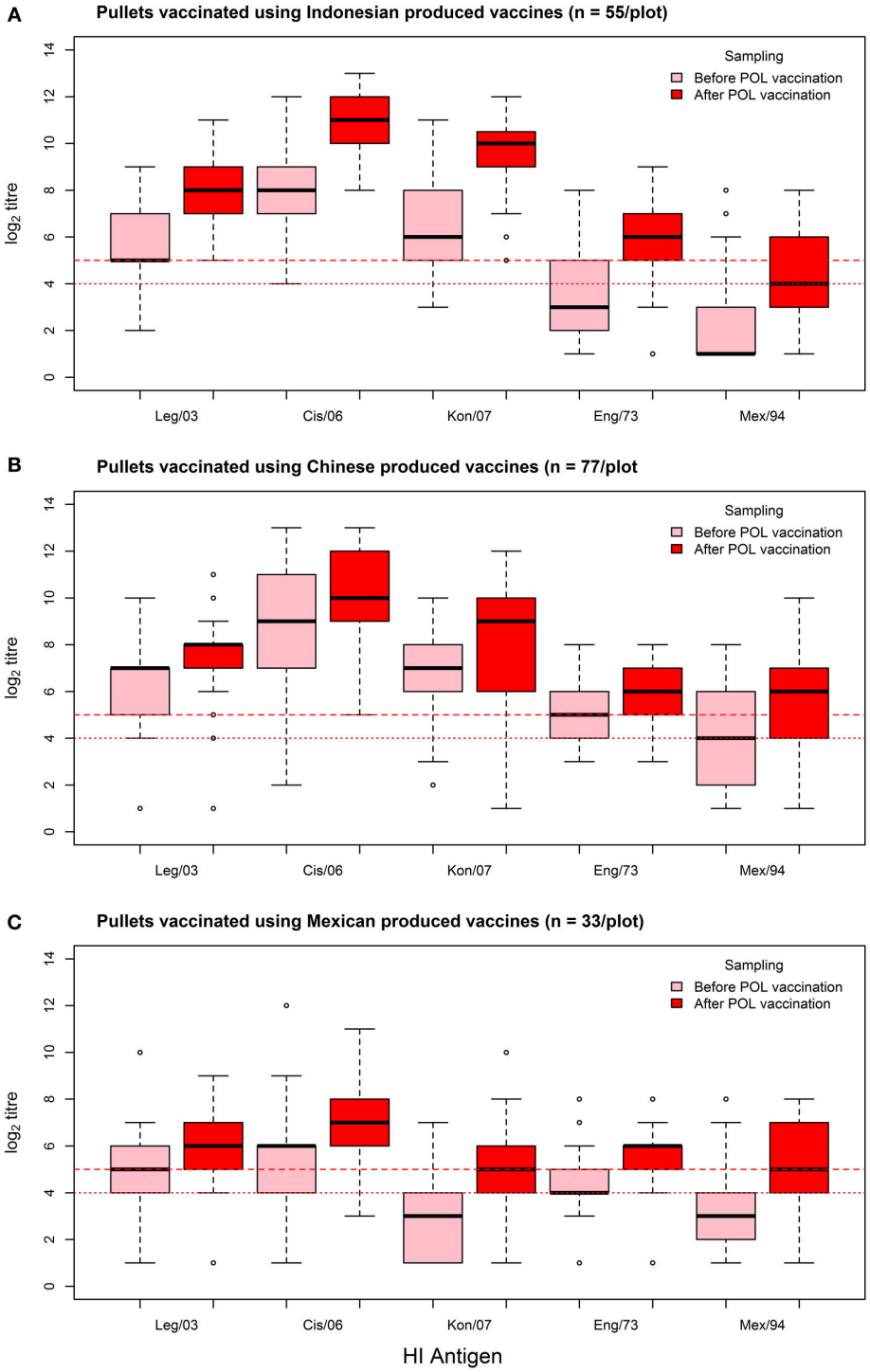

FIGURE 3 | Box-plots of individual bird $\log_2$ titer responses against the panel of HI test antigens for the sampling before and after the POL vaccination, grouped according to the country of origin of the POL vaccine: **(A)** Indonesian vaccines, **(B)** Chinese vaccines, and **(C)** Mexican vaccines. Threshold titres for partial ($1{:}2^4$) and full sero-protection ($1{:}2^5$) are indicated with red dotted and red dashed lines respectively.

these variables might either not be known, or else subject to a degree of uncertainty. The challenge is to develop methods to assess the field performance of HPAI vaccination in the endemically infected countries, which at the time of our study, and to a degree to this day, remains an under-researched topic (24–27).

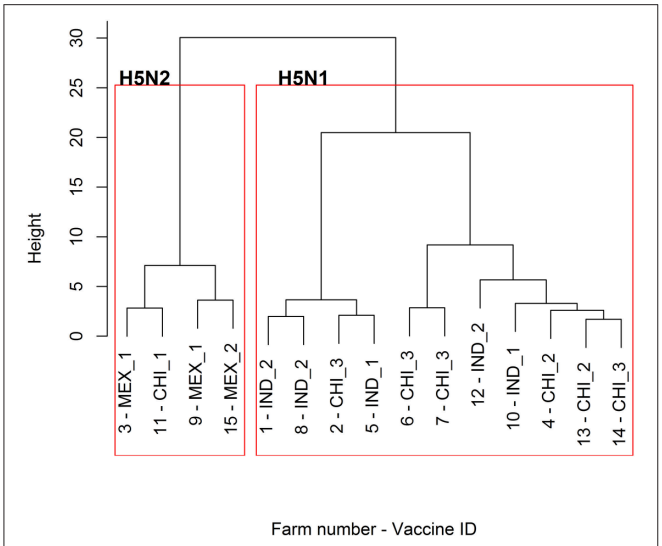

FIGURE 4 | Dendrogram of the hierarchical cluster analysis of the HI profiles of the 15 sampled farms. For the provenance of the vaccines used for the POL vaccination on each of the farms, refer to **Table 2**.

The approach we took was to assume that the vaccination system developed by the Indonesian poultry farmers, based on vaccinating the young birds several times before the commencement of laying, might be "fit for purpose," and to make an objective assessment of its performance. For this, we developed a sampling regime centered on the POL vaccination, based on the assumption that this would be of most interest to the participating farms, and therefore achieve greater farmer cooperation. Furthermore, we developed an Indonesian language questionnaire survey to ensure the capture of quality data of the management and vaccination practices. Using this data we were then able to select antigens which corresponded to the registered seed strain of the POL vaccine, and then systematically compare responses to a panel of homologous and heterologous antigens.

In retrospect it can be seen that the limitation of this study design was the presumption that the seed strain used in the POL vaccine would equate with that to which it was registered, and because mislabelled vaccines were used in 6 of the 15 sampled farms, this made interpreting the resulting post-vaccination responses initially problematic. Through a combination of careful rechecking the farm questionnaire data, repeating the HI testing using newly obtained H5N2 antigens and undertaking detailed statistical analyses we arrived at the hypothesis that some of the Chinese origin vaccines contained a H5N1 antigen. This hypothesis was subsequently confirmed with the publication by Swayne et al. (9), reporting that some of Chinese origin vaccines being used in Indonesia at the time were found by sequencing to contain a seed strain using an antigen derived from *A/goose/Guangdong/1/1996* (H5N1).

The use of these mislabelled vaccines is now largely of historical interest, as in 2012 the Indonesian veterinary authorities prohibited the further use of imported vaccines. However, this example does illustrate the capability of the HI test, when used against a panel of homologous and heterologous antigens, to reconstruct the previous exposure of birds to vaccines. Although not unexpected, this is as far as we are aware the first time this result has actually been shown. A more practical learning from this vaccine mislabelling is that if inconsistent results are found in vaccine efficacy or effectiveness trials, then sequencing of the vaccine (and possibly the test antigen) is recommended.

Once the test results could be reinterpreted with the true or probable vaccine seed strain, then it was possible to confirm that those flocks in which a H5N2 vaccine was used had significantly lower median titres than those using a H5N1 seed strain. The use of H5N2 (and H5N9) vaccines had initially been recommended for use in Indonesia on the basis of the experience in Italy during an outbreak of H7N1 LPAI/HPAI between 1999 and 2001, where the application of a heterologous vaccine enabled the differentiation of vaccinated from infected flocks, and this assisted in proving freedom from disease in the latter stage of eradication (28). Our post-vaccination serological results are consistent with those obtained in the experimental challenge trials using H5N2 vaccines available in Indonesia (9) and thus provide additional evidence that the use of heterologous vaccines are suboptimal for the H5N1 endemically infected countries, and if a DIVA strategy is desired, then this would be best to be based on other testing methodologies (29, 30).

Implications for Farm-Level Sero-Monitoring in Endemically Infected Countries

A learning from our field sampling that remains relevant to the Indonesian situation—and also to those endemically infected countries with multiple circulating HPAI viruses—is the appropriate selection of test antigens for the HI test in response to evolving H5N1 strains. The use of a panel of antigens in our study was based on the recommendation that only homologous antigens would provide a true estimate of the post-vaccination titer, and that heterologous antigens would give an underestimate (21). While obtaining accurate titres is important when vaccines are being assessed for both vaccine efficacy (31) and field effectiveness trials (32), they are of lesser direct interest when the purpose of the HI testing is simply to determine if the flock has an adequate post-vaccination level of sero-protection. This was clearly demonstrated in this study, where for the H5N1 vaccines, there was little difference in predictive value of a single standardized antigen as compared to the classification of the flocks using a homologous antigen and using an antigen matching the current circulating strain (**Table 7**). This has beneficial implications for the testing laboratories as using a single, standardized antigen is significantly more practical than varying the test antigen according to the vaccine used, as this avoids the complexity of keeping in stock various test antigens whilst assuring their quality over time. A similar argument applies to avoiding the need to change the test antigen to reflect the frequent identification of genetically variant H5N1 isolates, which have now been documented in all the endemic countries (18, 33–35). Nevertheless, there will be a need to carefully

TABLE 5 | Median pre- and post-POL vaccination HI log_2 titres for the 15 farms sampled in the study.

Farm ID	POL vaccine probable seed strain	Pre-POL vaccination median HI titer					Post-POL vaccination median HI titer				
		Leg/03	Cis/06	Kon/07	Eng/73	Mex/94	Leg/03	Cis/06	Kon/07	Eng/73	Mex/94
1	Leg/03 (H5N1)	**4.55**	6.45	5.36	2.09	1.09	**7.82**	10.36	9.27	5.00	3.82
2	rgGD/96 (H5N1)	5.18	6.73	5.55	3.55	1.82	8.27	10.45	9.00	5.82	5.36
3	Mex/94 (H5N2)	5.36	6.18	3.91	5.45	**4.27**	3.91	5.55	3.91	5.00	**3.91**
4	rgGD/96 (H5N1)	6.64	8.64	6.73	4.64	3.45	8.45	11.00	9.45	6.91	6.64
5	PWT-WIJ/06 (H5N1)	4.64	**6.64**	5.18	2.73	1.64	8.55	**11.27**	10.27	6.09	4.45
6	rgGD/96 (H5N1)	8.27	11.09	7.73	6.36	5.82	7.45	10.55	7.18	5.55	5.27
7	rgGD/96 (H5N1)	7.20	9.45	7.73	5.91	5.36	6.91	9.18	6.27	4.91	4.55
8	Leg/03 (H5N1)	**4.91**	7.64	6.27	2.09	1.09	**6.64**	10.45	9.09	4.73	3.64
9	Mex/94 (H5N2)	3.18	3.73	1.91	3.82	**2.18**	6.36	7.36	5.18	6.09	**5.45**
10	PWT-WIJ/06 (H5N1)	7.64	**10.45**	8.27	6.55	5.18	8.64	**11.64**	9.91	6.73	5.55
11	Eng/73 (H5N2)	5.36	6.27	4.73	**4.73**	3.27	5.82	6.45	4.73	**4.55**	3.27
12	Leg/03 (H5N1)	**6.73**	9.36	8.00	3.82	2.09	**8.27**	10.91	9.91	5.00	4.09
13	rgGD/96 (H5N1)	6.64	9.18	7.91	5.73	4.73	8.36	10.91	10.00	6.64	6.00
14	rgGD/96 (H5N1)	6.45	9.36	7.73	5.36	4.82	8.64	11.82	10.45	7.00	7.18
15	Mex/94 (H5N2)	5.09	5.91	2.55	4.00	**3.91**	6.45	8.18	5.55	5.55	**5.91**

Homologous titres (i.e., where the HI test antigen titer matches the probable vaccine seed strain) are indicated by bold, highlighted text, except for the Chinese origin vaccines that were subsequently determined to contain a seed strain using A/goose/Guangdong/1/1996 (rgGD/96), as this conclusion was reached subsequent to the serological testing.

TABLE 6 | Summary of median HI responses for the 15 sampled farms provided in **Table 5** with respect to two thresholds for sero-protection (1:16 and 1:32) and three scenarios of matching between the test antigen and the probable seed strain used in the POL vaccine.

Seed-strain vs. test antigen scenario	Sampling	Number of farms having a median HI titer above the two accepted thresholds for the HI test	
		≥1:16	≥1:32
Single standard test antigen (Leg/03) (n = 15 farms)	Pre-POL vaccination	14/15	11/15
	Post-POL vaccination	14/15	14/15
Test antigen matched to the vaccine seed strain (n = 9 farms)	Pre-POL vaccination	7/9	3/9
	Post-POL vaccination	8/9	3/9
Test antigen corresponding to presumed circulating strain at time of study (Kon/07) (n = 15 farms)	Pre-POL vaccination	12/15	11/15
	Post-POL vaccination	14/15	13/15

TABLE 7 | Cross tabulation of results of the post-POL vaccination serology provided in **Table 5** comparing the classification of the sampled flocks H5N1 HPAI sero-protective status using a standardized test antigen (Leg/03) against: **A**. one of the then commonest circulating strains (Kon/07); and **B**. the antigen homologous to the vaccine seed strain.

A. Leg/03 vs. Kon/07 AT TWO THRESHOLDS OF POSITIVITY (n = 15 FARMS) AT TWO THRESHOLDS FOR POSITIVITY

1. Threshold for positivity: ≥1:16		Circulating strain (Kon/07)		2. Threshold for positivity: ≥1:32		Circulating strain (Kon/07)	
Leg/03		NEG	POS	Leg/03		NEG	POS
	NEG	1	0		NEG	1	0
	POS	0	14		POS	1	13

B. A. Leg/03 vs. HOMOLOGOUS TEST ANTIGEN AT TWO THRESHOLDS OF POSITIVITY (n = 9 FARMS)

1. Threshold for positivity: ≥1:16		Homologous test antigen		2. Threshold for positivity: ≥1:32		Homologous test antigen	
Leg/03		NEG	POS	Leg/03		NEG	POS
	NEG	1	0		NEG	1	0
	POS	0	8		POS	1	7

Each cross-tabulation comparison uses the two thresholds applied to the HI test, i.e., ≥1:16 and ≥1:32.

monitor the evolution of the predominant circulating virus and determine if it has diverged sufficiently to warrant a change. In the specific case of Indonesia, this is assisted by the creation of an avian influenza virus laboratory network, supported by a web-enabled database system, *IVM Online* (36), but for the endemic countries without comparable systems, approximate monitoring might be based on a bioinformatics analysis of the HA1 sequence (**Table 4**).

A second learning that has relevance beyond the Indonesian situation is that in layers the POL vaccination defines a reference point for the assessment of the effectiveness of HPAI vaccination throughout the birds' production cycle. Being able to define such a reference point is essential, because as was shown from our questionnaire survey (**Table 1**), the small to medium commercial layer sector engages in a diversity of practice, with respect to the vaccines used, the number administered and the timing of the vaccination. However, after the POL vaccination, the majority of the birds—with the exception of those being vaccinated with the H5N2 seed strain—had titres >1:32 and thus it becomes possible to make comparisons between flocks of vaccination parameters, such as the length of time the birds were protected, and the sero-protection status at the time of their culling. This possibility was in fact realized in a follow-on study, using some of the same farms reported on here, in which we were able to make a detailed assessment of the field effectiveness of HPAI vaccination by following cohorts of bird individually marked and resampled (32).

Finally, it needs to be stressed the limitation of our study with respect to understanding the effect of variables other than the seed strain in determining the HI titer in the pullets, *viz*. the type of vaccine, age at vaccination, number of vaccinations, the interval between vaccination and sampling etc. As was found, the farms used a diversity of vaccination practices (**Table 1**), and the relatively small sample size of our study precluded a detailed analysis of these. We do however, fully recommend that future studies explore the impact of vaccination practice on vaccine responses, both through more systematic field studies as well as complementary laboratory trials.

CONCLUSION

Whilst the general principles for implementing sero-surveillance for the endemically infected countries relying on vaccination as the principal method of control are now established (7, 14, 27), it is also clear that each of these countries have unique challenges that require such sero-surveillance to be customized taking into account the specifics of the poultry production system, vaccination practices and permissible vaccines (5, 6, 37). This study assessing the use of the HI test system for POL pullets, and the follow-on one assessing sero-protection during and at the end of the layer production cycle (32), contribute to the evidence-base on which to provide recommendations for the commercial layer sector. It is however evident that developing robust sero-monitoring and sero-surveillance programs is a complex problem for which further research, both in Indonesia and beyond, is required.

AUTHOR CONTRIBUTIONS

PD: study design, data analysis, interpretation of results, writing of draft; RI: study design, farm visits, and specimen collection, data input; laboratory testing, interpretation of results; PS: laboratory testing, interpretation of results, editing of draft; AA: study design; TS: study design, farm visits, and specimen collection; data input; JI: study design, interpretation of results.

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