Surveillance for early detection of low pathogenicity avian influenza in poultry

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Surveillance for early detection of low pathogenicity avian influenza in poultry

Surveillance voor vroege opsporing van laagpathogene
aviaire influenza bij pluimvee

(met een samenvatting in het Nederlands)

Proefschrift

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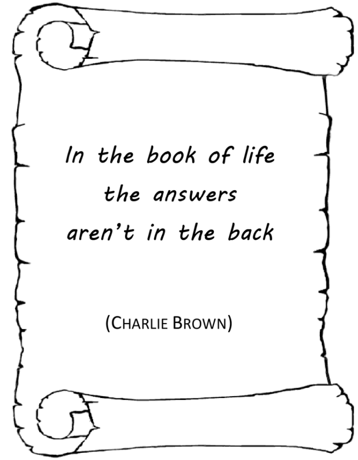
In the book of life
the answers
aren't in the back
(Charlie Brown)

CONTENTS

CHAPTER 1	General introduction	9
CHAPTER 2	Design and results of an intensive monitoring programme for avian influenza in meat-type turkey flocks during four epidemics in Northern Italy	17
CHAPTER 3	Epidemiology and control of low pathogenicity avian influenza infections in rural poultry in Italy	37
CHAPTER 4	Transmission dynamics of low pathogenicity avian influenza infections in turkey flocks	53
CHAPTER 5	Serological diagnosis of avian influenza in poultry: is the haemagglutination inhibition test really the “gold standard”?	75
CHAPTER 6	Evaluating surveillance strategies for the early detection of low pathogenicity avian influenza infections	91
CHAPTER 7	General discussion	109
ADDENDUM	Summary	122
	Samenvatting	126
	Curriculum vitae	129
	List of publications	130
	Acknowledgments	133

CHAPTER 1

General introduction

BACKGROUND ON AVIAN INFLUENZA VIRUSES

Avian influenza (AI) viruses belong to the Influenzavirus A genus of the Orthomyxoviridae family and can be divided into subtypes based on the antigenic relationships of their hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins. A total of 16 different HA subtypes (H1-H16) and 9 different NA subtypes (N1-N9) have been identified [19]. Virtually all HA and NA combinations have been isolated from birds, especially from waterfowl which are responsible for the perpetuation of these viruses in nature [2].

Influenza A viruses can be further divided into two distinct groups on the basis of their ability to cause disease, in particular, those that cause highly pathogenic avian influenza (HPAI), which are very virulent and can result in 100% flock mortality in poultry, and those that cause low pathogenicity avian influenza (LPAI), a much milder, primarily respiratory disease [7]. HPAI viruses can only be found among subtypes H5 and H7, although not all viruses that belong to these subtypes cause HPAI. The distinction between HPAI H5 and H7 subtypes and LPAI H5 and H7 subtypes is made on the basis of the mortality rate of the virus, as determined with an intravenous lethality test, and on whether multiple basic amino acids are present at the cleavage site of the precursor of the HA protein [29]. In fact, the HA of AI viruses requires post-translational cleavage by proteases of the host before it becomes functional and virus particles are infectious [14]. The cleavage motifs of LPAI viruses typically have only two basic amino acids, which limits the cleavage of the HA precursor to host proteases present in the respiratory and intestinal tracts. By contrast, the cleavage motifs of HPAI viruses have multiple basic amino acids, which allows the HA precursor to be cleavable by ubiquitous host proteases, enabling HPAI viruses to replicate systemically and damage vital organs and tissues [19].

After an LPAI virus has been introduced in poultry, it can mutate into an HPAI virus. [4,5]. Several genetic mechanisms seem to be responsible for this mutation, such as the insertion or substitution of basic amino acids at the HA cleavage site, due to a transcription fault of the polymerase complex [3]. However, the factors that bring about mutation from LPAI to HPAI are still not known, and it is impossible to predict if and when this mutation will occur. In some cases, mutation has been shown to occur at the primary site soon after the LPAI virus is introduced in poultry flocks from wild birds; in other cases, the LPAI virus circulated in poultry for months before mutating; in still other cases, mutation did not occur at all, though the possibility that it would have occurred eventually cannot be excluded. In any case, it can be reasonably assumed that the wider the circulation of LPAI in poultry, the higher the chance of mutation occurring [3].

BASIC PRINCIPLES OF AVIAN INFLUENZA CONTROL

The control of avian influenza has three main goals: prevention, management, and eradication [25]. The specific goal greatly depends on the infection status of the given area. If the area is free from AI, the goal should be to prevent the introduction of the virus from a wild bird reservoir or from infected poultry in an affected area. If there are infected flocks in the area, the goal should be disease management, followed by disease eradication [24].

Prevention is essentially based on biosecurity and surveillance. The term “biosecurity” refers to all procedures aimed at either reducing the likelihood of the introduction of the virus in a naïve population (bioexclusion) or, once the infection is present, at reducing the likelihood of spread to other premises or areas (biocontainment) [13]. At the farm level, biosecurity involves physical and temporal barriers to keep potentially infected animals and materials away from uninfected birds, together with cleaning and disinfection procedures to reduce environmental contamination [6]. The term “surveillance” is often used as a synonym for “monitoring” in animal health programs. Actually, monitoring is a continuous, adaptable process of collecting data about diseases and their determinants in a given population, without any immediate control activities, whereas surveillance is a specific type of monitoring in which control or eradication measures are implemented whenever certain threshold levels related to the infection or disease status are exceeded [22]. However, for the sake of simplicity, the two terms will be used interchangeably in this thesis. The purposes and the implementation of AI surveillance vary according to the goals of AI control. If the goal is prevention, surveillance is critical for promptly identifying new introductions of AI viruses and stopping the infection before a major epidemic occurs.

The management of AI infection is a process of decreasing the spread of the disease by reducing infection rates and allowing production to continue [24]. For example, the depopulation of infected poultry holdings may limit the spread of AI by decreasing the amount of virus released from the holding, though it cannot prevent it completely because some virus will have been released before culling and often before the disease is detected [13]. Other measures for interrupting the infection cycle include movement restrictions of birds and products (i.e., reducing the likelihood of transmission), controlled marketing (i.e., reducing bird density) and vaccination (i.e., decreasing the susceptibility of the poultry population) [8,16,18]. Finally, eradication, which is ideally the ultimate goal of AI control, is the complete elimination of the AI virus from an infected area [24].

CONTROL OF AVIAN INFLUENZA IN THE EUROPEAN UNION

Control measures for AI were first harmonized at the European Union (EU) level in 1992 by Council Directive 92/40/EEC [10], which established that a stamping-out policy should be applied for all HPAI outbreaks. According to this directive, suspected cases of HPAI infection in poultry had to be investigated, and in the case of confirmed cases, specific measures had to be applied to limit the spread of infection. In particular, the measures to be adopted at infected farms included: the stamping out and disposal of all poultry in the affected holdings; the cleaning and disinfection of the holding; and the destruction of contaminated equipment and manure. Moreover, veterinary authorities were required to conduct periodic clinical inspections in farms located within a radius of at least 3 km of the infected holding (known as “the protection zone”) and to implement movement restrictions on affected holdings and on all poultry farms located within a radius of at least 10 km (“the surveillance zone”). If no further outbreak occurred, it was possible to lift the control measures 30 days after the cleaning and disinfection of the infected premises [10].

For a number of years following the adoption of this directive, no major HPAI outbreaks occurred in the EU and the disease control measures were rarely implemented. However, the devastating HPAI crisis that began in late 1999 in Italy constituted a turning point, in that a low pathogenic LPAI virus strain of the H7N1 subtype, which had circulated in the densely populated poultry area of northern Italy for several months, eventually mutated into an HPAI virus, and the massive spread of the infection led to the culling of approximately 16 million birds to control the epidemic [20]. Following the 2003 Dutch H7N7 HPAI epidemic and an extensive debate with the veterinary authorities of the Member States and other stakeholders, Directive 92/40/EEC was replaced by Council Directive 2005/94/EC [11] to take into account the practical experiences gained in dealing with outbreaks [21]. The new directive also introduced notification and control measures for LPAI H5 and H7 subtypes. While the new directive has maintained the principle of a stamping-out policy, it allows for more flexibility in adapting the control measures to the prevailing epidemiological situation. For example, based on a risk assessment performed by the veterinary authorities in the given Member State, control measures can be applied more stringently, for instance, by applying a temporary ban for all movements of live poultry in the entire country and by culling poultry upon suspicion of disease without awaiting confirmation, as well as pre-emptive culling of poultry in holdings situated in the surrounding area. This aggressive approach will be mainly necessary in cases in which the AI virus is introduced in an area with a very dense poultry population, where it would probably spread extensively and explosively. The directive also allows for derogations from the culling of poultry and birds belonging to endangered species kept in non-commercial holdings, zoos or circuses where HPAI or LPAI has been confirmed, provided

that these birds be quarantined and subjected to laboratory testing until the risk of transmission is considered insignificant [11].

In the new directive, vaccination against AI plays a much more prominent role. In addition to its use in emergency situations to limit the spread of infection, it can now be applied as a preventative tool for poultry holdings deemed to be at higher risk for disease introduction (i.e., poultry in free-range farms or susceptible species such as turkeys). For both emergency and preventive vaccination, a “DIVA strategy” must be adopted (i.e., differentiating infected from vaccinated animals). Furthermore, to detect possible virus circulation in vaccinated poultry, comprehensive active surveillance of these poultry flocks must be implemented; this entails the use of diagnostic tests designed to detect antibodies against the field virus and the testing of unvaccinated sentinel birds [21].

SURVEILLANCE OF AVIAN INFLUENZA

Surveillance is the key to early warning of a change in the health status of a bird population (i.e., both poultry and wild birds). The goals of surveillance can vary depending on the infection status of the given country. In disease-free countries, the main objectives of surveillance are to detect incursions of disease as early as possible and to demonstrate the disease-free status to trading partners. In infected countries, the main objectives of surveillance are: 1) to describe the spatial and temporal distribution of the disease; 2) to assess the effectiveness of vaccination campaigns and other control measures; 3) to monitor antigenic drift; and 4) to achieve early identification of new virus incursions [24].

With regard to how surveillance is performed, the specific strategy varies from country to country. However, it generally includes a combination of the passive and active surveillance of wild and domestic bird populations. In wild birds, surveillance focuses on testing for antigens in bird species that are more susceptible (especially waterfowl), either found dead (passive surveillance) or hunted (active surveillance) [17]. In poultry, passive surveillance relies on the observation at the flock level of signs that could be indicative of infection (e.g., increased mortality, reduced feed and water consumption, reduced egg production, and respiratory symptoms). Active surveillance entails visiting farms and sampling animals for diagnostic tests, which can be performed either for detecting the disease by testing for viral antigens (virological surveillance) or for detecting the infection by looking for antibodies (serological surveillance) [29].

For HPAI, passive surveillance has been successful in early detection (e.g., Germany, 2007 [28]), given that infection induces clear clinical signs and high mortality in most poultry species [23]. However, for LPAI, the signs may go unnoticed; thus active surveillance should be carried out [12]. In the EU, current LPAI surveillance focuses on determining yearly the presence of infections with subtypes H5 and H7 in different

poultry species, rather than on the early detection of new incursions [9,11]. To this end, active (serological) surveillance has been based on the annual sampling of poultry holdings, with the aim of detecting a fixed design prevalence with a certain probability (i.e., assuming a design prevalence of 5% LPAI infected holdings and 30% infected animals within an infected holding). Although this strategy can be used to establish the presence of infected birds or freedom from infection, it does not take into consideration the dynamics of the infection in the population and may result in missed or delayed detection [15].

AIM AND OUTLINE OF THIS THESIS

The timeliness, effectiveness, and cost of AI control during an epidemic greatly depend on how soon the first cases are diagnosed, the level of biosecurity in the area, and how quickly control strategies can be implemented [24]. To promptly detect the introduction of an LPAI virus in poultry populations and thus prevent it from spreading before it mutates into an HPAI virus, active surveillance should be carried out. However, in the EU, active surveillance is currently based on classical sampling theory, which does not consider the dynamics of the infection and may thus result in missed or delayed detection, especially for LPAI. Although both the number of Member States that have implemented AI surveillance and the number of samples tested have increased over the years [15], evaluations of the effectiveness of LPAI surveillance have mainly focused on their performance in establishing the presence of infected birds or freedom from infection [1,27] and not on their performance as early warning systems for new introductions. To date, an optimal design for early warning surveillance has not been defined.

The main goal of this thesis was to develop a framework of surveillance strategies for the early detection of LPAI infections in poultry. As a starting point, the components of the Italian surveillance system and its resources were evaluated in terms of the capacity to detect LPAI outbreaks in poultry, in order to have a baseline of the current surveillance activity in the country with the longest experience in surveillance and outbreaks (**CHAPTER 2**). The involvement of the rural poultry sector in the 2007-2009 Italian LPAI epidemics was then investigated, in order to better understand the possibility that this sector will act as a source of LPAI virus introduction and amplification (**CHAPTER 3**). In fact, although the concern for AI infection is greatest for industrial poultry, the potential role of the rural poultry sector in the maintenance and spread of LPAI viruses should not be overlooked, as demonstrated by the circulation of H5 and H7 LPAI viruses for more than 10 years in the rural poultry premises belonging to the live bird market circuit in the New York City

area [26]. The next step was to provide quantitative information on key epidemiologic parameters of LPAI dynamics. The within-flock transmission of LPAI was investigated by means of simulation models fed with serosurveillance data from past epidemics in Italy and experimental infection data with outbreak strains (**CHAPTER 4**). Given that surveillance involves the detection of disease or infection based on the results of diagnostic tests, their accuracy may have an impact on the conclusions derived from surveillance itself. Therefore, the sensitivity and specificity of some diagnostic assays for detecting AI antibodies were estimated (**CHAPTER 5**). Information on baseline surveillance activities, within- and between-flock transmission of LPAI, and accuracy of diagnostic tools was entered into a simulation model to evaluate different surveillance strategies in terms of their performance as an early warning system (**CHAPTER 6**). Finally, the results, implications, and critical elements presented in the previous chapters are integrated and discussed (**CHAPTER 7**). The ultimate goal of this thesis is to provide new insight into the surveillance of LPAI infection, so as to improve and prioritize programs for early detection in industrial poultry.

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CHAPTER 2

Design and results of an intensive monitoring programme for avian influenza in meat type turkey flocks during four epidemics in northern Italy

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ABSTRACT

Surveillance programs for low pathogenicity (LPAI) and high pathogenicity avian influenza (HPAI) infections in poultry are compulsory for EU Member States, yet these programs have rarely been evaluated. In Italy, following a 1999 HPAI epidemic, control measures, including vaccination and monitoring, were implemented in the densely populated poultry area (DPPA) where all epidemics in Italy have been concentrated. We evaluated the monitoring system for its capacity to rapidly detect outbreaks in meat-type turkey flocks.

The evaluation was performed in vaccination areas and high-risk areas in the DPPA, in 2000-2005, during which four epidemics occurred. Serum samples and cloacal swabs were taken from vaccinated birds and unvaccinated ("sentinel") birds. We compared the detection rate of active, passive, and targeted surveillance, by vaccination status, using multinomial logistic regression.

A total of 13,275 samplings for serological testing and 4,889 samplings for virological testing were performed; 6,315 production cycles of different bird species were tested. The outbreaks detection rate in meat-type turkeys was: 61% for active surveillance (n=222/363 outbreaks), 32% for passive surveillance, and 7% for targeted surveillance. The maximum likelihood predicted values for the detection rates differed by vaccination status: in unvaccinated flocks, it was 50% for active surveillance, 40% for passive surveillance, and 10% for targeted surveillance, compared to, respectively, 79%, 17%, and 4% for vaccinated flocks.

Active surveillance seems to be most effective in detecting infection, especially when a vaccination program is in place. This is the first evaluation of the effectiveness of different types of surveillance in monitoring LPAI infections in vaccinated poultry using field data.

KEY WORDS

Avian influenza, surveillance, epidemics, field data

INTRODUCTION

Infection with highly pathogenic avian influenza (HPAI) viruses in poultry has had an enormous impact worldwide, causing massive epidemics involving millions of birds and resulting in great economic losses. It has also had implications for human health, with over 550 human cases reported, a proportion of which have been fatal [21].

Only avian influenza strains that carry haemagglutinin 5 or 7 (H5 or H7) with defined genome sequences are considered to be highly pathogenic [1, 16]. Outbreaks of HPAI most likely originate from low pathogenicity avian influenza (LPAI) strains of subtypes H5 or H7 circulating among wild waterfowl [1]. For example, in Italy, an outbreak of an LPAI virus (H7N1) began in the first half of 1999, and by the end of the year the virus had mutated into an HPAI virus [13]. In Chile, an LPAI virus (H7N3) circulating on a broiler farm in 2003 mutated to an HPAI virus within one month [18].

Following these outbreaks, in 2005 the World Organisation for Animal Health included LPAI infections caused by H5 and H7 subtypes among notifiable diseases (OIE, 2005). Given that outbreaks of infection with these subtypes must now be reported, surveillance programs for LPAI and HPAI have become compulsory for all Member States of the European Union (EU) [8, 11]. EU legislation also allows Member States to perform vaccination when there is a risk of the spread of infection, and this possibility needs to be considered in defining surveillance programmes. However, an optimal design for surveillance programs has not been defined, and the few evaluations of their effectiveness have mainly focused on their sensitivity in assessing freedom from disease [20], and not on the ability to promptly detect AI outbreaks.

In the EU, Italy is the country that has had the greatest number of outbreaks, which have spanned the longest period of time. From 1997 to 2005, there were seven waves of epidemics of various H5 and H7 subtypes, most of which occurred in the country's only densely populated poultry area (DPPA). Following the devastating H7N1 HPAI epidemic of 1999, which resulted in the death or slaughtering of 16 million birds, in 2000 a series of control measures began to be applied in this DPPA also in case of detection of LPAI of H5 and H7 subtypes. These measures included emergency and prophylactic vaccination [4] and an intensive monitoring system for AI, the first in the EU, which consisted of the routine laboratory testing of poultry farms located in the vaccination areas and high-risk areas in the DPPA. The objectives of the monitoring system were to verify the AI-free status of the vaccinated and unvaccinated poultry populations and to promptly detect newly infected farms. Since the system was made operational, four major LPAI epidemics have occurred, in particular, in 2000-2001, 2002-2003, 2004, and 2005, followed by two small epidemics in 2007 and 2009 mainly involving rural flocks.

Given that Italy’s monitoring program is the longest running in Europe, the experience gained could be useful in designing new programmes and improving existing ones. In fact,

the large quantity of data collected has been stored in a unique database which describes the evolving epidemiological situations. The objective of the present study was to describe and evaluate the monitoring system in terms of its capacity to detect outbreaks of LPAI infection in meat-type turkeys (i.e. the most affected species), taking into account the number and frequency of samplings and the time to detect infected flocks, comparing the detection rate of active, passive and targeted surveillance.

MATERIALS AND METHODS

The monitoring system

The monitoring system was implemented by the Regional veterinary authorities in the DPPA affected by the epidemics, which is located in northern Italy (in the adjoining Regions of Veneto and Lombardy) and is Italy's only DPPA. In this area, more than 2,200 industrial poultry farms (i.e. rearing more than 500 birds) are located, resulting in an average poultry density of more than 10,000 birds/km^2. Monitoring was implemented mainly in the vaccination area; however, in light of the risk of the spread of infection, it was extended to areas with a radius of 10 km around outbreaks near the edge of the vaccination area, hereafter referred to as the “risk area”. Vaccination areas varied depending on the location of the outbreaks and the risk of infection (Annex, Figures A1 – A4) [6]. The vaccinated species were those with long production cycles, in particular, meat-type turkeys, breeders, layers, cockerels and capons. In each vaccinated flock, 1% of the birds remained unvaccinated and were considered as “sentinel birds”, which were confined in a small fenced-in area in each of a given farm’s sheds. In 2004 and 2005 a “high monitoring zone” was also instituted, corresponding to the vaccination area of the previous vaccination campaign (Annex, Figures A3 and A4). The farms located inside this zone did not receive any vaccination but underwent a particular monitoring scheme because deemed at risk of infection. For our analyses, this zone was included into the “risk area”.

We categorised the surveillance into three types: active surveillance; passive surveillance; and targeted surveillance. The specific reasons for sampling according to each type of surveillance are summarized in Table 1. To start with the latter two, passive surveillance was performed when infection was suspected, mainly based on clinical signs reported by field veterinarians and farmers. Targeted surveillance was performed every time an outbreak was confirmed and included monitoring of all farms located in the protection/surveillance zones and those outside these zone having epidemiological links with the infected premise. Both passive and targeted surveillance resulted in collection of samples in accordance with existing EU provisions [10, 11], which prescribe additional

sampling (i.e. beyond the monitoring scheme) when a flock was: i) suspected of being infected (based on symptoms); ii) epidemiologically linked to an infected farm; or iii) located inside a protection or surveillance zone.

Table 1. Reason for which sampling was performed, by type of surveillance

Surveillance component	Reason for sampling
Active surveillance (**AS**)	Monitoring of unvaccinated birds (in unvaccinated flocks) Monitoring of sentinels (in vaccinated flocks) Monitoring of sentinels for international trade purposes Visit before slaughtering Monitoring at slaughterhouse
Passive surveillance (**PS**)	Suspected outbreak (based on symptoms)
Targeted surveillance (**TS**)	Epidemiological link with an infected farm Farm located within the protection zone of a confirmed outbreak Farm located within the surveillance zone of a confirmed outbreak
Unclassified	Further testing in a confirmed outbreak

Active surveillance consisted of the sampling of birds in accordance with the monitoring scheme (described in detail on Annex, Figures A5 – A8). Depending on the specific epidemiological situation, the number of birds tested per flock and the frequency of sampling (i.e. the monitoring scheme) varied (Annex, Figures A5 – A8). Generally speaking, the vaccinated poultry farms were visited every 30-75 days to test the health status of the birds, with particular reference to the sentinels, and to collect serum samples from at least 10 sentinels. Unvaccinated poultry farms in the vaccination area and the risk area were sampled every 30-60 days, collecting serum samples from 10-30 randomly selected birds. Cloacal swabs for virological testing were also collected from 30 birds (i.e., ducks, geese and ostriches) every three months in the vaccination area, but not for all of the epidemics.

In the particular case of meat-type turkeys, all the flocks located in the vaccination area should have been vaccinated. However during the 2000-2001 and 2002-2003 epidemics the vaccination campaign started two to three months after the onset of the epidemics (Table 2), leading to the presence of unvaccinated flocks within the vaccination area. Nonetheless, these unvaccinated turkey flocks underwent the same monitoring scheme set up for vaccinated ones. In 2000-2001 turkey flocks were sampled every 30 days, collecting 10 sera from unvaccinated sentinels/birds. Additional tracheal and/or cloacal swabs were taken in case of positive serological findings (Annex, Figure A5). The same scheme was applied in 2002-2003, but in this case samples were collected every 45

days and the subsequent testing (following a positive serological finding) could be either serological or virological (Annex, Figure A6). In 2004, the turkey flocks within the vaccination area were visited every 75 days to collect 10 sera from unvaccinated sentinels. In case of positive findings, a subsequent serological or virological test was applied. In addition, the high monitoring area (corresponding to the vaccination area of the previous vaccination campaign) surrounding the vaccination zone was instituted; all the (unvaccinated) meat turkey flocks within this area were monitored at slaughterhouse, collecting blood samples from 10 birds per flock (Annex, Figure A7). In 2005, the turkey flocks within the vaccination area were visited twice during a production cycle: at 70 or 100 days of age (for female and male turkeys, respectively) and at loading for slaughter, collecting ten blood samples from unvaccinated sentinels each time. Within the high monitoring area (in which only layers were vaccinated for three months), all the (unvaccinated) turkey flocks were monitored at slaughterhouse, testing serologically 5 birds per flock (Annex, Figure A8).

Table 2. Description of the epidemics (2000-2005).

Epidemic	1	2	3	4
Period	2000-2001	2002-2003	2004	2005
Virus subtype	H7N1	H7N3	H7N3	H5N2
Beginning of epidemic	14/08/2000	10/10/2002	15/09/2004	11/04/2005
End of epidemic	26/03/2001	30/09/2003	10/12/2004	15/05/2005
Duration of epidemic (in days)	224	355	86	34
Beginning of vaccination program	15/11/2000	10/12/2002	01/07/2004	01/07/2004
Number of monitored farms in vaccination areas	506	1440	615	326
Number of monitored farms in risk areas	276	779	350	269
Total number of monitored farms	782	2219	965	595
Number of monitored production cycles in vaccination areas	682	3016	730	377
Number of monitored production cycles in risk areas	279	1052	379	283
Total number of monitored production cycles	961	4068	1109	660
Number of outbreaks[1] in vaccination area	44	322	30	2
Number of outbreaks in risk area	21	19	1	2
Total number of outbreaks	65	341	31	4

[1] outbreak = AI viruses detected in production cycle

Note: For some production types (i.e., layers or breeders), a production cycle may have spanned two consecutive epidemics; in this case, it was counted twice (once for each epidemic).

All samples were collected by veterinarians from the Regional veterinary authorities. The serum samples were tested for the presence of HI antibodies, and the cloacal swabs were examined for the presence of AI [10, 11]. Laboratory data were stored in a central database at the National Reference Centre for AI at the *Istituto Zooprofilattico Sperimentale delle Venezie* (IZSVe), and linked with data from the poultry farm registry.

Suspected infections were reported by field veterinarians and farmers, and a farm was considered as "infected" (i.e., an "outbreak" was defined) based on isolation of the virus, PCR assay, or serological testing. In particular, during the early stages of an epidemic (i.e., when the first few cases were identified), a farm was considered as infected when two consecutive samplings provided positive results. In later stages of the epidemic (i.e., when there was an evident epidemic in the area), one positive sampling was sufficient to define an outbreak.

Dataset building and editing

We extracted all 56,803 records for the controls performed in the periods from 2000 to 2005 in which the four major epidemics occurred. The details of the epidemic periods are reported in Table 2. A total of 6,102 poultry farms were sampled: 1,376 in 2000-2001; 2,499 in 2002-2003; 1,524 in 2004; and 703 in 2005. A total of 497 LPAI outbreaks were identified. Records with missing information on the dates of stocking and slaughtering (both necessary for defining a precise production cycle on a farm), the sampling day, the species, the number of samples collected, or the test results were excluded (n=38,639); thus the final number of records was 18,164, which refer to 2,312 poultry farms, 6,315 production cycles and 441 LPAI outbreaks, 90% of which involving meat-type turkeys (n=398). More information on virus subtypes, evolution of the epidemics and applied control measures is available in Capua and Marangon (2007) [6].

Data analysis

The description of the results of the monitoring system referred to all the species, but the specific analyses on the sampling day, age at detection and detection rates were restricted to only meat-type turkey flocks, because the majority of outbreaks occurred in this production type. In fact, if they had been analysed for all of the species, such unbalanced data along with the different lengths of production cycles and the different sampling schemes among species would have generated bias and made comparisons difficult.

For each of the three types of surveillance (active, passive, and targeted), the detection rate was determined using multinomial logistic regression, to evaluate the ability to detect an outbreak in meat-type turkey flocks. The type of surveillance with which the outbreak was detected (i.e. the surveillance component associated to the first positive sampling in confirmed infected flocks) was considered as the dependent variable,

whereas the possible explanatory variables were vaccination status and the specific epidemic. Multinomial *logit* models allow nominal variables to be handled by defining one value as the reference category and comparing the probability of membership in this category to that in other categories. In our case, if active surveillance detects outbreaks with probability π_A, passive surveillance with probability π_P, and targeted surveillance with probability π_T, then:

$$\ln\left(\frac{\pi_P}{\pi_A}\right) = \alpha_P + \beta_{Pk} X_{Pk} \quad \text{and} \quad \ln\left(\frac{\pi_T}{\pi_A}\right) = \alpha_T + \beta_{Tk} X_{Tk}$$

where:

- α is the intercept (i.e., baseline containing reference groups);
- β is the vector of k coefficients associated with the k explanatory variables; and
- X is the vector of k explanatory variables.

The three probabilities were derived by solving the equations, considering that $\pi_A + \pi_P + \pi_T = 1$:

$$\pi_A = \frac{1}{1 + e^{\alpha_P + \beta_{Pk} X_{Pk}} + e^{\alpha_T + \beta_{Tk} X_{Tk}}}, \quad \pi_P = \frac{e^{\alpha_P + \beta_{Pk} X_{Pk}}}{1 + e^{\alpha_P + \beta_{Pk} X_{Pk}} + e^{\alpha_T + \beta_{Tk} X_{Tk}}}, \quad \pi_T = \frac{e^{\alpha_T + \beta_{Tk} X_{Tk}}}{1 + e^{\alpha_P + \beta_{Pk} X_{Pk}} + e^{\alpha_T + \beta_{Tk} X_{Tk}}}$$

Of the 398 outbreaks involving meat-type turkey flocks , 35 were discarded because the information about the type of surveillance with which the outbreak was detected was missing. 363 outbreaks, referring to 283 different farms, were finally included in the analysis.

The models, implemented using the statistical software SAS 9.1.3. (SAS Inst., Inc., Cary, NC), were selected by backward elimination, considering both Akaike’s information criterion (AIC, "smaller is better") and the overall significance of each effect, measured with the Wald χ^2–test. The effect of the specific epidemic was significant but associated with huge standard errors, probably because of the extremely unequal distribution of cases among epidemics, especially in relation to vaccination. We then excluded it and its elimination lowered the AIC of the model.

RESULTS

The characteristics of the four LPAI epidemics and the monitoring activity are summarized in Tables 2 and 3. The identified virus subtypes were H7N1 in 2000-2001 epidemic, H7N3 in 2002-2003 and in 2004 epidemics, and H5N2 in 2005 [6]. Of the monitored holdings, 65% were located in the vaccination areas and 35% in the risk areas.

The second epidemic (2002-2003) was the most severe in terms of the number of outbreaks (77% of the total), duration (355 days) and geographic extension, with more than 4,000 monitored production cycles. It follows that most of the results of the monitoring activity are referred to this epidemic (Table 2).

A total of 6,315 different production cycles were tested during the four considered epidemics. The flocks mainly consisted of meat-type turkeys (39%), broilers (37%), and layers (12%). Half of the meat-type turkeys and layers flocks had been vaccinated. Of the 441 outbreaks included in this study, 398 (90%) occurred on meat-type turkey farms. The infection rate on vaccinated meat-type turkey flocks (10%; n=153/1,466; CI95%=8.9%-12.1%) differed from that on unvaccinated meat-type turkey flocks (24%; n=245/1,028; CI95%=21.2%-26.5%).

For serological testing, 13,275 samplings were performed (173,598 individual serum samples); for virological testing, 4,889 samplings were performed (Table 3). Most of the serological samplings (n=12,388) were carried out as part of active surveillance, and 4% of the samplings were positive; 207 were carried out as part of passive surveillance (63% positive) , and 644 as part of targeted surveillance (6% positive). Concerning virological testing, 4,469 of the 4,889 samplings were carried as part of active surveillance (3% of the samplings were positive), 263 samplings for passive surveillance (40% positive samplings), and 136 for targeted surveillance (7% positive samplings).

Table 3. Number of positive samplings (i.e., visit to a farm to collect samples) for serological and virological testing, by production type and epidemic

Production types	**Serological testing (positive*/total)**				
	Epidemic 1	Epidemic 2	Epidemic 3	Epidemic 4	Total
Breeders	1/97	18/924	3/222	4/147	26/1390
Broilers	0/325	2/2543	0/185	0/42	2/3095
Layers	1/146	44/1459	15/596	8/221	28/2422
Meat type Turkeys	69/930	350/3024	52/796	29/474	185/5224
Other species	10/219	34/820	1/88	0/17	45/1144
Total	81/1717	448/8770	71/1887	41/901	286/13275
Production types	**Virological testing (positive°/total)**				
	Epidemic 1	Epidemic 2	Epidemic 3	Epidemic 4	*Overall*
Breeders	1/12	6/404	0/12	0/26	7/454
Broilers	0/2	7/697	0/18	0/1	7/718
Layers	1/19	3/435	0/97	0/44	4/595
Meat type Turkeys	26/88	155/2012	16/388	2/299	59/2787
Other species	0/22	1/289	0/21	0/3	1/335
Total	28/143	172/3837	16/536	2/373	78/4889

*positive sampling for serological testing = at least one sampled bird tested positive

°positive sampling for virological testing = at least one pool of 5 birds tested positive

Monitoring of meat-type turkey flocks started one week after the start of the production cycle. These flocks were more frequently sampled at the end of the production cycle; in particular, most of the sampling days were during slaughtering (90-95 days for female turkeys and 138-145 days for male turkeys). Infections mainly occurred in the second half of the production cycle, with no differences between vaccinated and unvaccinated flocks, though the first infections were observed between days 22 and 28 on unvaccinated and between days 43 and 49 on vaccinated meat-type turkey flocks (Figure 1)

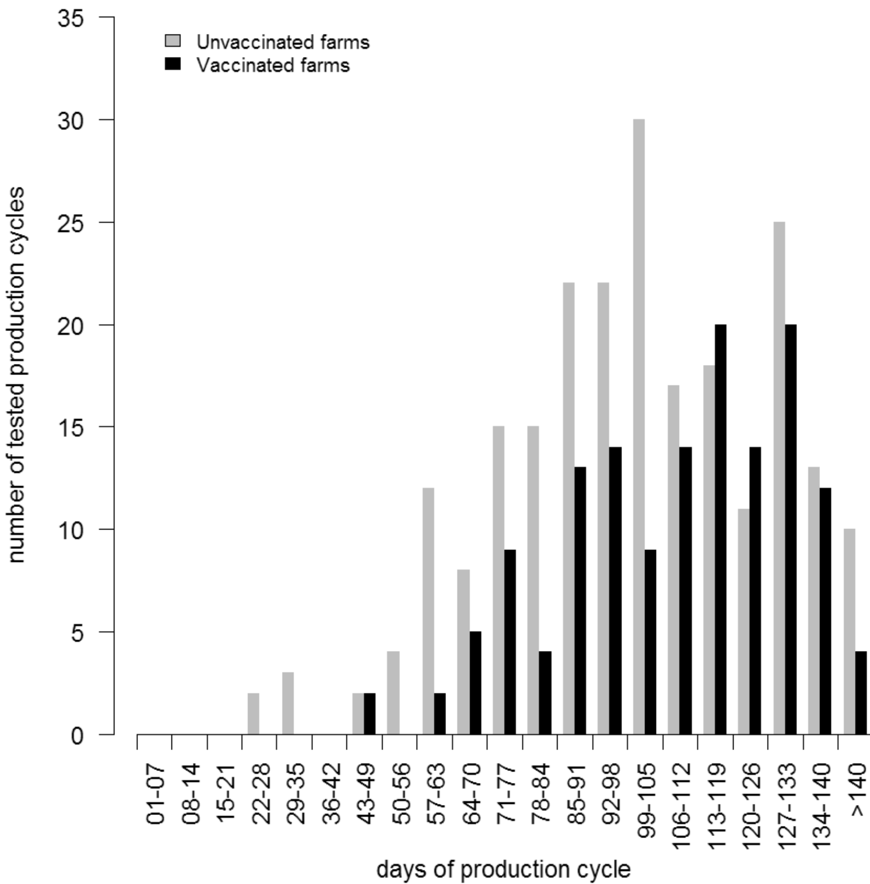

Figure 1. Sampling day (starting from stocking of birds) corresponding to first positive results in meat-type turkey outbreaks, by vaccination status. Grey and black bars indicate the frequency (number of farms) of unvaccinated and vaccinated flocks that tested positive on that day, respectively.

Active surveillance resulted in the identification of 222 (61%) of the 363 outbreaks available in meat-type turkey flocks (108 vaccinated and 114 unvaccinated infected meat-type turkey flocks). Passive surveillance identified 114 outbreaks (32% of the total; 90 and 24 in unvaccinated and vaccinated flocks, respectively). Targeted surveillance detected 7% of the outbreaks (22 and 5 in unvaccinated and vaccinated flocks, respectively) (Table 4). The maximum likelihood predicted values for the detection probabilities differed between vaccinated and unvaccinated flocks. In unvaccinated infected flocks, the detection probability was 50% for active surveillance (π_A), 40% for passive surveillance (π_P), and 10% for targeted surveillance (π_T), whereas in vaccinated flocks it was 79%, 17%, and 4% for, respectively, active, passive, and targeted surveillance. The 95% confidence intervals for the estimates of detection probability were quite narrow and did not overlap among the three components (Table 4).

Table 4. Number of outbreaks (n) identified, estimated detection probabilities (π), and 95% confidence intervals, by type of surveillance and vaccination status in meat-type turkey flocks.

Type of surveillance that identified the first positive result	outbreaks						Total*		
	Unvaccinated flocks			Vaccinated flocks					
	n	π	95%CI	n	π	95%CI	n	π	95%CI
Active surveillance	114	0.50	[0.47;0.59]	108	0.79	[0.72;0.86]	222	0.61	[0.56;0.66]
Passive surveillance	90	0.40	[0.33;0.46]	24	0.17	[0.11;0.24]	114	0.32	[0.27;0.36]
Targeted surveillance	22	0.10	[0.06;0.13]	5	0.04	[0.01;0.07]	27	0.07	[0.05;0.10]

*The total number of outbreaks in meat-type turkey flocks reported in this table (363) is lower than the total number of outbreaks considered in this study for that species (398) because for 35 premises the information about the type of surveillance with which the outbreak was detected was missing.

DISCUSSION

We evaluated Italy's monitoring programme for LPAI infection in terms of its capacity to rapidly detect outbreaks in meat-type turkey flocks. We found that active surveillance detected 61% (222/363) of the outbreaks that occurred in the study period and that passive surveillance detected 32% (114/363), whereas targeted surveillance detected only 7% (27/363). These findings are consistent with other studies that have shown that the large majority of LPAI-infected poultry farms can be detected by routine serological or virological testing alone [7, 17]. They also indicate the need to improve the rapidity with which AI outbreaks are reported, which could be achieved by training farmers and veterinarians in recognising the clinical signs of LPAI infection and increasing the level of

awareness of all stakeholders in disease control.

Based on evidence that farms located near an HPAI-infected premise have a higher chance of becoming infected [12], we expected targeted surveillance to be effective in detecting outbreaks. However, the additional samplings performed on farms located in protection zones did not increase the detection rate, possibly because of the farmers' awareness of clinical signs, as demonstrated by the results of passive surveillance. Moreover, the specific control measures implemented during the epidemics resulted in a significant reduction of between-flock transmission [14] and could have contributed to reducing the probability of detecting infection through targeted surveillance.

Active surveillance was the most effective means of detecting infections in meat-type turkeys. However, the relative probability of outbreak detection differed when comparing vaccinated and unvaccinated flocks. The probability of detection with active surveillance was higher in vaccinated flocks, whereas for passive and targeted surveillance it was higher in unvaccinated flocks. While during the first two epidemics vaccinated and unvaccinated flocks underwent the same sampling scheme, in 2004 and 2005 there was a difference in sample size that could have resulted in an enhanced performance of AS in vaccinated flocks. Nonetheless this eventuated only in the last two epidemics, which contributed in a reduced manner both in terms of time (120 days in total) and number of outbreaks (25 in 2004 and 9 in 2005, in turkeys).

Regarding the better performances of PS on unvaccinated flocks, this could be due to the effect of vaccination, which can reduce the severity of clinical manifestations and thus the sensitivity of passive surveillance [2, 5, 19]. These findings indicate the need to implement active surveillance when vaccination is performed and the importance of a careful and regular inspection of unvaccinated sentinels birds in vaccinated flocks for prompt detection of the disease [9].

Although the performance of targeted surveillance may have been influenced by disease-control measures, these measures cannot explain the differences in the effectiveness of the different types of surveillance between vaccinated and unvaccinated flocks, which could also be associated with vaccination itself. It could thus be inferred that the lower probability of detection of targeted surveillance in vaccinated flocks was also due to the effect of vaccination, which has been shown to reduce the incidence of LPAI outbreaks [4] and thus could have protected susceptible flocks from neighbouring infection. Nonetheless, it can be argued that whereas active surveillance was carried out inside vaccination and intensive monitoring zones, targeted surveillance was only applied in protection/surveillance zones or in farms epidemiologically linked to infected premises, leading to different populations surveyed. However, we rather think of these selection criteria as being part of the surveillance schemes, so that the detection rate of TS measures selection of farms at risk plus detection of outbreaks among those farms. The result is the contribution of TS to the detection of outbreaks.

To evaluate the monitoring system, we relied on field data, which have the advantage of providing a good representation of reality yet the disadvantage of partial or missing information. In fact, because of partial or missing data, we discarded many records, which could have biased our results. Moreover, the field data were rather unbalanced, in that 90% of the outbreaks involved meat-type turkey flocks, which made it difficult to make comparisons among species. For this reason, we evaluated the timing of outbreak detection and the detection rate of the three types of surveillance for turkeys only.

In our analyses the unit of interest was the production cycle rather than the farm, being an outbreak defined as an infected production cycle. Nonetheless, it happened that more than one production cycle of the same farm was affected by LPAI during the four considered epidemics. In fact, of the 363 available outbreaks in as many turkey production cycles, a total of 283 different farms were involved. 213 of the affected farms experienced LPAI infection only once during the four epidemics whereas 70 farms were infected more than once, involving two to four different production cycles. However, when trying to correct for repeated measurements in the multinomial logistic regression, SAS software reported that the number of repeated data was too low. Furthermore, different production cycles in the same farm can be considered poorly related, because the reared animals are not the same and also the vaccination status of the flock or the species may change from a production cycle to another. To validate our results, we did the same analysis taking into account only one affected production cycle per farm and results were identical to the ones obtained from the whole dataset and presented here.

This paper illustrates the capricious nature of surveillance programmes carried out during several epidemics over a number of years. Nevertheless, it brings together a wealth of information and shows the effectiveness of different types of surveillance for the monitoring of LPAI in (partly) vaccinated poultry populations.

In conclusion, this study highlights the importance of surveillance systems in monitoring the introduction and spread of LPAI viruses in DPPAs. Given that LPAI infection is characterized by mild symptoms and a low mortality rate, and taking into account that HPAI could have a different clinical picture in vaccinated flocks, the routine testing of meat-type turkey farms (active surveillance) can be considered as the most effective means of detecting new outbreaks, especially when a vaccination program is in place.

ACKNOWLEDGEMENTS

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ANNEX

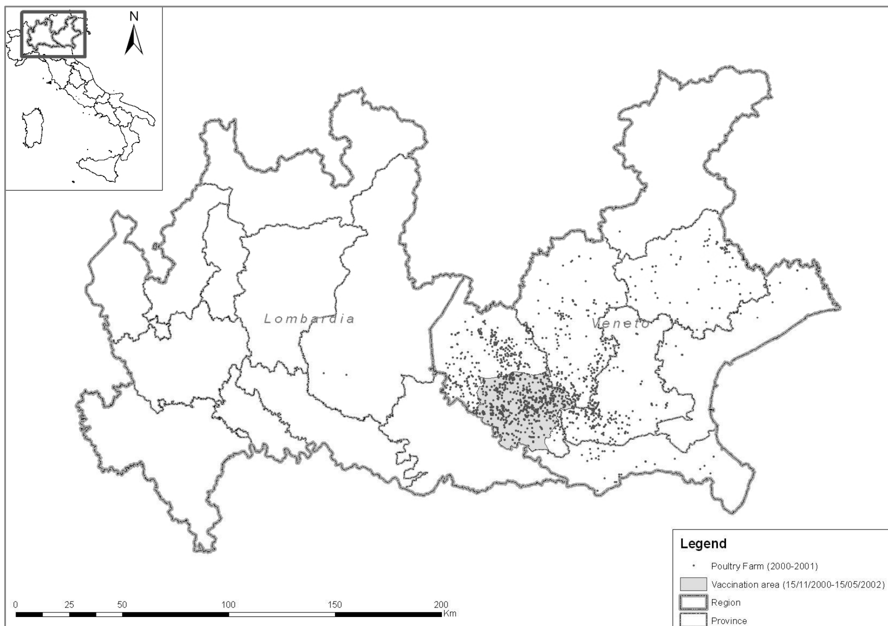

Figure A1. Vaccination area during the 2000-2001 epidemic

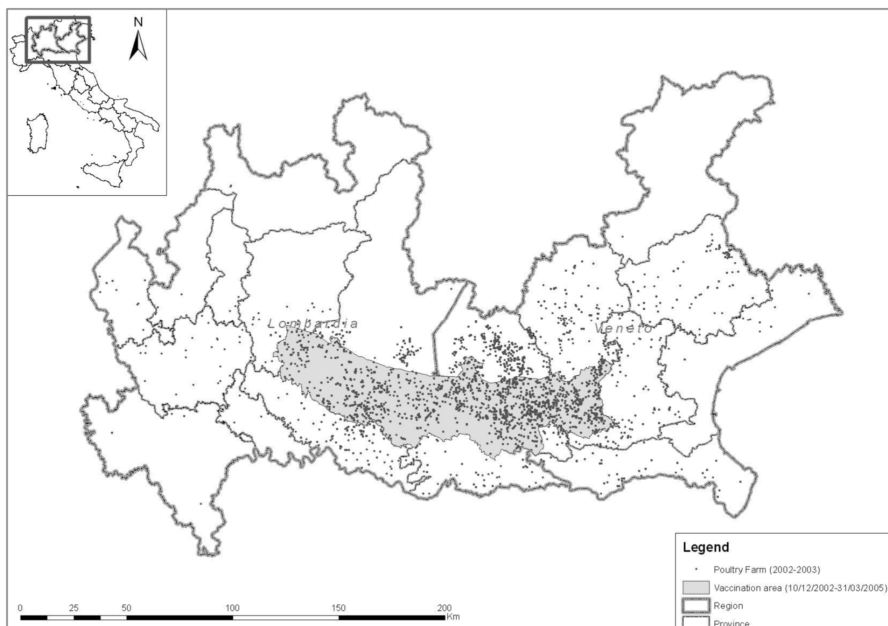

Figure A2. Vaccination area during the 2002-2003 epidemic

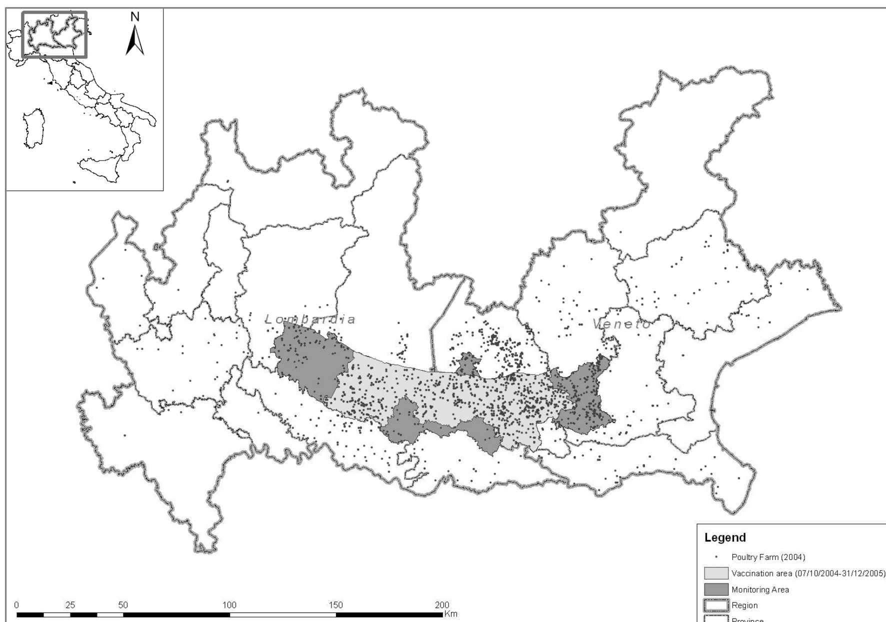

Figure A3. Vaccination area during the 2004 epidemic

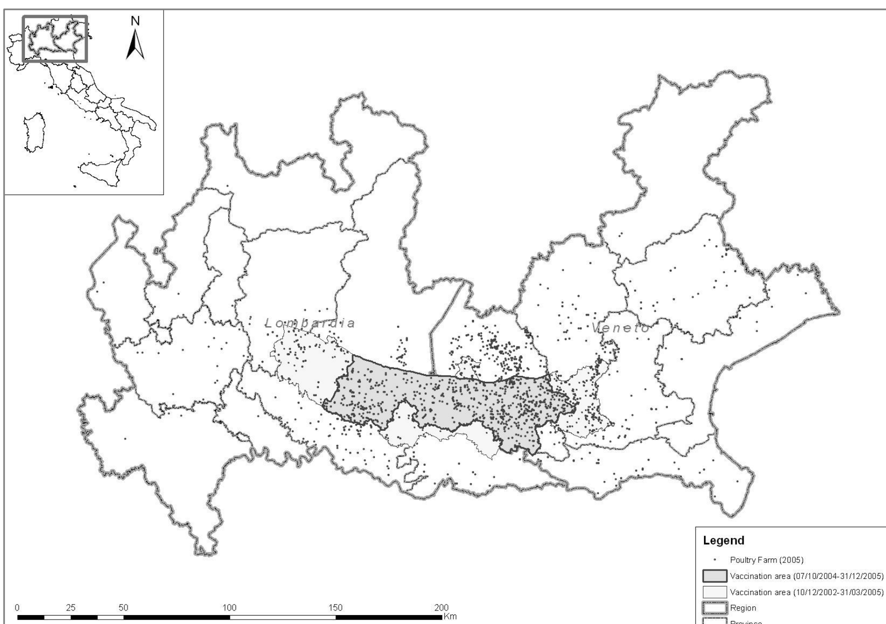

Figure A4. Vaccination area during the 2005 epidemic

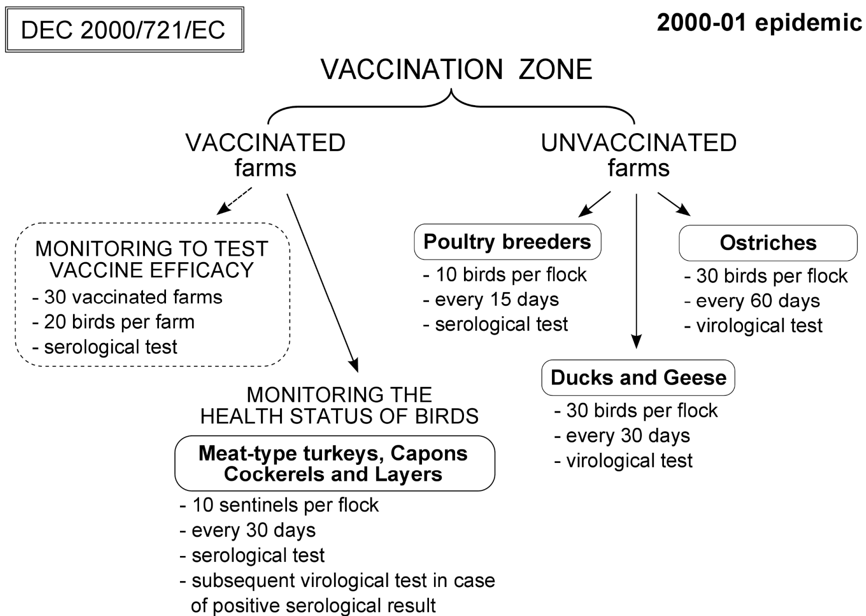

Figure A5. Surveillance programme in place during the 2000-2001 epidemic

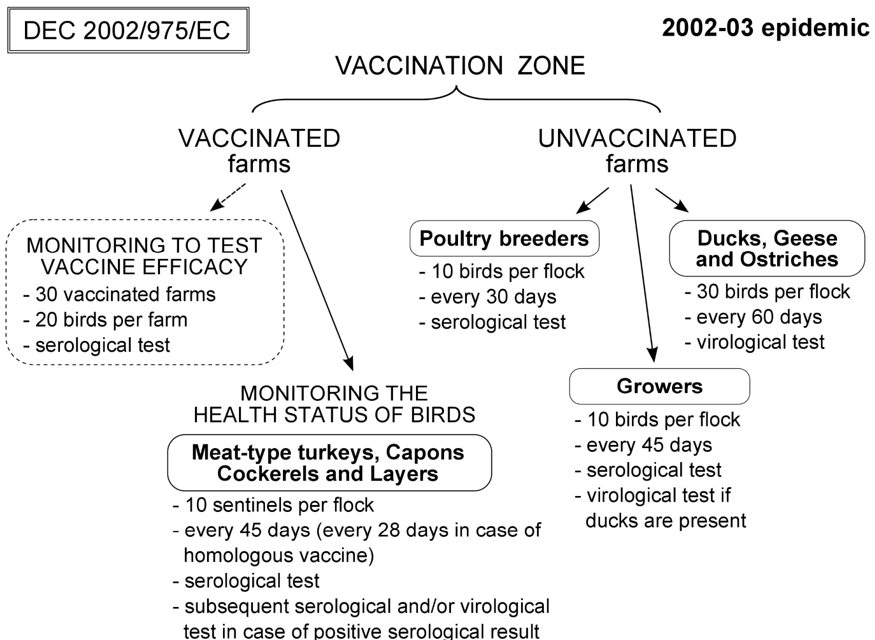

Figure A6. Surveillance programme in place during the 2002-2003 epidemic

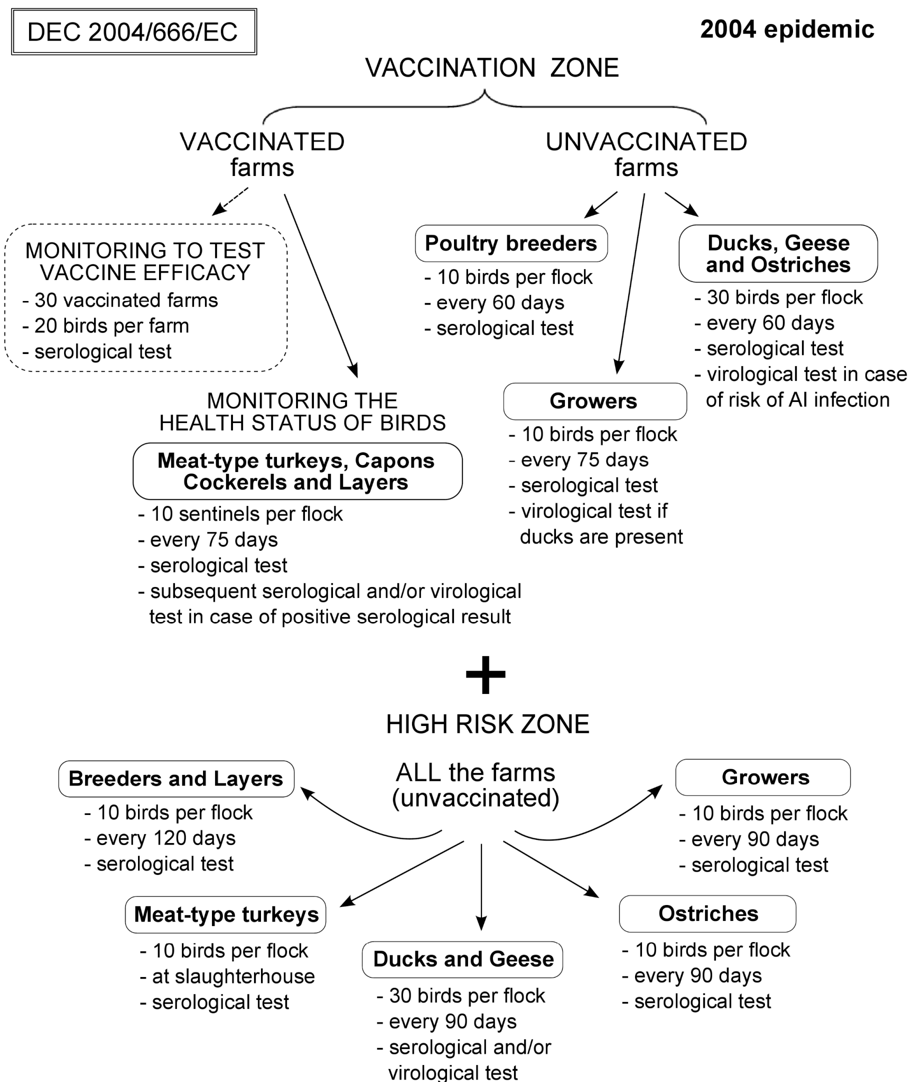

Figure A7. Surveillance programme in place during the 2004 epidemic

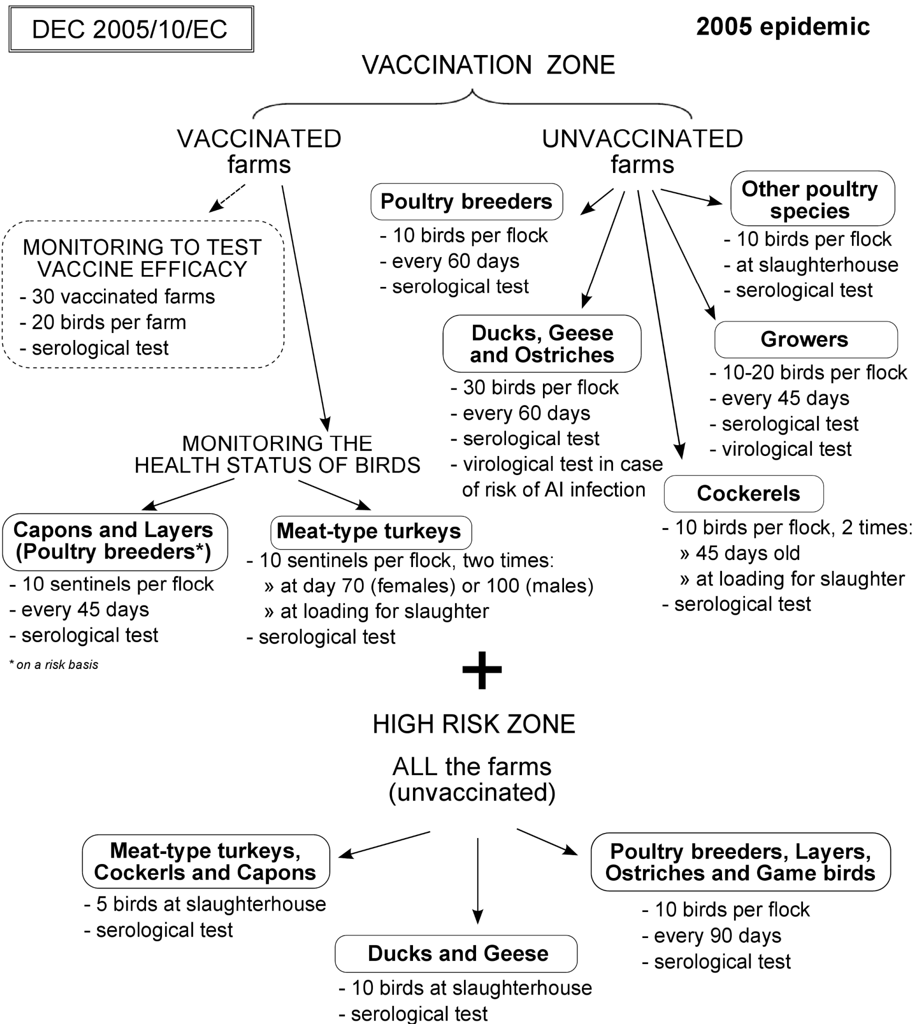

Figure A8. Surveillance programme in place during the 2005 epidemic

CHAPTER 3

Epidemiology and control of low pathogenicity avian influenza infections in rural poultry in Italy

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ABSTRACT

We analyzed the involvement of the rural poultry sector in outbreaks of low pathogenicity avian influenza (AI) in Italy in 2007–2009 and discuss possible measures for improving monitoring and control. A description of how the rural poultry sector is organized also is provided. Data were obtained by the AI surveillance system established in the areas affected by the outbreaks. The surveillance activities identified two H7N3 epidemics, in 2007 and 2009, both of which mainly involved the rural sector, yet these activities did not allow for the prompt eradication of the disease. Additional strategies could be adopted to avoid the persistence of AI within the rural sector, based on the regulation and control of poultry holdings at the top of the production chain.

KEY WORDS

Avian influenza, rural poultry, surveillance, control measures, Italy

INTRODUCTION

In Italy, several outbreaks of avian influenza (AI) viruses of the H5 and H7 subtypes have occurred since 1997. The outbreaks were caused by highly pathogenic AI (HPAI) viruses in domestic poultry [7], which in some cases had mutated from low pathogenicity AI (LPAI) virus strains after widespread circulation in poultry [18] and by LPAI viruses that did not mutate into HPAI viruses [5]. Nearly all AI outbreaks occurred in a densely populated poultry area (DPPA) in the Po Valley in North-Eastern Italy, where approximately 70% of the national poultry production is concentrated, leading to severe economic losses to both the poultry sector and the state. Based on a simple qualitative risk analysis, the following risk factors for the introduction and spread of AI viruses were identified in the DPPA:

1) high densities of turkeys (*Meleagris gallopavo*), which are highly susceptible to AI viruses [28],
2) presence of extensive wetlands and resting sites for migratory waterfowl [16], and
3) occurrence of multiple introductions in domestic poultry holdings of LPAI viruses from the wild reservoir [4].

The epidemiologic studies that were carried out to identify these risk factors were based on data referring to the industrial poultry sector, given that the outbreaks mainly affected this sector [3,4,22]. However, there are significant concentrations of rural poultry holdings in the DPPA, which could play a role in introducing AI viruses into the industrial sector. In fact, rural poultry are mainly kept in free-range, multispecies, multiage holdings that have low biosecurity levels and are thus exposed to many at-risk contacts, and they could act as the epidemiologic link between the wild reservoir of AI viruses and industrial poultry.

To control and eradicate LPAI viruses introduced in the poultry population, Italy's veterinary authorities developed coordinated preventive and control measures in 2000 that included emergency and prophylactic vaccination, and based on their effectiveness, these measures were progressively modified as subsequent epizootics occurred [19,20]. In the first few years of implementation, the monitoring of infection mainly focused on the early detection of AI introduction in the industrial sector; in 2004, it was extended to the rural sector, in which several LPAI outbreaks were detected [26]. In fact, the H7N3 LPAI outbreaks in 2007 and 2009 mainly involved this sector.

Our objectives were to analyze the involvement of the rural poultry sector in the 2007–2009 outbreaks and to discuss the possible measures for improving the monitoring and control of infection in this sector. We also provide a description of how the rural poultry sector is organized in Italy.

MATERIALS AND METHODS

Rural poultry sector in Italy.

According to current European Union (EU) legislation on AI, a ''holding'' is defined as ''*any agricultural or other premises, including hatcheries, circuses, zoos, pet-bird shops, bird markets, and aviaries where poultry or other captive birds are being bred or kept*'' [10]. In Italy, poultry holdings can be further classified based on the commercial circuit (i.e., industrial or rural sector) and the final destination of the birds (i.e., commercial purposes or self-consumption [21]). The industrial sector includes holdings where birds are kept only for commercial purposes and that in many cases are part of a vertically integrated system, with adequate biosecurity measures. The rural sector includes those holdings where birds are reared, kept, traded, or a combination of these measures, with other rural holdings. The main categories of rural producers are listed below (although other intermediate categories also exist, they have not been listed for the sake of simplicity):

Growers. Any rural premise where poultry or other captive birds (e.g., ornamental birds) are bred for part of their production life and then sold to any component of the rural sector.

Dealers. Anyone who keeps poultry or other captive birds for a limited time (maximum 72 hr) to be sold to any component of the rural sector.

Backyard flocks. Any rural premise where poultry or other captive birds are kept either for self-consumption or for hobby purposes (i.e., for exhibition or just as pets); in Italy, the majority of backyard flocks are for the production of poultry meat.

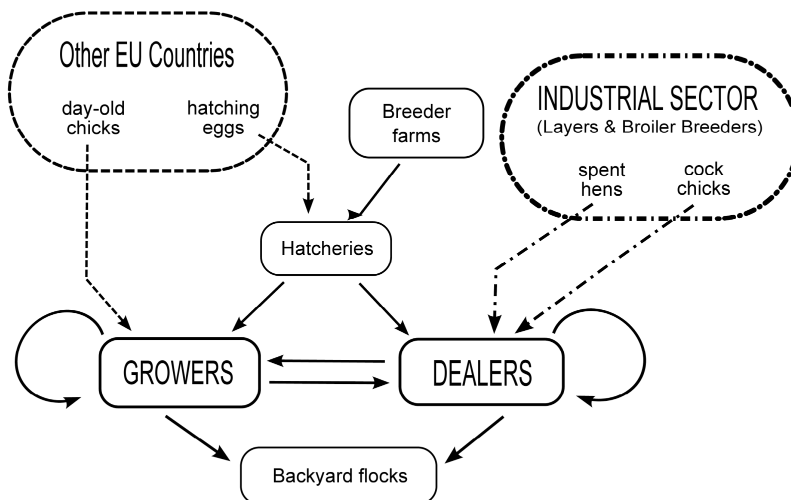

Figure 1. Outline of poultry trade in the rural sector in Italy.

The rural sector is a very complex segment of the poultry industry. In Italy, breeding birds is a widespread custom in rural and periurban areas, and it has considerable socioeconomic importance. The rural sector has composite relationships, which have been outlined in Figure 1. Breeder holdings and hatcheries (in both Italy and other countries) provide day-old chicks of different poultry species directly to growers, or to dealers, which immediately retail the chicks to growers or backyard flocks. Growers breed both day-old chicks and older poultry until they become pullets or adult birds. The birds can be sold at any stage of their production life, mainly to dealers or backyard flocks, directly or through markets and fairs. Dealers can sell poultry to growers, other dealers, or backyard flocks, and the sale can take place through 1) markets and fairs, 2) retailers, or 3) door to door. In backyard flocks, which are usually free-range, the birds are kept only for self-consumption; thus, these holdings represent only a minor concern for AI diffusion.

To date, the connections that are known to constitute a risk for the spread of AI viruses between the rural and industrial sectors are as follows:

1) trade of spent hens and cock chicks from layer and broiler breeder holdings to rural holdings,
2) feed purchase from the same feed-mill (e.g., sharing of trucks), and
3) personnel of industrial poultry holdings who keep backyard poultry at home.

Regarding the size of the rural sector, although no official data are available, it has been estimated that approximately 55 million birds are traded yearly. For chickens (Gallus *gallus*), there are nine large breeder holdings (with a total of approximately 500,000 birds) and 10 hatcheries, which supply day-old-chicks to growers and dealers. There are no turkey breeder holdings, but two hatcheries receive turkey hatching eggs from France (approximately 700,000 eggs/yr). Approximately 70% of geese (*Anser* spp.) and ducks (*Anas platyrhynchos*) are imported at 2–3 days of age from France; the remaining geese and ducks are produced by three breeder holdings. Approximately 1,500,000 layer hens per year are commercialized by two firms, although industrial companies are also marginally involved in this market, selling their spent hens. Guinea fowls (*Numida meleagris*) also are bred for the rural market.

AI surveillance system.

AI surveillance in Italy began to be implemented in 2000 after the severe AI epizootics that involved the industrial sector. Surveillance of the industrial sector was based on the guidelines in EU legislation ([9], and further amendments), which provides indications of the number of holdings and of birds per holding for each poultry category to be inspected and sampled within a country.

The surveillance of dealers, growers, and backyard flocks was only implemented in eight of Italy’s 20 regions. The rural holdings covered by surveillance were identified by

the regional veterinary services, taking into account the main risk factors for the introduction of AI viruses (e.g., free-range housing, proximity to wetlands or other resting sites for migratory birds, multispecies and multiage holdings) and for the spread of these viruses (e.g., live bird markets, location in a DPPA). Depending on the region, the number of sampled birds and the sampling frequencies in each at-risk holding varied; in particular, 10–40 blood samples were collected for serologic testing every 15–60 days for growers and dealers and once or twice a year (during the migratory period) for backyard flocks. Growers and dealers also were monitored 7 days before any bird movement (e.g. fairs and markets). The sampled birds were preferably those coming from markets or fairs and those kept for long periods in the flock. In multispecies holdings, samples were preferably taken from ducks, geese, and turkeys. When ducks and geese were present in a flock, tracheal or cloacal swabs or pools of feces also were collected for virologic testing.

From 2007 to 2009, the AI surveillance in the rural sector involved approximately 3000 poultry holdings per year, which were not homogenously distributed among the regions [17]. Sampling of rural poultry holdings was more intensive in northern regions (Piemonte, Lombardia, Veneto, and Emilia Romagna), where the DPPA is located, and in Marche and Sicily.

A resampling activity for both virologic and serologic testing was performed in each farm where positive findings occurred. All laboratory tests were conducted in accordance with the EU AI diagnostic manual [11]. In particular, the hemagglutination inhibition for H5 and H7 has been performed as a serologic test. A preliminary screening with M-gene real-time reverse transcriptase (RRT)-PCR to confirm the presence of an influenza A virus, followed by screening of positive samples for H5 and H7 using RRT-PCR, was performed on RNA extracted from swabs or pool of feces. The RRT-PCR-positive samples were inoculated in embryonated chicken eggs for virus isolation.

RESULTS

During the national AI surveillance in 2007–2009, five different LPAI virus subtypes were detected in industrial and rural poultry sectors (i.e., H7N1, H7N3, H5N1, H5N2, and H5N7). However, in this study, only H7N3 outbreaks were considered because the majority of the outbreaks that occurred in the rural sector were caused by this virus subtype.

2007 H7N3 LPAI epizootic.

From May to October 2007, AI surveillance identified 17 LPAI H7N3 outbreaks in the following regions: Veneto (*n* = 2 outbreaks), Lombardy (*n* = 10), Piemonte (*n* = 2), Emilia Romagna (*n* = 2), and Basilicata (*n* = 1). Of these outbreaks, 11 occurred in the rural

sector: backyard flocks (four flocks, rearing 4239 birds in total) and dealer and grower flocks (seven flocks, with 52,308 birds), which contained different poultry, ornamental bird species, or a combination. The remaining six outbreaks were detected at industrial meat-turkey holdings (73,158 turkeys in total) situated in the DPPA (Lombardy region; Fig. 2).

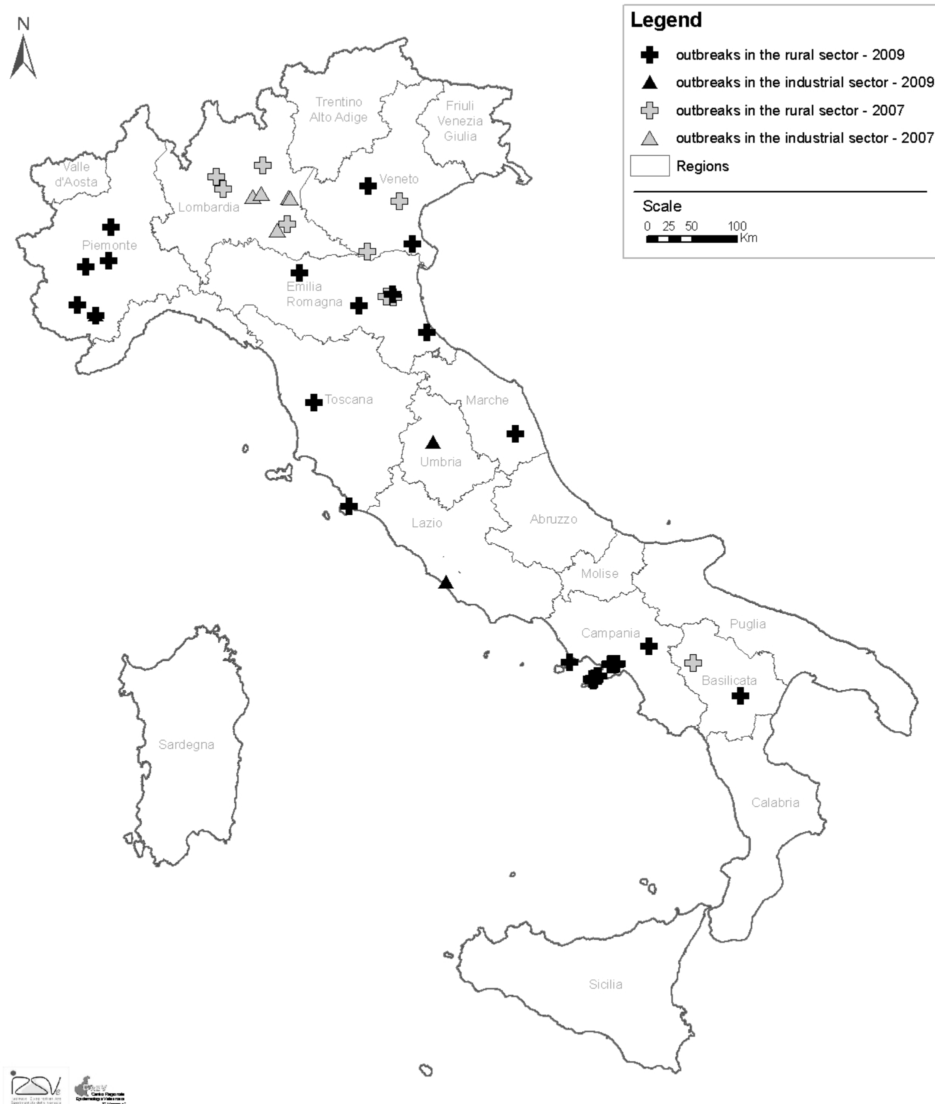

Figure 2. Geographical distribution of H7N3 LPAI outbreaks in Italy in 2007 and 2009.

At the time of sampling, none of the birds at the rural holdings showed any clinical symptoms, whereas at the industrial meat-turkey holdings, a mild respiratory disease associated with anorexia and a slightly increased mortality rate were observed. Epidemiologic investigations revealed that there were direct contacts (i.e. movement of live birds) among eight of the 11 infected rural holdings. Furthermore, seven flocks (four layer flocks, two backyard flocks, and one dealer or grower flock) in the Campania region (southern Italy) were found to have been serologically positive for H7; yet, the presence of antibodies alone was deemed to be insufficient for confirming the outbreaks, given the established absence of virus circulation in the area [23].

Phylogenetic analysis performed on the HA and NA genes indicated that all identified viruses were part of the Eurasian H7 lineage and presented percentages of similarities lower than 96% with HA and NA gene sequences of 2002/04 H7N3 Italian LPAI viruses [5]. All of the H7N3 viruses detected in 2007 showed a high level of similarity with each other (98.7%–99.8% in the HA gene), and all of them clustered together, with the exception of the two virus strains isolated on May 27 from the first LPAI-affected backyard flock (A/chicken/Italy/2837-54/2007 and A/chicken/Italy/ 2837-58/2007; Fig. 3).

To control and eradicate the infection, disease control measures were implemented, as specified in EU legislation [10]. These measures mainly consisted of the stamping out of all birds reared at the infected holdings, the cleaning and disinfection of the infected holdings, and the establishment of monitoring and restriction measures. In total, 129,386 birds were stamped out. In three of the infected holdings, there also were endangered species of birds (n = 244); however, as specified in EU legislation [10], these birds were not killed and underwent periodic official inspection and virologic and serologic testing, in accordance with the EU diagnostic manual for AI [11]. Because an LPAI strain was introduced in the industrial sector in the DPPA, it was required to perform emergency vaccination in industrially reared poultry in the areas at higher risk of AI [12]. To differentiate vaccinated from field-exposed birds and holdings, two heterologous vaccines (a monovalent H7N1 subtype and a bivalent H7N4 and H5N9 subtype vaccine) and a suitable companion discriminatory test were applied [8]. Vaccination was carried out from October 2007 to March 2008 at industrial holdings located in the DPPA, in the provinces of Verona, Brescia, and Mantova. The targeted species were ‘‘long-lived’’ birds such as meat turkeys and table eggs layers. In total, 380 flocks were vaccinated and 14,530,000 vaccine doses were distributed. The field application of emergency vaccination required the implementation of an intensive surveillance program aimed at the prompt identification of infected flocks. Serologic monitoring also was enforced in unvaccinated flocks located both inside and in proximity to the vaccination area.

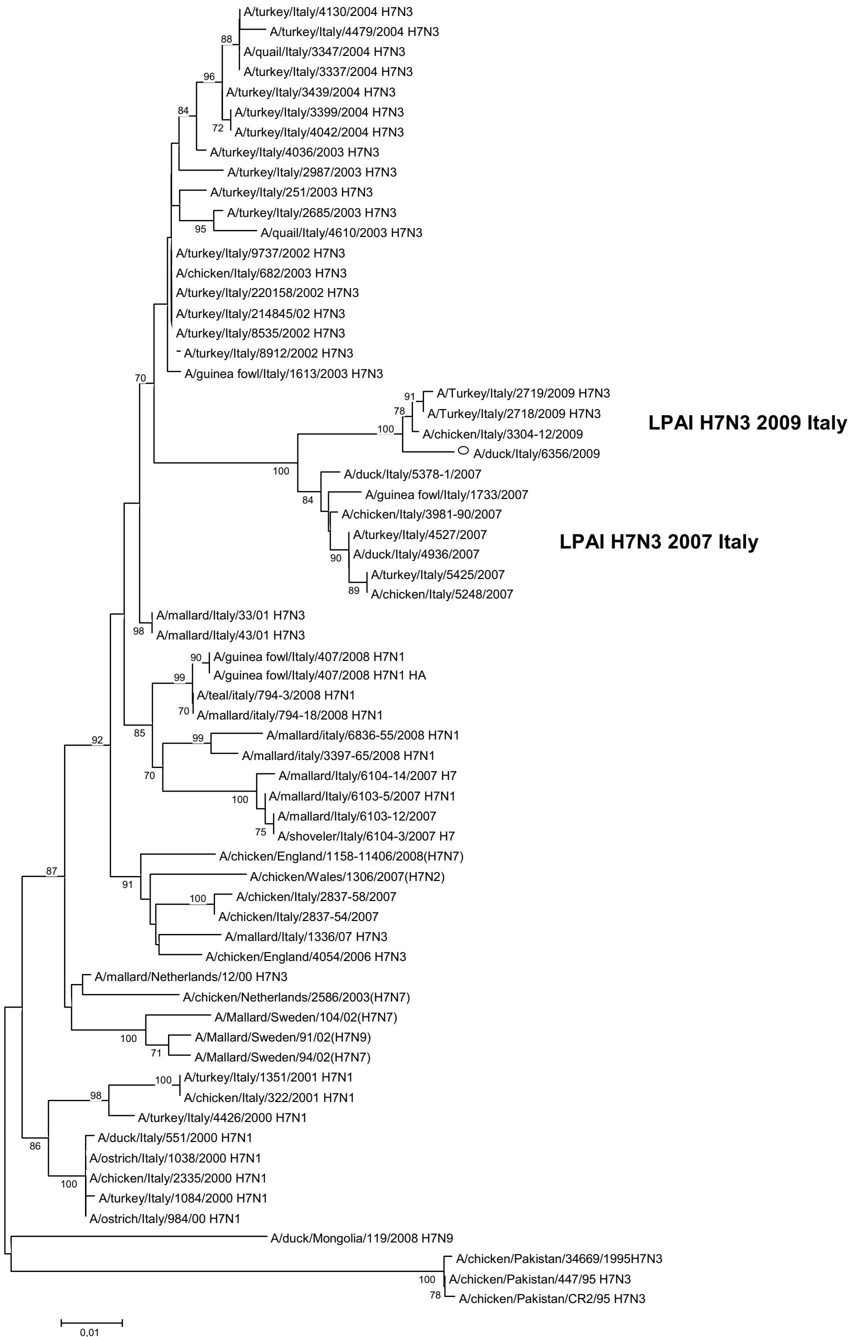

Figure 3. Phylogenetic tree of the genome segment encoding for the HA of H7 strains constructed using neighbour joining method and bootstrap analysis (1000 replicates) by MEGA 3 software.

2008 H7 serologically positive poultry flocks.

In 2008, no outbreaks of H7N3 were identified, yet 11 flocks (two industrial and nine rural) were found to have been seropositive for the H7 AI virus subtype. All of the flocks were located in southern Italy, in the regions of Basilicata (*n* = 2), Calabria (*n* = 1), and Campania (*n* = 8). Both of the industrial holdings reared layer hens (72,874 birds), whereas the backyard flocks were mainly multispecies (each rearing 10–70 birds), and all of them reared a few layer hens. The control measures included stamping out at infected holdings and monitoring of the rural and industrial sectors at the local level. The epidemiologic investigation did not provide any useful information.

2009 H7N3 LPAI epizootic.

In May 2009, two strains of H7N3 LPAI virus (A/turkey/Italy/2719/2009 H7N3 and A/turkey/Italy 2718/2009 H7N3) were isolated from industrial meat turkeys at a slaughterhouse in the region of Umbria (central Italy). The meat turkey holding that had provided the slaughterhouse with these birds, also located in Umbria, reared both female and male turkeys, and the female turkeys (which are usually slaughtered 2 mo before the males) were found to be virologically positive at the slaughterhouse. Both female turkeys (n = 13,970) and male turkeys (n = 19,328) were culled and the carcasses were disposed. A sequences analysis of the HA and NA genes revealed high similarity (97.8%–98% and 98.4%–98.6%, respectively) of this virus with those isolated during the 2007 Italian H7N3 LPAI epizootic. One week after the outbreak, another H7N3 LPAI outbreak was detected (by means of RRT-PCR assay) at an industrial broiler breeder holding in the Piemonte region (rearing 11,717 birds). The epidemiologic investigation did not reveal any connections with the previous outbreak.

From May to December 2009, another 31 outbreaks caused by the same LPAI virus subtype were reported in the rural sector (18 backyard flocks and 13 dealers or growers). These outbreaks occurred in eight regions (Fig. 2) and involved 41,434 birds. These holdings reared multispecies (i.e., layers, turkeys, chickens, ducks, geese, guinea fowls, quails (*Coturnix coturnix*), peacocks (*Pavo cristatus*), ornamental birds, and pigeons (*Columba livia*) and multiage poultry. No clinical signs were observed in the infected flocks. Several direct and indirect connections among 21 of the infected holdings were identified (Fig. 4). In particular, a direct connection (i.e. the purchase of live poultry) was identified between one large grower and dealer holding and another 19 infected premises (five dealers and 14 backyard flocks), which were subsequently found to be positive. Moreover, H7N3 LPAI virus infection was detected twice in the above-mentioned holding: the first time in July and the second time in December, despite the stamping out, cleaning, disinfection, and negative serologic and virologic findings performed after the previous outbreak.

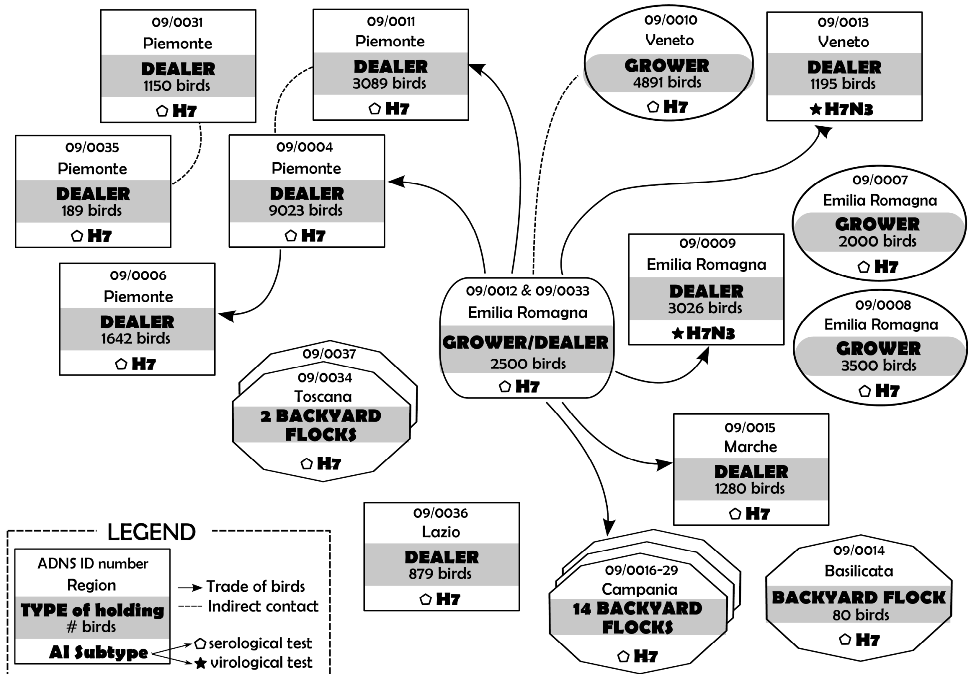

Figure 4. Epidemiologic links among H7 positive rural holdings in 2009. A progressive number (ID) referred to notification of the Italian outbreaks to the EU via Animal Disease Notification System (ADNS) are reported.

Of the 31 LPAI outbreaks in rural holdings, sequencing of the HA gene was possible only in two holdings (A/chicken/Italy/3304-12/ 2009 H7N3 and A/duck/Italy/6356/2009 H7N3). Analysis of the HA gene sequences of these strains revealed a similarity of 98.6%–99.9% with the respective gene sequence of viruses previously isolated in the industrial sector in 2009. The percentage of similarity between the available HA sequences of H7N3 LPAI viruses isolated in 2009 and the gene sequences of the LPAI H7N3 viruses isolated in 2007 ranged between 96.5% and 97.3%. The phylogenetic analysis revealed that all of the LPAI H7N3 strains belonged to the same cluster, forming two separate subclusters related to the year of isolation (Fig. 3).

Control measures were applied according to EU legislation [10]. At one infected holding, derogation for endangered species was applied: 61 birds were not stamped out, and they were regularly tested as prescribed previously [11]. Different additional control measures also were implemented in different Italian regions according to the local epidemiologic situation. These measures included:

1) pre-movement sampling of birds in all industrial turkey, breeder, and layer holdings;
2) sampling of all dealer holdings (serologic samples and tracheal swabs from 10 birds per shed, up to a maximum of 60 samples per holding);
3) a 3-wk temporary ban of bird movements from dealer and grower holdings;
4) prohibition of markets and fairs; and
5) prohibition of the trading in markets and fairs of some bird species known to be reservoirs of AI viruses (i.e., ducks, geese, and quails).

DISCUSSION

Two AI epizootics caused by an LPAI virus of the H7N3 subtype were identified in Italy in 2007 and 2009, both of which mainly affected rural poultry holdings. Given that previous outbreaks affected almost exclusively the industrial sector, or, in the 1997 H5N2 HPAI outbreak, only the rural sector, it was hypothesize that these sectors could be considered as two separate poultry compartments. However, the findings described here raised the issue of the permanent presence of multiple epidemiologic connections that could result in transmission between the two sectors. For example, we can hypothesize as at-risk connections:

1) the trade of birds from the industrial to the rural poultry production chain (e.g. trade of spent hens and cock chicks);
2) staff of industrial poultry holdings possessing backyard poultry, as reported by Bonfanti et al. [2]; and
3) illegal contacts, such as the direct trade of live poultry from industrial holdings to rural growers or dealers.

Seven LPAI outbreaks in 2007 and two outbreaks in 2009 were identified in industrial meat-turkey holdings by the controls carried out as part of the national AI surveillance program, confirming the program's effectiveness in terms of the early detection of the introduction of AI viruses in the domestic bird population. Furthermore, the prompt identification of these outbreaks and the immediate application of adequate eradication measures, including emergency vaccination, allowed the disease to be eliminated from the industrial sector. The spread of AI infection was thus limited, and the outbreaks were eliminated in a shorter period of time than in the previous AI epizootics in Italy, with a marked reduction in economic losses [24].

Epidemiologic and phylogenetic data indicate the possible persistence in a domestic reservoir of the 2007 H7N3 LPAI virus, which reemerged in 2009, mainly in rural poultry. In fact, in both the 2007 epizootic and the 2009 epizootic, rural poultry was extensively involved in the spread of the virus. The complex structure of the rural sector makes it very

difficult to control the transmission of AI infections in the dealer circuit, with the possible onset of a huge number of outbreaks in backyard poultry, as observed in the 2000 epizootic of Newcastle disease in Italy [6]. The role of growers and dealers in disseminating and perpetuating the infection in the rural poultry production chain was highlighted in the aforementioned epizootics, during which they were identified as the source of infection for several rural holdings (Fig. 4). The high genomic homology between the H7N3 LPAI virus strains isolated in 2007 and 2009 and the detection of H7 seropositive poultry holdings in 2008 support the hypothesis of the continuous circulation of the H7N3 LPAI virus in rural poultry. Grower and dealer holdings probably play the main role in perpetuating the infection, because they are characterized by a high number of at-risk contacts with other types of rural poultry holdings. In fact, two dealer holdings linked with a large grower and dealer holding were found to be infected both in 2007 and 2009, and this grower and dealer holding was affected twice in 2009, despite the cleaning and disinfection carried out after the first case of AI infection.

Although backyard or hobby flocks, where poultry are frequently reared in the open, may represent the epidemiologic link between the wild reservoir and domestic poultry [14,25], in the majority of cases they probably play only a marginal role in the spread of AI. In fact, AI outbreaks in backyard flocks are frequently self-limiting, and it is very unlikely that the infection could spread further. This view is consistent with the scientific opinion of the panel of experts of the European Food Safety Authority [13], in which it was reported that backyard or hobby flocks may not play a role in the transmission of AI to commercial holdings in the EU. Although it was demonstrated that backyard poultry played a marginal role in the 2003 H7N7 HPAI epizootic in the Netherlands [1], this conclusion could be due to the backyard poultry population having different characteristics in different EU member states. For example, in the Netherlands most of the backyard poultry population consists of hobby or pet-bird flocks, whereas in Italy the rural poultry production chain is almost exclusively orientated toward the production of poultry meat in rural or suburban areas.

In Italy, the major concern is posed by growers and dealers, whose peculiar husbandry and trade practices facilitate the persistence and circulation of AI viruses. The 2009 H7N3 LPAI epizootic, in which multiple connections among grower and dealer holdings were identified, demonstrated that the biosecurity and surveillance activities implemented at grower and dealer holdings are not sufficient. In fact, although increasing the sampling frequency may contribute to earlier and more efficient detection in industrial premises [15], this approach is not applicable to Italian grower and dealer holdings, given that these holdings are characterized by the continuous trade and mixing of birds of different species and ages, so that the amount and the frequency of samples per holding needed for early detection would be unaffordable. It follows that one possible strategy to bring

these holdings under control could be the application of stricter biosecurity measures and rules on the movement of live poultry.

To reduce the risk of spread of AI virus in the rural poultry population, the level of control at the holdings at the top of the production chain (breeders, dealers, and growers) should be increased, through the accreditation of these types of poultry operations according to well-defined managerial, structural, and sanitary requirements. Greater attention also should be placed on the rural poultry operations that trade live poultry outside of their region, because they might spread AI viruses to the whole country. In such premises, the following should be guaranteed:

1) traceability of traded birds;
2) enforcement of well-defined biosecurity measures; e.g. ducks, geese, and quails should be reared separately from other bird species; live poultry brought to markets and fairs should not be reintroduced at the holding; once a year, an empty period should be applied; and cleaning and disinfection should be regularly performed; and
3) regular testing of birds for AI.

Less restrictive measures could be implemented in rural premises that trade live poultry within the region only. To participate at markets and fairs, these premises should guarantee the traceability of traded birds and the regular testing of birds for AI. Moreover, accreditation should be given by the local veterinary authorities after inspections carried out twice a year. Rural holdings not meeting the criteria would lose accreditation, with consequent limitations on the trade and movement of live poultry. An analogous approach was successfully applied to live bird markets in the United States [27], allowing the H7N2 LPAI virus that had persisted for approximately 13 yr to be eliminated.

In conclusion, the recent H7N3 LPAI epizootics highlighted the important role of particular components of the Italian rural sector (i.e., growers and dealers) in the maintenance and spread of AI viruses. The current surveillance activities led to the identification of the infected premises but did not allow the prompt eradication of the disease. In this work, we suggest strategies that could be adopted to avoid the persistence of AI within the rural sector, based on the regulation and control of poultry holdings at the top of the production chain.

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CHAPTER 4

Transmission dynamics of low pathogenicity avian influenza infections in turkey flocks

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ABSTRACT

Low pathogenicity avian influenza (LPAI) viruses of H5 and H7 subtypes have the potential to mutate into highly pathogenic strains (HPAI), which can threaten human health and cause huge economic losses. The current knowledge on the mechanisms of mutation from LPAI to HPAI is insufficient for predicting which H5 or H7 strains will mutate into an HPAI strain, and since the molecular changes necessary for the change in virulence seemingly occur at random, the probability of mutation depends on the number of virus replicates, which is associated with the number of birds that acquire infection. We estimated the transmission dynamics of LPAI viruses in turkeys using serosurveillance data from past epidemics in Italy. We fitted the proportions of birds infected in 36 flocks into a hierarchical model to estimate the basic reproduction number (R0) and possible variations in R0 among flocks caused by differences among farms. We also estimated the distributions of the latent and infectious periods, using experimental infection data with outbreak strains. These were then combined with the R0 to simulate LPAI outbreaks and characterise the resulting dynamics. The estimated mean within-flock R0 in the population of infected flocks was 5.5, indicating that an infectious bird would infect an average of more than five susceptible birds. The results also indicate that the presence of seropositive birds does not necessarily mean that the virus has already been cleared and the flock is no longer infective, so that seropositive flocks may still constitute a risk of infection for other flocks. In light of these results, the enforcement of appropriate restrictions, the culling of seropositive flocks, or pre-emptive slaughtering may be useful. The model and parameter estimates presented in this paper provide the first complete picture of LPAI dynamics in turkey flocks and could be used for designing a suitable surveillance program.

KEY WORDS

Within-flock transmission, LPAI, meat turkeys, field data, Bayesian approach, Stochastic epidemic model

INTRODUCTION

Infection with low pathogenicity avian influenza (LPAI) viruses is widespread and in many countries has led to outbreaks in domestic birds [1]. Although LPAI strains do not pose a serious concern for animal health, LPAI subtypes H5 and H7 may mutate into highly pathogenic strains (HPAI) [2], outbreaks of which can threaten human health [3], in addition to causing huge economic losses due to high bird-mortality rates and to the cost of control measures [4].

Although influenza viruses have been extensively studied, the current knowledge on the mechanisms of mutation from LPAI to HPAI is insufficient for predicting which H5 or H7 strains will mutate into an HPAI strain. Moreover, given that the molecular changes necessary for the change in virulence seem to occur at random [5], the probability that an LPAI strain will mutate into an HPAI strain depends on the extent of viral replication, which in turn is associated with the number of birds that acquire infection. Hence knowledge of the disease dynamics of LPAI viruses is important for better understanding their reversion to virulence. This knowledge can also contribute to optimizing surveillance systems and improving the effectiveness of control measures for reducing transmission and thus the number of virus replicates, reducing the probability of mutation into HPAI viruses.

Studies conducted on the disease dynamics of LPAI viruses under experimental conditions have provided rough estimates of the parameters of bird-to-bird transmission for a H5N2 LPAI [6] and a H7N1 LPAI [7] virus strains. For instance, the basic reproduction number (R_0), which is defined as the mean number of secondary cases per primary case in a susceptible population [8] and is a key epidemiological parameter, was estimated to be between 0.6 and 4.0. However, in experimental conditions it is impossible to assess the variability in transmission that occurs among flocks in field conditions. Using outbreak data, the transmission dynamics of HPAI strains have been studied by applying compartmental models and using mortality data to extrapolate the moment of virus introduction [9, 10]. However, for LPAI epidemics, such data cannot be used because infections result in only mild symptoms and low mortality rates.

In the period 2000-2005, Italy experienced four epidemics of LPAI, all of which occurred in the north and most of which involved meat turkeys. In the present study, we used serosurveillance data from these epidemics [11] to estimate the R_0 of LPAI in turkeys; this is the first time that field data have been used to evaluate the transmission dynamics of LPAI. We fitted the proportions of birds ultimately infected in 36 flocks into a hierarchical model to estimate R_0 and the possible variation in R_0 among flocks caused by differences among farms. To obtain a more complete picture of LPAI transmission, we used experimental infection data with outbreak strains to estimate the distributions of the latent and infectious periods. These were then combined with the R_0 to simulate LPAI

outbreaks, characterise the resulting dynamics, and discuss the implications for surveillance.

RESULTS

Basic reproduction number (R_0)

Using data from the 2000-2005 LPAI outbreaks in northern Italy, we estimated the R_0 based on the seroprevalence in selected flocks after the outbreaks had come to an end (referred to as the "final size"). In other words, we considered only those flocks that tested negative to antigen detection ±5 days from the earliest positive serological finding in the flock. The selected farms consisted of those with unvaccinated meat-turkey flocks housed in a single shed. This resulted in 36 selected flocks (Table 1): 27 were infected by H7N3 and 9 by H7N1 LPAI strains. The mean seroprevalence (i.e., final size) in the selected flocks was 89.3% (Exact Fisher's 95% confidence interval: 85.7-92.2), which was significantly higher than the seroprevalence in the flocks that were positive for antigen detection (i.e., 61.7%; 95%CI: 50.3-72.3, data not shown), confirming the validity of this inclusion criterion (i.e., negative for antigen detection).

Table 1. Outbreak data included in the analyses.

outbreak ID	virus strain	sampled birds	positive findings	outbreak ID [continue]	virus strain	sampled birds	positive findings
1	H7N3	10	10	19	H7N1	10	8
2	H7N3	10	10	20	H7N1	10	9
3	H7N3	10	5	21	H7N1	10	10
4	H7N3	10	10	22	H7N3	10	10
5	H7N3	10	9	23	H7N1	15	12
6	H7N3	10	10	24	H7N3	10	10
7	H7N3	10	9	25	H7N1	10	1
8	H7N3	10	9	26	H7N3	10	10
9	H7N3	10	10	27	H7N1	10	10
10	H7N3	10	9	28	H7N1	10	8
11	H7N1	10	10	29	H7N3	10	10
12	H7N3	10	10	30	H7N3	10	9
13	H7N3	10	9	31	H7N3	10	7
14	H7N3	10	10	32	H7N3	10	10
15	H7N3	8	8	33	H7N3	10	10
16	H7N3	10	10	34	H7N3	10	10
17	H7N3	10	10	35	H7N3	10	2
18	H7N1	20	20	36	H7N3	10	9

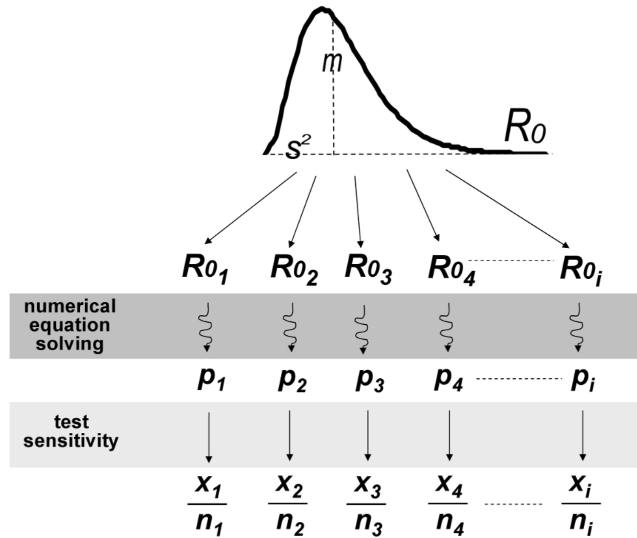

Figure 1. Hierarchical model linking serosurveillance data with R_0 in the population of infected flocks, through the final size equation

Legend: ***m***, mean R_0 in the population of infected flocks; $\boldsymbol{s^2}$, variance of R_0 in the population of infected flocks; $\boldsymbol{R_{0i}}$, basic reproductive number of each infected flock *i*; ***p***, final size of the epidemic; ***x***, proportion of positive samples; ***n***, total number of samples.

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The final size data were fitted into a Bayesian hierarchical model (Figure 1) to estimate the distribution of R_0 among flocks, resulting in a mean value of 5.5 (95% posterior credible interval: 3.4–18.3) and a variance of 11.3 (95%PCI: 1.7-298).

The sensitivity of the diagnostic test (i.e., haemoagglutination inhibition) was estimated in the same model and was equal to 0.977 (95%PCI: 0.953-0.992) (Table 2), which was insensitive to the choice of prior distribution (0.975 with uninformative prior).

Table 2. Estimation of R_0. Median and 95% credibility intervals of the posterior densities of shape and rate (i.e., the parameters defining the gamma distribution of R_0 in the population of infected flocks), mean and variance of R_0 and test sensitivity.

	median	95% posterior credibility interval
ρ, rate	2.73	[0.9839 – 7.47]
κ, shape	0.4909	[0.06023 – 2.07]
m, mean R_0	5.535	[3.357 –18.33]
s^2, variance of R_0	11.29	[1.684 – 298.8]
Se, test sensitivity	0.9768	[0.9532 – 0.9924]

Estimation of latent and infectious periods

Given that field data were not available for estimating the duration of the latent and infectious periods, we used previous data from experimental infections with outbreak strains. The data were available for 18 unvaccinated commercial turkeys challenged with two different LPAI strains (H5N2 and H7N3, 9 birds per strain) and swabbed at days 3, 5, 7, 10, 12, 15 and 20 post inoculation. Infectivity was tested by means of both PCR and virus isolation assays. The test results are given in Table 3. Because sensitivity was higher for PCR, compared to virus isolation assays, we used the PCR results for our default analysis. However, given that positive virus isolation may better reflect infectivity, we repeated the analysis with the virus isolation results to assess the sensitivity of this choice for the outbreak simulations described below.

Table 3. Test results of swabbed turkeys at different days post infection.

	results of PCR assay (dataset A)								**results of virus isolation (dataset B)**							
ID of challenged birds	days p.i.*	3	5	7	10	12	15	20	days p.i.	3	5	7	10	12	15	20
	k1	+	+	+	−	−	−	−	k1	+	−	−	−	−	−	−
	k2	−	+	+	+	−	−	−	k2	−	−	+	−	−	−	−
	k3	−	+	+	−	−	−	−	k3	−	−	−	−	−	−	−
	k4	−	+	+	+	−	−	−	k4	−	−	+	−	−	−	−
	k5	−	+	+	−	−	−	−	k5	−	−	−	−	−	−	−
	k6	+	+	+	−	−	−	−	k6	−	−	−	−	−	−	−
	k7	−	+	+	−	−	−	−	k7	−	−	+	−	−	−	−
	k8	−	+	+	−	−	−	−	k8	−	+	−	−	−	−	−
	k9	−	+	+	+	−	+	−	k9	−	+	+	−	−	−	−
	k10	−	+	+	+	−	+	−	k10	−	−	−	−	−	−	−
	k11	+	+	+	+	+	−	−	k11	−	−	−	−	−	−	−
	k12	+	+	+	+	−	−	−	k12	−	−	−	−	−	−	−
	k13	+	+	+	+	+	+	−	k13	−	−	−	−	+	+	−
	k14	+	+	+	+	−	+	−	k14	−	−	−	−	−	+	−
	k15	+	−	−	−	−	−	−	k15	+	−	−	−	−	−	−
	k16	+	+	+	+	+	+	−	k16	+	−	−	−	−	+	−
	k17	+	+	+	+	−	−	−	k17	−	−	−	−	−	−	−
	k18	+	+	+	+	+	−	−	k18	−	−	−	−	+	−	−

Birds k1 to k9 were challenged with H5N2 LPAI virus and birds k10 to k18 with H7N3 LPAI virus. *Days p.i. = days post inoculation.

The estimates of latent and infectious periods were calculated using a Bayesian model, and the results varied according to the definition of "infected animal". When the definition was based on the quantity of viral genome in faeces (identified by PCR), the mean latent period was 2.9 days (95%PCI: 2.4-3.4), and the mean infectious period was

8.2 days (95%PCI: 6.5-10.6). When the definition was based on the isolation from faeces of a live virus capable of replication (detected by virus isolation), the mean latent period was 8.7 days (95%PCI: 3.9-33.8) and the mean infectious period was 2.3 days (95%PCI: 1.3-3.5) (Table 4).

Table 4. Estimates of the latent (LP) and infectious (IP) periods. Median and 95% credibility intervals of the posterior densities of κ_L, ρ_L, κ_I and ρ_I (i.e., the parameters defining the gamma distribution of LP and IP), and the mean latent and infectious periods.

	dataset A		dataset B	
mean latent period (days)	2.932	[2.407; 3.388]	8.650	[3.847; 33.780]
mean infectious period (days)	8.161	[6.454; 10.580]	2.323	[1.303; 3.530]
κ_L	17.480	[3.011; 128.20]	0.878	[0.240; 3.458]
ρ_L	5.954	[1.096; 43.110]	0.102	[0.011; 0.533]
κ_I	4.640	[2.036; 9.634]	3.803	[0.672; 53.210]
ρ_I	0.568	[0.233; 1.228]	1.723	[0.332; 18.100

Outbreak simulations

To characterize the dynamics of LPAI outbreaks, we simulated 1,000 outbreaks in flocks of 10,000 turkeys each, with a SEIR stochastic model using the posterior median transmission parameters (Tables 2, 4, 5), with the estimates of latent and infectious periods derived from the PCR results (dataset A).

Table 5. Input parameters and assumptions for the three simulation models.

	model 1	model 2*	model 3
input parameters	$m, \kappa_L, \rho_L, \kappa_I, \rho_I$	$\kappa, \rho, \kappa_L, \rho_L, \kappa_I, \rho_I$	$\kappa_{[i]}, \rho_{[i]}, \kappa_{L[i]}, \rho_{L[i]}, \kappa_{I[i]}, \rho_{I[i]}$
basic reproduction number	$R_{0[i]} = m$	$R_{0[i]}$ ~ Gamma(κ, ρ)	$R_{0[i]}$ ~ Gamma($\kappa_{[i]}, \rho_{[i]}$)
latent period	$LP_{[i]}$ ~ Gamma(κ_L, ρ_L)	$LP_{[i]}$ ~ Gamma(κ_L, ρ_L)	$LP_{[i]}$ ~ Gamma($\kappa_{L[i]}, \rho_{L[i]}$)
infectious period	$IP_{[i]}$ ~ Gamma(κ_I, ρ_I)	$IP_{[i]}$ ~ Gamma(κ_I, ρ_I)	$IP_{[i]}$ ~ Gamma($\kappa_{I[i]}, \rho_{I[i]}$)

**baseline model*

Legend: $\boldsymbol{m}$, mean R_0; $\boldsymbol{\kappa_L}$ and $\boldsymbol{\rho_L}$, parameters describing the gamma distribution of the latent period; $\boldsymbol{\kappa_I}$ and $\boldsymbol{\rho_I}$, parameters describing the gamma distribution of the infectious period; $\boldsymbol{\kappa}$, *shape* parameter of the gamma distribution of R_0; $\boldsymbol{\rho}$, *rate* parameter of the gamma distribution of R_0; i = 1 to 1,000 (i.e., number of simulated outbreaks).

The descriptive statistics of the simulated outbreaks using the baseline model (model 2) are shown in Table 6. The quoted intervals are the 2.5th and 97.5th percentiles. The duration of outbreaks (i.e., from the first to the last infected turkey) ranged from 56 to

337 days (i.e., 2 to 11 months), although 90% of the infections were observed in a period of 10-150 days. The epidemic peak (i.e., the day that the peak number of infective birds was reached) occurred at a median of 45 days after the first case, which is only 7 days after a serological sample of 10 turkeys would be detected with 50% probability (median $T_{det50\%}$ is 38 days). At the peak, a median of about 50% of the turkeys were infected, though this percentage greatly varied among farms (3% – 74%). At that time, 15.8% – 28.3% of the turkeys were already seropositive, indicating a period of overlap where both antigen and serological tests were able to detect infection. As expected, the seroprevalence at the end of the outbreak (R_{final},) (i.e., the final size) was high, even higher than 99.4% in half of the cases.

Table 6. Descriptive statistics for 1,000 simulated outbreaks using the baseline model (model 2) and reference dataset (dataset A) (i.e., PCR results).

parameter	mean	median	2.5th percentile	97.5th percentile
Duration (days)	106	83	56	337
T_{peak} (days)	57	45	28	164
$D_{90\%}$ (days)	32	20	10	150
I_{peak} (%)	46.4	49.8	3.4	74.1
R_{peak} (%)	28.8	29.6	15.8	28.3
R_{final} (%)	93.5	99.4	41.9	100
$T_{det50\%}$ (days)	47	38	25	130

Note: Model 2 assumed that all 1,000 simulated outbreaks had the same value of mean latent and infections periods (estimated using the results of PCR assay), whereas values of R0 were all sampled from the same gamma distribution with parameters κ and ρ at the median value of the posterior distributions.

Legend: Duration, duration of the epidemic in days; $\mathbf{T_{peak}}$, time of the epidemic peak (days after infection); $\mathbf{D_{90\%}}$, time interval (days) during which the mid-90% of the cases occur (90% incidence interval); $\mathbf{I_{peak}}$, peak number of infective birds; $\mathbf{R_{peak}}$, seroprevalence at the epidemic peak; $\mathbf{R_{final}}$, seroprevalence at the end of the outbreak; $\mathbf{T_{det50}}$, time by which a serological sample of 10 turkeys would result in detection with 50% probability (days).

Sensitivity analysis

To investigate the possible sources of variation in the outbreaks' descriptive statistics, we compared the above-mentioned results (obtained with the baseline model) with simulations derived from models with different levels of uncertainty (Table 5). In particular, model 1 was used to investigate only stochastic effects, model 2 (i.e., the baseline model) to investigate stochastic effects and variation in R_0 among flocks, and model 3 to investigate stochastic effects, variation in R_0 among flocks, and uncertainty about the parameters that defined the distribution of the R_0, latent and infectious periods. The results are provided in Table 7.

Table 7. Sensitivity analysis: descriptive statistics for 1,000 simulated outbreaks under different model assumptions and datasets.

	Model 1 – dataset A			Model 3 – dataset A			Model 2 – dataset B		
Parameter	median	2.5^{th} percentile	97.5^{th} percentile	median	2.5^{th} percentile	97.5^{th} percentile	median	2.5^{th} percentile	97.5^{th} percentile
Duration (days)	79	72	90	86	42	400	135	100	400
T_{peak} (days)	43	39	51	47	17	235	41	18	175
I_{peak} (%)	52.4	51.3	53.4	46.7	1.4	87.2	11.3	1.2	16.2
R_{final} (%)	99.6	99.5	99.7	99.3	38.4	100	99.5	49.7	100

Note: Model 1 assumed that all 1,000 simulated outbreaks had the same R_0, κ_L, ρ_L, κ_I and ρ_I, all medians from the posterior distributions. Model 2 assumed all simulations with the same κ_L, ρ_L, κ_I and ρ_I, but with different R_0. 3In model 3 all simulations had different κ_L, ρ_L, κ_I, ρ_I, and R_0. Dataset A includes the results of PCR assay, whereas dataset B includes the results of virus isolation.

Legend: Duration, duration of the epidemic in days; $\mathbf{T_{peak}}$, time of the epidemic peak (days after infection); $\mathbf{D_{90\%}}$, time interval (days) during which the mid-90% of the cases occur (90% incidence interval); $\mathbf{I_{peak}}$, peak number of infective birds; $\mathbf{R_{peak}}$, seroprevalence at the epidemic peak; $\mathbf{R_{final}}$, seroprevalence at the end of the outbreak; $\mathbf{T_{det50}}$, time by which a serological sample of 10 turkeys would result in detection with 50% probability (days).

The estimated median values obtained with model 1 were very similar to those obtained with the baseline model, though with a marked narrowing of the 95% credible intervals. This is clearly visible for the peak number of infective birds (median Ipeak: 52.4% in model 1 versus 49.8% in model 2), for which the precision of the estimation in model 1 reached a very narrow interval (51.3%-53.4%). This implies that most variation in the field is due to intrinsic differences among flocks and not to stochastic effects. Obviously, model 3 added more uncertainty to the estimates, resulting in broader credible intervals; however the median results of model 3 were similar to those obtained with model 2 (Tables 6 and 7). The differences between models 2 and 3 were relatively small, indicating that more precise parameter estimates would improve the predicted dynamics of LPAI outbreaks only to a limited extent. This can also be seen in Figure 2, in which an example of the impact of the three models on the time of the epidemic peak is illustrated. Whereas the median estimates were quite similar, the higher precision of model 1 led to a sharper distribution compared to the distributions resulting from models 2 and 3, which encompassed more uncertainty.

To investigate the effect of the definition of "infectious bird" on the disease dynamics, we performed additional simulations using the posterior estimates derived from virus isolation results (i.e., dataset B) (Table 4). Table 7 shows the descriptive statistics of the 1,000 simulated outbreaks using model 2 and dataset B. The final size of the epidemic was the same (99.5%), yet the duration of the epidemic was longer (135 days), the epidemic peak occurred slightly earlier (41 days after infection), and the proportion of infectious birds at the epidemic peak was lower (11.3%) The different disease dynamics associated with different definitions of "infective birds" is shown in Figure 3. The different

assumptions of infectivity (i.e., based upon different diagnostic assays) led to different peak prevalences; however, the timing of the peak prevalences was very close (41 versus 45 days after infection).

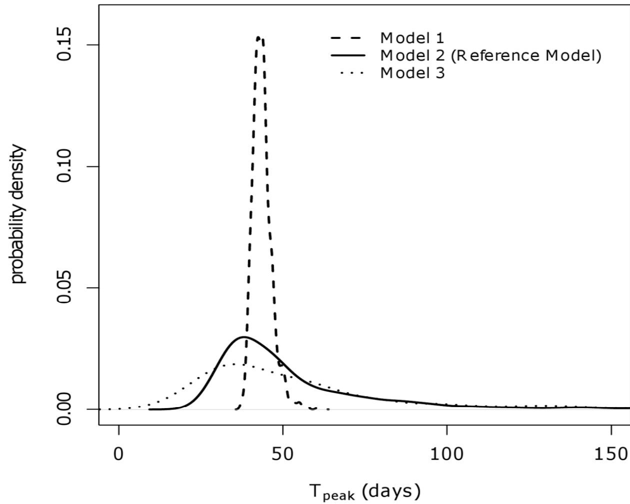

Figure 2. Sensitivity analysis: estimates of time of the epidemic peak (T_{peak}) resulting from 1,000 outbreak simulations using the three different models and the reference dataset (i.e., PCR data)

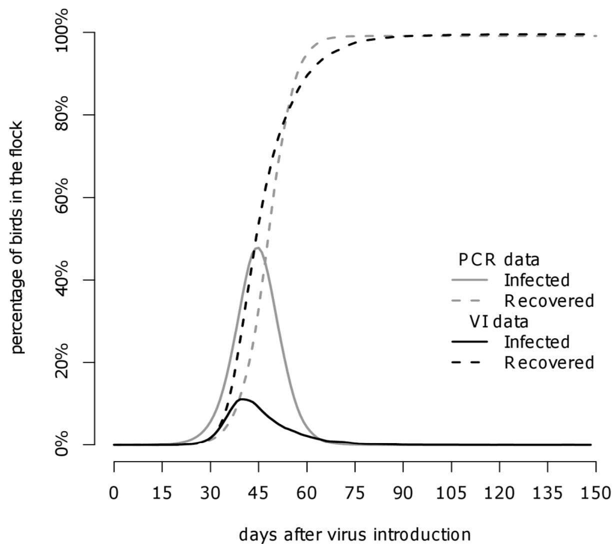

Figure 2. Outbreak simulation in a flock of 10,000 turkeys using the baseline model (model 2): comparison of disease dynamics assuming different definitions of "infective birds" [i.e., based on PCR data or virus isolation (VI) data]

DISCUSSION

In this study, we provide quantitative information on key epidemiological parameters of LPAI dynamics in turkeys, which is the first time that this has been done using outbreak data. We first estimated the basic reproduction number of LPAI infections using the final size equation. To do so, some conditions had to be met. First, the data had to refer to a single population with homogeneous mixing. Because data from farms with multiple sheds were not stratified by shed, only the flocks consisting of a single shed were included in the analyses. Second, the seroprevalence in the samples needed to be representative of the entire flock, so that the seroprevalence in each sample could be considered to have a binomial distribution depending on the final size and the sample size. In accordance with the surveillance plan, sampled animals were randomly selected within each flock. Third, the outbreaks in the flocks had to have ended (i.e., no virus should have still been circulating). For this reason we included only flocks with negative virus tests ± 5 days from the day of serological positivity. The validity of this inclusion criterion was indicated by the lower seroprevalence in virus-positive flocks, although absolute certainty about the final size status of the flocks can never be obtained. If a virus had still been circulating in some flocks, the R_0 would have been underestimated.

Based on these assumptions, the estimated mean within-flock R_0 in the population of infected flocks was 5.5, meaning that on average an infectious bird would infect more than five susceptible animals. In an experimental study of van der Goot et al. (2003) [6], estimates of R_0 for LPAI H5N2 in chickens were much lower, ranging from 0.6 to 1.2. On the other hand, Gonzales et al. (2011) [7] recently estimated R_0 for LPAI H7N1 in experimentally infected chickens to be about 4.0, demonstrating a high variability in virus transmission among different strains. Another possible explanation for the difference between our results and the estimates reported in literature could be due to differences in susceptibility between chickens and turkeys, which has been reported in comparative experimental studies [12, 13] that show that turkeys are highly susceptible to LPAI infections and that chickens are less susceptible. Lastly, the difference could also be due to differences between experimental and field conditions, as reported by Bos et al. (2010) [14] for HPAI; in particular, whereas experiments take place under controlled settings, in field conditions other factors can enhance transmission, such as concurrent infections, climatic and environmental factors, and factors related to management.

In several studies on within-flock transmission of HPAI based on outbreak data [9, 10, 14], only a single R_0 was estimated, based on the assumption that there is only one R0 that is common to all flocks. However, in the field a number of factors can result in differences among flocks. First of all, there are differences between LPAI virus strains [6, 7], such as the amount of virus excreted by infected birds and the minimum infectious dose [15]. Furthermore, we should also consider the differences in the characteristics of

the farms and the age of the birds when the outbreak occurs. For example, the density of birds, which is mainly related to the birds' size and thus their age, can affect the contact rate among animals. Moreover, the time at which the virus enters a flock may instead influence the infectivity and/or susceptibility of the birds, which is related to their age, immunological competence and eventual stress due to intensive production cycles. Our approach took into account this variability by modelling R_0 as a probability distribution and thus allowing the transmission dynamics to vary from flock to flock. Furthermore, in our model, the sensitivity of the test was estimated together with R_0, and the median sensitivity was 97.6%, which is fairly close to the sensitivity suggested by laboratory experience (98%). Uncertainty about test performance allowed us to better account for the fact that data came from a serosurveillance program, whose results depend on the true infectious status of the flock, the sampling scheme and the accuracy of the diagnostic assays.

To investigate the within-flock dynamics of LPAI viruses, we needed to know the temporal window of infectivity, defined by the mean lengths of the latent and infectious periods. Because this information was unavailable from field data, we based our estimates on earlier experimental infections with the outbreak strains. The infection status of single birds was tested by means of both PCR and virus isolation assays. If PCR results reflect infectivity (default), the infectious period of 8.2 days would be longer than that reported for chickens: 4.5 to 7.7 days for LPAI [6, 7] and 1.3-2.5 days for HPAI [16]. This may be related to the higher R_0 in turkeys and to the virus strain.

In our experimental data, a positive PCR result indicated the presence of viral genome in faeces, which may not be sufficient for replication and the infection of new hosts, possibly resulting in an overestimate of the length of the infectious period. For this reason, we also estimated the infectious period using the results of virus isolation assay, to assess the effect on the predicted outbreak dynamics. In fact, a positive virus isolation implies that the virus can replicate and may thus better reflect infectivity. The difference between the two tests can be seen in Table 3: for example, bird k13 tested positive to PCR from day 3 to day 15 (dataset A) but showed a detectable amount of virus only starting from day 12 (dataset B). Thus, based on virus isolation, the latent period would be longer and the infectious period shorter. However, the difference in terms of mean generation time was small: 7.9 and 10.1 days for PCR and virus isolation results, respectively. The comparison of the prevalence between the two datasets is unfair because it is based on different diagnostic tests and assumptions regarding infectivity. The important difference lies in the timing of the peak prevalence and the increase in seroprevalence, which were rather similar when comparing PCR and virus isolation data (Figure 3), indicating that the results are not very sensitive to the choice of diagnostic assay.

The comparison of the three simulation models showed that the variation in R_0 among flocks plays an important role in the variation among outbreaks (Tables 6 and 7). In Figure 2, it appears that model 1, which only accounts for differences due to the stochastic process, resulted in only a limited variability in the timing of the epidemic peak (median: 43 days, 95%PCI: 39–51). Adding uncertainty related to possible variation of R0 among flocks (model 2) resulted in a similar median estimate (45 days), yet it remarkably increased the variation (95%PCI: 28–164), as demonstrated by the much flatter density distribution of the parameter. The inclusion of further uncertainty about the parameter estimates (model 3) led to an additional widening of the interval (95%PCI: 17–235), but the difference was limited extent when compared to model 2. (Figure 2, solid versus dotted line). We could thus argue that our estimates of latency, infectivity and the mean and variance of R_0 in the population of infected flocks are sufficiently precise, though we cannot overlook the variation in R_0 among flocks, which seems to play the most important role in the variation among infected premises.

The simulations showed that the finding of seropositive birds does not necessarily mean that the flock is no longer infective: Table 6 shows that at the epidemic peak about 50% of the turkeys were infected, yet that 16% to 28% of the turkeys were already seropositive, indicating a period of overlap where both antigen and antibodies are detectable (Figure 3, grey lines). This implies that seropositive flocks may still pose a risk for other flocks; thus the enforcement of appropriate restrictions, the culling of seropositive flocks or pre-emptive slaughtering may be useful in preventing farm-to-farm transmission. On the other hand, sero-sampling for early disease detection may be difficult, because the time by which a serological sample of 10 turkeys would result in detection with 50% probability (Tdet50%) is only 7 days before peak infectivity (Tpeak).

The model and parameter estimates presented in this paper provide the first complete picture of LPAI dynamics in turkey flocks and could as such be used for the design and optimization of a suitable surveillance program.

MATERIALS AND METHODS

The within-flock disease dynamics of LPAI were investigated using field data from outbreaks and data from experimental infections and combining these data in a stochastic simulation model. The investigation was conducted in three steps:

Estimation of within-flock R0 for LPAI infections using field data and a Bayesian hierarchical model based on the final size equation, provided below;

Estimation of the distribution of latent and infectious periods of LPAI in turkeys, using pre-existing data from experimental infections; and

Simulation of outbreaks using the estimates in points 1 and 2 and characterisation of LPAI outbreaks and their uncertainty (sensitivity analysis).

Estimation of the basic reproduction number (R_0)

Data source

The field data were provided by the intensive surveillance system which was in place during the LPAI epidemics in 2000-2001, 2002-2003, 2004 and 2005 [17]. During and around the time of the epidemics, a total of 6,102 poultry farms were routinely visited; 495 infected premises (i.e., outbreaks) were identified; 429 (87%) of these premises reared meat turkeys. Of the 429 outbreaks, we included only those that had occurred among unvaccinated flocks (n=204). Although it would have been interesting to have investigated the disease dynamics in vaccinated birds, this was not possible because in the vaccinated flocks only unvaccinated sentinels were sampled. To fulfil the assumption of homogeneous mixing of the animals required for the analysis, we only included those farms on which the birds were housed in a single shed (n=64).

Inclusion criteria

At the 64 farms, multiple samplings had been carried out. In each flock, a median of 10 (range: 8-20) birds per sampling were considered. We considered the earliest sampling that revealed a positive serological finding and determined whether an antigen detection assay had been performed ±5 days from this finding; antigen detection had been performed on mixed samples (pools) of five birds. If the flock was negative, then the outbreak was assumed to be over, and the seroprevalence in the sample was considered to represent the proportion of the population that had been infected by the end of the outbreak (defined as the “final size”). This resulted in the identification of 36 outbreaks (Table 1 and Table S1).

Model building

The final size of an epidemic (p, the proportion of a population that had been infected by the end of an outbreak) and the basic reproduction number (R0) are related through the final size equation:

$$p = 1 - \mathrm{e}^{(-\mathrm{p}\mathrm{R}_0)} \qquad \text{(Eq. 1)}$$

which is considered to be valid under very general circumstances [18]. Serosurveillance data were fitted to a hierarchical model (Figure 1), assuming that R_0 in the population of infected flocks followed a gamma probability distribution, with mean m and variance s2. Each R_{0i} of flock i corresponds to a final size pi, calculated numerically from Eq. 1. The observed number of positive samples xi in each flock was then considered

to be a sample from a binomial distribution with n = ni (sample size) and p = pi (final size)*test sensitivity (which represents the apparent prevalence in each flock i). The gamma distribution of R_0 was defined by the parameters shape ($\kappa > 0$) and rate ($\rho > 0$), which are related to the mean (m) and variance (s2) of R_0 as:

$$m = \frac{\kappa}{\rho} \quad \text{and} \quad s^2 = \frac{\kappa}{\rho^2}$$

Furthermore, the use of an imperfect diagnostic test was assumed in the detection of seroprevalence, with sensitivity modelled as a Beta distribution. This model is the result of a careful preliminary investigation in which several alternatives have been compared. The initial assumption of a single R_0 value common to all the infected flocks did not fit our field data and we thus modelled R_0 as a probability density distribution. Different hypothesis on R_0 distribution and test sensitivity were then explored and evaluated by means of the deviance information criterion (DIC) [19]. The currently presented model is the one which resulted in the best fit of the field data.

The model was implemented in WinBUGS software version 1.4.3; posterior distributions were obtained using the default internal Gibbs sampler [20]. Uninformative prior distributions were used for the parameters κ and ρ [i.e., Gamma(0.01,0.01)]. Informative prior information, based on laboratory experience (but no solid data), was used for the distribution of test sensitivity. Using the R function beta.prior (available at http://skoval.bol.ucla.edu/beta.prior.R), we derived the parameters of the Beta distribution that corresponded to a most likely sensitivity of 0.98 and to a 95% certainty that the sensitivity would be greater than 0.95 [i.e. Beta(151.77,4.08)]. Posterior inferences were based on 30,000 iterations with a sampling lag of 10, after a burn-in of 15,000 iterations was discarded. Convergence was assessed by running multiple chains from dispersed starting values and using the Gelman-Rubin statistic.

Estimation of latent and infectious periods

Data source

The data used for this analysis were taken from a vaccine trial performed in 2004 at the Italian National Reference Laboratory for Avian Influenza (unpublished data). Eighteen unvaccinated commercial turkeys (i.e., the controls of the trial) were challenged with two LPAI strains at 12 weeks of age via the intranasal route. Nine birds were challenged with H5N2 LPAI virus A/TK/IT/80 and 9 birds with H7N3 LPAI virus A/TK/IT/8000/02. The infective dose was 104 EID50. For each bird, cloacal swabs were taken at day 3, 5, 7, 10, 12, 15 and 20 post-inoculation and tested using a real-time RT-PCR assay and virus isolation in SPF fertile eggs. The results are given in Table 3. Because of the higher sensitivity, we used the PCR results for our default analysis. However, given

that a positive virus isolation may better reflect infectivity, we repeated the analysis with the virus isolation results to assess the sensitivity of this choice for the simulation output. Thus two different datasets were built: dataset A (PCR assay) and dataset B (virus isolation).

Model building

We assumed that infectivity was indicated by a positive test result and that, based on individual test results (Table 3), the infectious period was preceded by a latent period. This latent period began immediately after virus inoculation (day 0) and ended at a time point (T1) between the last negative and the first positive test result. Consequently, the infectious period started just after the latent period and ended in the period (T2) between the last positive test and the subsequent negative test. For example, bird k2 in Table 3 (dataset A) showed a latent period starting at day 0 and lasting to somewhere between day 3 and 5 ($3 < T1 < 5$); the infectious period lasted from T1 to between day 10 and 12 ($10 < T2 < 12$). We then assumed that both the latent period (LP) and infectious period (IP) in the population of infected birds followed a gamma distribution, characterized by parameters κ and ρ, as follows:

$$LP \sim \text{gamma}(\kappa_L, \rho_L) \quad \text{and} \quad IP \sim \text{gamma}(\kappa_I, \rho_I)$$

To have an estimate of the latent and infectious periods in the population, we built a Bayesian model to link the distributions of these periods in the population of infected birds with the test results of the 18 challenged turkeys. We noted that for each bird (k1–k18) and at each sampling day (D = 3, 5, 7, 10, 12, 15 and 20) the test result y could be either positive (1) or negative (0). It follows that y has a Bernoulli distribution, depending on the success probability π:

$$y \sim \text{Bernoulli}(\pi)$$

Assuming that the diagnostic test perfectly reflects infectivity, the success probability (i.e., positive test result) depends on whether or not the sample was taken during the infectious period (i.e., when the bird sheds the virus with faeces). We thus assumed 100% probability of a positive test result ($\pi = 1$) if the sampling day D was within the infectious period and 0% probability of a positive test result ($\pi = 0$) if the sample was collected before or after the infectious period:

$$\pi = 1 \quad \text{if} \quad T1 \le D \le T2 \qquad \text{and} \qquad \pi = 0 \quad \text{if} \quad D < T1 \text{ or } D > T2$$

Due to the limited amount of data, it was impossible to obtain reliable estimates of LP and IP for H5N2 and H7N7 strains separately. However, preliminary investigations showed that the overall generation time was a good average of the two separately, which were not that far apart indeed. We thus decided to estimate LP and IP using all the available data, given that the further infection model will encompass enough variability to allow for different virus transmission characteristics.

The model was implemented in WinBUGS software using the default internal Gibbs sampler [20]. Uninformative prior distributions were used for the parameters κL, ρL, κI and ρI [i.e., Gamma(0.01,0.01)]. Posterior inferences were based on 30,000 iterations with a sampling lag of 10, after a burn-in of 15 000 iterations was discarded. Convergence was assessed by running multiple chains from dispersed starting values and using the Gelman-Rubin statistic.

Outbreak simulations

Model building

Estimates of R_0, κ_L, ρ_L, κ_I and ρ_I were combined to simulate and characterize the course of LPAI outbreaks in turkey flocks. Simulations were carried out in R statistical software [21]. Simulations started with one index case infected at time = 0 and 9,999 susceptible birds. The end of the latent and infectious periods of the index case were sampled and stored. At each time step of 0.02 days, the number of infected birds I was calculated; then the number of new infections C was sampled from a binomial distribution (n = number of susceptible birds; p = 0.02 β I/10,000; β = transmission rate = $R_0 \cdot \lambda_I$); finally, the latent and infectious periods of the new cases were sampled and stored.

The simulated outbreaks were summarized by calculating six descriptive statistics: the time of the epidemic peak T_{peak}, the peak number of infective birds I_{peak}, the seroprevalence at the epidemic peak R_{peak}, the seroprevalence at the end of the outbreak R_{final}, the time interval during which the mid-90% of the cases occur (90% incidence interval) $D_{90\%}$, and the time by which a serological sample of 10 turkeys would result in detection with 50% probability (assuming a test sensitivity of 97.7%) T_{det50}.

Sensitivity analysis

To distinguish between sources of variation and uncertainty, three sets of 1,000 simulations each were performed (Table 5):

- **model 1**: all simulations with the same R_0, κ_L, ρ_L, κ_I and ρ_I, all medians from the posterior distributions. Variation among these simulations only reflects stochastic effects;

- **model 2**: all simulations with the same κ_L, ρ_L, κ_I and ρ_I, but with different R_0. The values of R_0 were all sampled from the same gamma distribution with parameters κ and ρ at the median value of the posterior distributions. Variation among these simulations reflects stochastic effects plus variation in R_0 among flocks;
- **model 3**: all simulations with different κ_L, ρ_L, κ_I, ρ_I, and R_0. For each simulation, a random quartet of κ_L, ρ_L, κ_I and ρ_I was sampled from their multiivariate posterior distribution. A random couple of κ and ρ was also sampled, and R_0 was sampled from the corresponding gamma distribution. Variation among these simulations reflects stochastic effects and variation in R_0 among flocks, plus uncertainty about the parameter values.

Model 2 was our baseline model because it reflects the course of outbreaks and variation therein, according to our best estimate. The estimates of κ_L, ρ_L, κ_I and ρ_I included in the above-mentioned models came from dataset A (i.e., PCR results) because we selected PCR as our default analysis, given its higher sensitivity. To investigate the role of the virus detection assay as a further source of uncertainty, a fourth set of 1,000 simulations was performed applying model 2 to dataset B (i.e., virus isolation results).

ETHICS STATEMENT

This study was carried out in strict accordance with the requirements of Italian Law n. 1,1.6 of 27 January 1.992 (OJ.LR. 18 February 1.992, n.40, O.S.) and further amendments referring to Council Directive 86/609/FEC of the European Community (OJ.E.C. 18 December 1986, n. 358) on the protection of animals used for experimental and other scientific purposes. The protocol met the requirements outlined in Annex 4 to Circular n. 8 of the Italian the Ministry of Health, 22 Apr-J. 1994. All animal manipulations were performed under Tiletamine HC1-Zolazepam HC1 anesthesia and all efforts were made to minimize suffering. At the end of the experiment, the animals were euthanized by terminal anaesthesia. The handling and publication of the data generated from such experiment have been approved by the Institutional Ethics Committee of the Istituto Zooproflattico Sperimentale delle Venezie (Permit Number: CE.IZSVE.01/2011).

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SUPPLEMENTARY MATERIAL

Table S1. Raw data used to estimate the probability density distribution of R_0.

flock ID	flock size	virus strain	first sampling: day	first sampling: serology result (positive/tested)	first sampling: virology result (pool)	second sampling: day	second sampling: serology result (positive/tested)	second sampling: virology result (pool)	third sampling: day	third sampling: serology result (positive/tested)	third sampling: virology result (pool)	fourth sampling: day	fourth sampling: serology result (positive/tested)	fourth sampling: virology result (pool)
1	8900	H7N3	92	0/10	not done	97	not done	negative	125	**10/10**	**negative**			
2	13300	H7N3	79	0/10	not done	103	not done	negative	127	**10/10**	**negative**			
3	4300	H7N3	133	**5/10**	**negative**									
4	11000	H7N3	58	0/10	negative	105	**10/10**	**negative**	147	7/10	not done			
5	8000	H7N3	82	0/10	negative	124	0/10	positive	128	0/10	negative	135	**9/10**	**negative**
6	6400	H7N3	64	0/10	negative	92	0/10	negative	99	**10/10**	**negative**			
7	13000	H7N3	73	0/10	not done	107	**9/10**	**negative**	149	not done	negative			
8	11000	H7N3	78	0/10	negative	112	**9/10**	**negative**	154	not done	negative			
9	15000	H7N3	101	not done	positive	107	0/10	positive	**109**	**10/10**	not done	**114**	not done	**negative**
10	16800	H7N3	89	0/10	negative	126	0/10	negative	131	**9/10**	**negative**			
11	10000	H7N1	77	0/10	not done	96	**10/10**	not done	99	not done	**negative**			
12	10900	H7N3	34	0/10	not done	49	not done	negative	83	**10/10**	**negative**			
13	6116	H7N3	63	0/10	not done	106	**9/10**	**negative**						
14	5200	H7N3	66	0/10	negative	117	**10/10**	**negative**	144	not done	negative			
15	5000	H7N3	73	0/10	negative	109	**8/8**	**negative**	116	9/10	negative	132	not done	negative
16	6800	H7N3	80	0/10	not done	84	not done	negative	118	**10/10**	**negative**	151	not done	negative
17	16000	H7N3	43	0/10	negative	79	**10/10**	**negative**						
18	6580	H7N1	87	0/10	positive	102	**20/20**	**negative**	110	not done	negative			
19	12600	H7N1	81	**9/10**	**negative**	93	6/10	not done						
20	12600	H7N1	81	**8/10**	**negative**	93	6/10	not done						
21	12600	H7N1	81	**10/10**	**negative**	93	3/10	not done						
22	3600	H7N3	56	not done	negative	101	**10/10**	**negative**						
23	25600	H7N1	71	**12/15**	**negative**	78	8/20	not done						
24	7600	H7N3	55	0/10	not done	76	0/10	negative	98	**10/10**	**negative**	111	10/10	not done
25	7500	H7N1	51	0/10	not done	64	**1/10**	**negative**						
26	7800	H7N3	84	0/10	negative	102	**10/10**	**negative**						
27	4000	H7N1	81	0/10	not done	86	0/15	negative	106	0/10	not done	110	**10/10**	**negative**
28	6400	H7N1	**87**	**8/10**	not done	**91**	not done	**negative**	98	8/10	not done			
29	12000	H7N3	88	10/10	not done	92	**10/10**	**negative**	112	not done	negative	115	not done	negative
30	17760	H7N3	65	0/10	not done	131	**9/10**	**negative**	145	7/10	negative	147	not done	negative
31	17760	H7N3	65	0/10	not done	131	**7/10**	**negative**						
32	12000	H7N3	42	not done	negative	77	0/10	negative	106	**10/10**	**negative**			
33	12320	H7N3	103	0/10	not done	125	0/10	negative	137	not done	negative	152	**10/10**	**negative**
34	16000	H7N3	56	not done	negative	84	0/10	negative	112	**10/10**	**negative**			
35	9800	H7N3	71	0/10	negative	113	0/10	negative	136	**2/10**	**negative**	137	10/10	negative
36	15300	H7N3	91	0/10	not done	112	0/10	negative	140	**9/10**	**negative**			

Legend: **day** indicates the day of sampling, starting from the onset of the production cycle; the **shaded cells** corresponds to the data included in the analysis (i.e., earliest positive serological result associated to a negative virological result within ±5 days).

CHAPTER 5

Serological diagnosis of avian influenza in poultry: is the haemagglutination inhibition test really the "gold standard"?

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ABSTRACT

The serological diagnosis of avian influenza (AI) can be performed using different methods, yet the hemagglutination inhibition (HI) test is considered as the ‘‘gold standard’’ for AI antibody subtyping. Although alternative diagnostic assays have been developed, in most cases their accuracy has been evaluated in comparison to HI test results, whose performance for poultry has not been properly evaluated.

We estimated the sensitivity (Se) and specificity (Sp) of the HI test and six other diagnostic assays for the detection of AI antibodies in the absence of a gold standard.

We applied a Bayesian version of Latent Class Analysis to compare the results of multiple tests from different study settings reported in the literature.

The results showed that the HI test has nearly perfect accuracy (i.e., 98.8% sensitivity and 99.5% specificity). It performed well in both chickens and turkeys yet was less accurate in experimentally infected poultry, compared to naturally infected. Blocking ELISA and the indirect immunofluorescence assay also performed very well.

Given its very high Se and Sp, the HI test may be effectively considered as a gold standard. In the framework of LPAI surveillance, where large numbers of samples have to be processed, the blocking ELISA could be a valid alternative to the HI test, in that it is almost as sensitive and specific as the HI test yet quicker and easier to automate.

KEY WORDS

Avian influenza, diagnostic test evaluation, haemagglutination inhibition test, latent class analysis, serological tests

INTRODUCTION

The majority of avian influenza (AI) infections are caused by low pathogenicity avian influenza viruses (LPAIV) and appear as mild respiratory diseases. However, LPAIV subtypes H5 and H7 can mutate into highly pathogenic avian influenza viruses (HPAIV)[1], outbreaks of which can threaten human health [2] and cause huge economic losses, given the high mortality in poultry and the cost of control measures [3].

The most effective means of identifying and controlling AI viruses in poultry is a constant and global surveillance [4]. The surveillance of LPAIV infection aims to detect the causative agent (i.e., the replicating virus or viral RNA) or antibodies against AI viral proteins. Generally speaking, LPAIV or its genome can be detected in an individual bird for only a few days, depending on the virus strain, the bird species, the infectious dose and the method of detection, whereas LPAIV antibodies are often present for the entire production life of the infected poultry [5]. Moreover, LPAIV infection is often clinically asymptomatic. It follows that, in areas at high risk of AI exposure, adequate surveillance based on the serological detection of LPAIV antibodies is of vital importance for the early detection of LPAIV and, consequently, the prevention of mutation into HPAIV [6]. Although serological LPAIV surveillance can be performed using various diagnostic assays, the hemagglutination inhibition (HI) test, which detects antibodies to the hemagglutinin (HA) antigen, is considered to be the ‘‘gold standard’’ for AI antibody subtyping, and it is recommended by both the European Union (EU) [7] and the World Organization for Animal Health [8]. However, the HI test is quite laborious because it needs manual reading of the results. For this reason, alternative assays have been developed. The accuracy of these assays has in most cases been evaluated in reference to the HI test. However, to the best of our knowledge, only one evaluation of the HI test in poultry has been published, and this evaluation was based on a small dataset, so that there were wide ranges for the estimated sensitivity (Se) and specificity (Sp) (95.7−100% for Se and 59.3−99.6% for Sp) [9].

The Se and Sp of a test are usually estimated by comparison with a reference test (i.e., the “gold standard”), which is supposed to determine the true disease state of the animals unambiguously [10]. However, the true disease state is rarely known in practice. An alternative way of evaluating diagnostic tests when the infection status is unknown is Latent Class Analysis [11]. This approach is based on the analysis of multiple populations with different disease prevalences, to obtain estimates of Se and Sp of two (or more) tests without requiring a gold standard.

The objective of the present study was to estimate the diagnostic Se and Sp of various serological assays for detecting AIV antibodies – with emphasis on the HI test – without assuming a gold standard. To do so, we applied a Bayesian version of Latent Class Analysis.

MATERIALS AND METHODS

Data collection

To determine which assays would be included in the analysis, we considered studies published in peer-reviewed journals in which serological assays for detecting AIV antibodies in poultry were evaluated. We initially identified a number of such studies by interviewing experts, performing a PubMed search, and looking at the reference lists of previously identified published papers. To be included in our analysis, the studies had to have included the HI test and to have clearly reported test results in 2 x 2 tables; we included only studies based on serum samples (i.e., no egg yolk) and with results for individual bird species (i.e., no mixed results from multiple species).

We initially identified 36 studies (see Appendix). In eight of these studies, the results of the HI test were compared with those of another AI serological test. However, two of these studies were discarded because it was not possible to distinguish the results for individual species [12] or the results based on serum samples [13]. The main features of the six selected studies are summarized in Table 1. Four studies were conducted on chickens alone, one on turkeys alone, and one on chickens, turkeys and ducks, yet for the latter study, we did not consider the duck population, for the sake of homogeneity among studies.

Table 1. Main features of the studies included in the comparative analysis.

ID	country	avian species	developed test	target protein	type of test	source of infection	tested AIV subtypes	HI antigen vs. tested antigen	ref.
A	Italy	turkey	iIFA	NA	N1-specific	natural	H7N1	not indicated	[29]
B	Taiwan	chicken	bELISA°	HA	H5-specific	natural	H5N3	heterologous	[21]
C	Taiwan	chicken	bELISA°	HA	H6-specific	natural	H6N1	homologous	[22]
D	Ohio	chicken, turkey, duck*	cELISA°	HA	H5-specific	experimental	H5N1, H5N2, H5N3, H5N9	heterologous	[14]
E	China	chicken	NP-ELISA	NP	Type A-specific	experimental	H5N1, H9N2	not indicated	[30]
F	Japan	chicken	AGP	NP	Type A-specific	natural	H5N2	homologous	[9]

* For the sake of comparison, we considered only chicken and turkey populations.

Note: In studies A and F, the reference antigen for the HI test was not provided; in studies B and D, the antigen for the HI test was different from circulating/inoculated virus strains; in studies C and F, the HI test was set up using the same antigen isolated from the first outbreaks.

In all of the six studies included in our analysis the HI test has been performed according to the protocol prescribed by the OIE Diagnostic Manual [8] (i.e., using 4 hemagglutinin units of virus antigen and 1% chicken erythrocytes, diluted in PBS) and titres ≥ 1:16 were considered positive. The diagnostic assays that were compared to the HI test in the six studies were: indirect immunofluorescence assay (iIFA), subtype-specific

blocking enzyme linked immunosorbent assay (bELISA), competitive ELISA (cELISA), nucleoprotein-based specific indirect ELISA (NP-ELISA), and agar gel precipitation (AGP). Given that the authors of the study on cELISA [14] pointed out that this assay performs quite differently in chickens and turkeys, we considered it as two separate tests (herein referred to as "cELISA_C" for chickens and "cELISA_T" for turkeys). Thus seven different tests were considered in the analysis (Table 2).

Table 2. Cross-tabulated test results included in the analysis. Joint tests outcome (y) is coded as 1=positive, 0=negative. cELISA is assumed to perform differently for chickens and turkeys.

	study				combination of test results			
k	ID	n_k	serological tests		y_{11}	y_{10}	y_{01}	y_{00}
1	A	247	T_1=HI	T_2=iIFA	105	2	6	134
2	B	478	T_1=HI	T_3=bELISA	232	4	10	232
3	C	400	T_1=HI	T_3=bELISA	184	0	6	210
4	D	172	T_1=HI	T_4=cELISA_C*	95	39	14	24
5	D	94	T_1=HI	T_5=cELISA_T°	80	2	10	2
6	E	150	T_1=HI	T_6=NP-ELISA	99	8	19	24
7	F	114	T_1=HI	T_7=AGP	64	32	0	18

*cELISA_C = cELISA used for chickens; °cELISA_T = cELISA used for turkeys

Data analysis

We applied a Bayesian version of Latent Class Analysis to compare the results of tests in different populations, as proposed by Branscum et al. (2000) [15]. The association between the different tests, the poultry species and how the birds were infected (i.e., natural vs. experimental infection) considered in the model are illustrated in Figure 1.

The model was implemented in WinBUGS [16] which uses a Markov Chain Monte Carlo (MCMC) sampling algorithm to obtain a Monte Carlo (MC) sample from the posterior distribution. For this analysis, there was no reliable prior information on prevalences, and prior knowledge of the Se and Sp of the tests was scarce. We thus chose to use uninformative priors to avoid the potential distortion of posterior estimates due to misleading prior information. Prior information was modelled using the Beta(1,1) distribution, which is uniform for the interval between zero and one (i.e., uninformative priors). For the analysis, the first 10,000 MC samples were discarded as a burn-in, and the successive 150,000 iterations were used for posterior inference. Potential autocorrelation was removed by storing one MC sample every 50 iterations. As suggested by Toft et al. (2007) [17], convergence of the MCMC chains was assessed both by visual inspection of the time-series plots and by computing the Gelman-Rubin convergence diagnostic plots using three MCMC chains with different starting values.

Posterior inference was performed by calculating the median and the 95% posterior

credible intervals (PCI) of the Se and Sp of the seven tests. To compare the different parameters in a manner more similar to traditional frequentist statistical methods, Bayesian posterior probabilities (POPR) were calculated and used to decide in favour of or against several hypothesis (e.g., H_0: $Se_{cELISA_C} < Se_{cELISA_T}$). The POPR used to test H0 was calculated as the proportion of MC samples for which H0 was true.

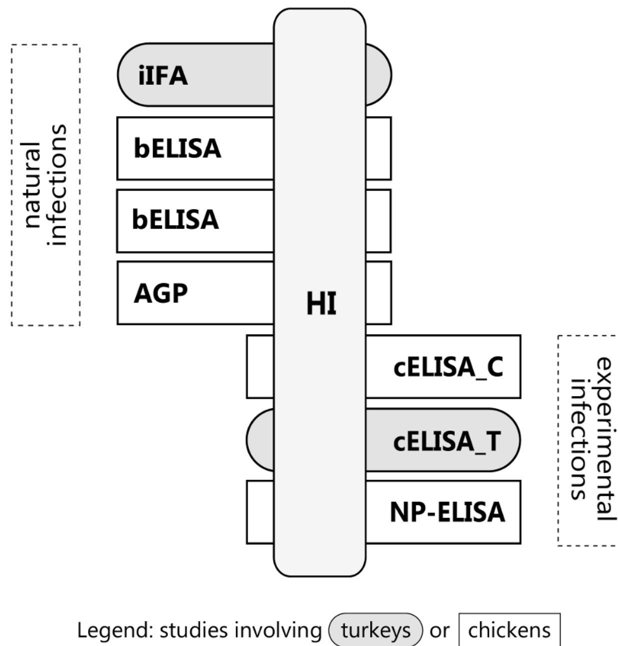

Figure 1. Graphical description of the association among the different tests evaluated, the poultry species and the source of infection considered in the Latent Class Analysis model.

Note: The HI test is evaluated against one of the alternatives in each study, with natural/experimental infections in chickens or turkey.

Sensitivity analysis

To investigate whether the available prior knowledge would have affected the posterior estimates of the parameters, we repeated the analysis, including informative priors on the Se and Sp of the iIFA and NP-ELISA tests (only for these tests were other studies with comparable settings available in the literature). Prior distributions of the Se and Sp of the two tests were modelled as Beta(α,β) distributions, whose specific parameters α and β were derived based on the most likely value (mode) and the 5th percentile of the Se and Sp reported in the literature [18,19] (Table 3). The two models (i.e., with and without informative priors) were further compared by means of the

deviance information criterion (DIC, smaller is better [20]).

Given that the bELISA had been used in two studies, these studies could have had a disproportional impact on the estimated performance of the HI test. To test this, we repeated the analysis, including only the datasets of the two studies with the HI and bELISA [21,22].

Table 3. Available information and corresponding prior distributions for the sensitivity (Se) and specificity (Sp) of two of the diagnostic tests evaluated

Diagnostic test	Parameter	Mode	5th percentile	Prior distribution	Reference
iIFA	Se	95.0	89.0	Beta(75.959, 4.945)	van der Goot *et al.* (2010)[18]
	Sp	92.0	87.0	Beta(119.426, 11.298)	van der Goot *et al.* (2010)[18]
NP-ELISA	Se	99.9	85.0	Beta(18.634, 1.018)	Upadhyay *et al.* (2009)[19]
	Sp	97.0	65.0	Beta(7.771,1.210)	Upadhyay *et al.* (2009)[19]

As a last step, we investigated the stability of the HI test by looking at possible external factors capable of influencing its performance, such as the bird species and how the birds were infected. To assess the influence of the species, we estimated the species-specific Se and Sp of the HI test in chickens and turkeys. The same approach was adopted for investigating the performance of HI in naturally and experimentally infected birds.

RESULTS

The posterior estimates of the Se and Sp of the seven tests are given in Table 4. The HI test had an Se of 98.8% and an Sp of 99.5%. The Se and Sp of the bELISA and iIFA were not significantly different from that of the HI test. The NP-ELISA had a high Se (92.1%) yet a low Sp (57.5%). By contrast, the AGP had a high Sp (96.3%) but a low Se (66.2%). The Se and Sp of cELISA were lower than those of the HI; they also differed significantly among species. In particular, the Se of cELISA was 70.8% in chickens (Se_{cELISA_C}) and 96.8% in turkeys (Se_{cELISA_T}). Assuming H_0: $Se_{cELISA_T} < Se_{cELISA_C}$, the Bayesian POPR < 0.0001 (which can be interpreted as statistical significance in a one-sided test) indicated that Se_{cELISA_C} was significantly lower than Se_{cELISA_T}. By contrast, the Sp of the cELISA was significantly higher in chickens than in turkeys (POPR = 0.0086).

The use of informative priors on the Se and Sp of the iIFA and the NP-ELISA did not affect the posterior estimates of any of the parameters (Table 4). Furthermore, the DIC slightly favoured the model with uninformative priors (DIC = 126.2 for the model with uninformative priors, compared to 127.3 for the model with informative priors).

The estimated Se and Sp of the HI test derived from the two studies in which the HI test and the bELISA were evaluated [21,22] were very close to the estimates obtained when including all of the studies (Table 5).

Table 4. Posterior median and 95% posterior credible intervals (PCI) of the sensitivity (Se) and specificity (Sp) of the serological tests evaluated, according to the specified prior information.

	uninformative priors [DIC = 126.2]				informative priors on Se and Sp of iIFA and NP-ELISA [DIC = 127.3]			
test	Se	95%PCI	Sp	95%PCI	Se	95%PCI	Sp	95%PCI
HI	98.8	[96.0 ; 100]	99.5	[98.4 ; 100]	99.0	[96.4 ; 100]	99.6	[98.5 ; 100]
iIFA	98.1	[94.0 ; 99.9]	96.3	[91.8 ; 99.4]	96.6	[93.3 ; 98.7]	94.0	[90.6 ; 96.5]
bELISA	99.3	[98.0 ; 100]	97.6	[95.2 ; 99.8]	99.3	[97.9 ; 100]	97.4	[95.0 ; 99.7]
cELISA_C*	70.8	[62.8 ; 78.0]	64.6	[48.0 ; 80.0]	70.7	[62.7 ; 78.0]	64.3	[48.0 ; 79.4]
cELISA_T°	96.8	[91.6 ; 99.3]	22.0	[5.2 ; 52.4]	96.8	[91.6 ; 99.3]	21.6	[5.2 ; 51.0]
NP-ELISA	92.1	[86.0 ; 96.3]	57.5	[42.2 ; 72.6]	93.2	[87.9 ; 96.8]	63.0	[48.9 ; 76.6]
AGP	66.2	[56.4 ; 75.1]	96.3	[81.9 ; 99.9]	66.2	[56.5 ; 75.1]	96.3	[81.9 ; 99.9]

*cELISA_C = cELISA used for chickens; °cELISA_T = cELISA used for turkeys

Table 5. Posterior median and 95% posterior credible intervals (PCI) of the sensitivity (Se) and specificity (Sp) of the HI test and bELISA, considering using only the two studies in which these tests were evaluated [21, 22].

Test	Se	95%PCI	Sp	95%PCI
HI	97.9	[95.1 ; 99.9]	99.5	[98.2 ; 100]
bELISA	99.4	[98.0 ; 99.9]	98.2	[95.5 ; 99.9]

The results of the stability analysis of the HI test are reported in Table 6. The HI test appeared to perform the same for the two species considered: the estimated Se and Sp of $HI_{chicken}$ did not differ significantly from the estimates of HI_{turkey} (POPR=0.3539 for Se and POPR=0.3597 for Sp). However, the HI test was more accurate in naturally infected birds than in experimentally infected birds: POPR = 0.022 for Se of $HI_{natural}$ > Se of $HI_{experimental}$ and POPR = 0.0002 for Sp of $HI_{natural}$ > Sp of $HI_{experimental}$.

Table 6. Posterior median and 95% posterior credible intervals (in square brackets) of the sensitivity (Se) and specificity (Sp) of the HI test, assuming different performance by species ($HI_{chicken}$ and HI_{turkey}) and source of infection ($HI_{natural}$ and $HI_{experimental}$).

		Test evaluated				*null hyphotesis (H_0)*	*POPR**
Species		$HI_{chicken}$		HI_{turkey}			
	Se	98.9	[96.1 ; 100]	98.1	[92.6 ; 99.9]	Se $HI_{chicken}$ < Se HI_{turkey}	0.3539
	Sp	99.5	[98.1 ; 100]	99.1	[96.0 ; 100]	Sp $HI_{chicken}$ < Sp HI_{turkey}	0.3597
Source of infection		$HI_{natural}$		$HI_{experimental}$			
	Se	99.3	[97.0 ; 100]	91.1	[85.1 ; 98.9]	Se $HI_{natural}$ < Se $HI_{experimental}$	0.0222
	Sp	99.6	[98.5 ; 100]	81.2	[59.5 ; 98.6]	Sp $HI_{natural}$ < Sp $HI_{experimental}$	0.0002

*POPR = Bayesian posterior probability

DISCUSSION

Using Latent Class Analysis and published data, we estimated the accuracy of the HI test and six other diagnostic assays in detecting AIV antibodies, without making reference to a gold standard. Because the HI test is commonly considered as the gold standard for type-specific AIV antibody detection, its performance has rarely been questioned. Compared to the only previous study in which the accuracy of the HI test was estimated for poultry [9], we found a similar Se and a much higher Sp, as well as much narrower credible intervals. This comparison might seem unfair, since we included the data of the previous study in our model. However, according to a sensitivity analysis (data not shown), the estimated Se and Sp of the HI test remained basically unvaried when excluding the study from the analysis, suggesting that the data from such study are consistent with those of the other studies.

The present study is the first attempt to estimate the Se and Sp of the HI test based on data collected in different study settings. This allowed us to investigate possible sources of variation in the performance of the HI test. Our results confirmed that the HI test is very accurate and that it performs well in both chickens and turkeys. However, the HI test was less accurate in experimentally infected birds compared to naturally infected birds. Although this result was rather unexpected, it could be explained by differences in the laboratory settings of the studies considered in our analysis. In fact, for two out of the three experimentally infected populations, the virus antigen of the HI test was different from the virus strains used to inoculate the birds [14]. Since the performance of the HI test is strongly influenced by the homology between the reference viral antigens and the virus isolates to be tested [23], this may have biased the estimates of the HI test accuracy in experimentally infected birds. However, the HI test reference antigen differed from the field AI strains also in one of the four naturally infected populations [21], though this apparently did not lower the estimated accuracy of the HI test in naturally infected birds, which was very high. To this regard, It would have been interesting to further investigate the role antigenic relatedness in the performance of HI test, unfortunately it was not possible because two of the six studies considered in our analysis [29,30] did not report any detailed information about the HI antigen and/or the tested antigen. This implies that such studies would have been excluded from the analyses, reducing the number of data points and the degrees of freedom and making the model unidentifiable.

The bELISA seems to be a good alternative to the HI test: apart from being quicker and easier to automate, it has a very high Se and a good Sp. These results are consistent with those of other studies in which the accuracy of the bELISA was calculated based on HI test results on field sera [24] or on a combination of different test results in experimentally infected birds [25]. The iIFA test showed satisfactory accuracy, which was slightly higher than that estimated by van der Goot at al.(2010) [18] in the absence of a gold standard

yet a little lower than the accuracy reported by Cattoli et al. (2006) [26], who used a gold standard defined by a combination of virological and serological test results on field sera. The NP-ELISA seems to be quite sensitive, yet it lacks specificity, which differs from the results of the study by Upadhyay et al. (2009) [19], who estimated both high Se and Sp. However, that study used a commercial ELISA as the gold standard. Based on our analysis, the cELISA has the lowest accuracy, and it differs significantly among species. When applied to chickens, the cELISA showed decent Se and moderate Sp, whereas in turkeys it was more sensitive yet less specific. These differences were also reported by the authors of the original study [14], whereas other authors did not find any important differences by poultry species in the performance of the cELISA, using the HI test as the gold standard [27].

The estimates obtained with and without informative priors were very similar, confirming that the data themselves were quite robust and rather insensitive to the choice of the prior information. Furthermore, the DIC slightly favoured the model with uninformative priors.

A key assumption in Latent Class Analysis is that the results of the diagnostic tests are independent given the disease status. The disease status often refers to the presence of the pathogen. However, since we evaluated serological tests, the definition of the disease status for AIV infection must be considered as a measure of the presence of AIV antibodies. Conditional to such disease status, the seven tests may be assumed as conditionally independent. Furthermore, a model that assumes that all of the tests are conditionally dependent given the disease status is unidentifiable and should as such not be evaluated without specifying informative priors for all (or at least a large proportion) of the parameters [28].

This is the first study in which all of these tests were compared in a single analysis. However, some limitations should be acknowledged. Although six studies met the inclusion criteria, apart from the HI test only the bELISA was evaluated in more than one study. For this reason, the two studies that included the bELISA could have influenced the estimates of the HI accuracy more than the other studies. Another potential limitation is that we did not take into account factors such as the potential differences in accuracy related to the virus subtype (i.e., H5, H6, H7 or H9) or the timing of testing. However, given that the HI test is considered as the gold standard for type-specific AIV antibody detection, its accuracy would have been difficult to estimate using the traditional method for test evaluation. Thus, using Latent Class Analysis, we were able to evaluate for the first time the performance of the HI test under different conditions.

CONCLUSIONS

The HI test has a near perfect accuracy and may be considered as a gold standard, provided that the reference viral antigen is close enough to the virus isolate to be tested or a panel of different antigens are used. In the framework of LPAI surveillance, in which large amounts of samples have to be processed and the virus subtype for which there is a risk of exposure may not be known, the use of subtype-specific bELISA as a screening test could be a valid alternative to HI, given that it is almost equally accurate yet quicker and easier to automate.

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APPENDIX

List of peer-reviewed publications initially deemed as potentially relevant and further reviewed

1. Beard CW. 1970. Demonstration of type-specific influenza antibody in mammalian and avian sera by immunodiffusion. Bulletin of the World Health Organization. 1970;42(5):779-85.
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Model Description

For each population k two tests T_1 and T_j are applied, so that there are n_k tested samples giving y_k joint test results: $y_k = (y_{11k}, y_{10k}, y_{01k}, y_{00k})$ where 1 and 0 indicates the outcome (1=positive, 0=negative) of the two tests in population k. The counts of joint test results are assumed to follow a multinomial distribution:

$$y_k \sim \text{multinomial}(n_k, (p_{11k}, p_{10k}, p_{01k}, p_{00k}))$$

in which the probabilities of observing the individual combinations of test results for population k are given by:

$$p_{11k} = \pi_k\, Se_1\, Se_j + (1 - \pi_k)(1 - Sp_1)(1 - Sp_j)$$
$$p_{10k} = \pi_k\, Se_1\, (1 - Se_j) + (1 - \pi_k)(1 - Sp_1)\, Sp_j$$
$$p_{01k} = \pi_k\, (1 - Se_1)\, Se_j + (1 - \pi_k)\, Sp_1\, (1 - Sp_j)$$
$$p_{00k} = \pi_k\, (1 - Se_1)\, (1 - Se_j) + (1 - \pi_k)\, Sp_1\, Sp_j$$

where π_k is the seroprevalence in population k, Se_1 and Se_j are the sensitivities of T_1 and T_j, and Sp_1 and Sp_j are the specificities of T_1 and T_j . For the purpose of this study, T_1 represents the HI test and T_j can be either iIFA, bELISA, cELISA_C, cELISA_T, NP-ELISA or AGP. The analysis combined the test results in seven poultry populations, i.e. k = 7.

CHAPTER 6

Evaluating surveillance schemes for early detection of low pathogenicity avian influenza infections

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ABSTRACT

In recent years, the early detection of low pathogenicity avian influenza (LPAI) viruses in poultry has become increasingly important, given their potential to mutate into highly pathogenic viruses. However, evaluations of LPAI surveillance have mainly focused on prevalence and not on the ability to act as an early warning system. We used a simulation model based on data from Italian LPAI epidemics in turkeys to evaluate different surveillance strategies in terms of their performance as early warning systems. The strategies differed in terms of sample size, sampling frequency, diagnostic tests, whether or not active surveillance (i.e. routine laboratory testing of farms) was performed, and were also tested under different epidemiological scenarios. We compared surveillance strategies by simulating within-farm outbreaks. The output measures were the proportion of infected farms that are detected and the farm reproduction number (R_h). The first one provides an indication of the sensitivity of the surveillance system to detect within-farm infections, whereas R_h reflects the effectiveness of outbreak detection (i.e. if detection occurs soon enough to bring an epidemic under control). Increasing the sampling *frequency* was the most effective means of improving the timeliness of detection (i.e., it occurs earlier), whereas increasing the sample *size* increased the likelihood of detection. Surveillance was only effective in preventing an epidemic if actions were taken within two days of sampling. The strategies were not affected by the quality of the diagnostic test, although performing both serological and virological assays increased the sensitivity of active surveillance. Early detection of LPAI outbreaks in turkeys can be achieved by increasing the sampling frequency for active surveillance, though very frequent sampling may not be sustainable in the long term. We suggest that, when no LPAI virus is circulating yet and there is a low risk of virus introduction, a less frequent sampling approach might be admitted, provided that the surveillance is intensified as soon as the first outbreak is detected.

KEY WORDS

Surveillance – early detection − avian influenza – outbreak data – simulation model

INTRODUCTION

The surveillance and control of avian influenza (AI) have historically focused on the detection and eradication of infections with highly pathogenic avian influenza (HPAI) viruses in poultry populations. However, since low pathogenicity AI (LPAI) viruses of the H5 and H7 subtypes can mutate into HPAI viruses, as occurred for example in 1999 in Italy [1] and in 2003 in Chile [2], the detection and control of LPAI has become compulsory in the European Union (EU) [3]. Moreover, in 2008, the rapid mutation of an LPAI H7N7 virus strain (2–3 weeks after its introduction) that resulted in the HPAI outbreak in the United Kingdom and evidence of multiple incursions of different AI viruses in EU in recent years have highlighted the need for early detection [4].

For HPAI, early detection has been successfully based on passive surveillance (i.e., the observation of signs and symptoms), given that infection induces clear clinical signs and high mortality in most poultry species. However, for LPAI, the signs may go unnoticed by passive surveillance and mortality is low [5]. For this reason, active surveillance is performed (i.e., visiting farms and sampling animals for diagnostic tests) [6].

In the EU, current AI surveillance focuses on determining the presence of infections with subtypes H5 and H7 in different poultry species [3,6]. To this end, sampling strategies have been set up to detect a fixed design prevalence with a certain probability (i.e., assuming minimum detectable prevalences (i.e., design prevalences) of 5% LPAI infected holdings and 30% infected animals within an infected holding). However, these methods neglect the dynamics of the infection in the population and may result in missed or delayed detection [4].

Over the years, both the number of EU Member States that have implemented AI surveillance and the number of samples tested have increased. However, evaluations of the effectiveness of LPAI surveillance programs have mainly focused on their performance in establishing the presence of infected birds or assessing freedom from infection [7,8] and not on their performance as early warning systems for new introductions. For this reason, an optimal design for early warning surveillance has not been defined.

Italy is the EU country with the largest number of AI outbreaks, and it was the first to implement an AI surveillance system, which also consisted of the routine laboratory testing of poultry farms [9]. Since the system was made operational, four major LPAI epidemics have occurred, in 2000-2001, 2002-2003, 2004, and 2005, in addition to two small epidemics in 2007 and 2009, which mainly involved non-industrial flocks. Given that this is the longest running surveillance system in Europe, a large quantity of data has been collected, which, together with the experience gained, could be useful in evaluating and improving surveillance programmes. In fact, in a previous study [10] we investigated the transmission dynamics of LPAI within turkey farms using serosurveillance data from the

above epidemics and experimental infection data. That study showed that the basic reproduction number (R_0) within farms was on average 5.5, meaning that an infectious turkey infects on average 5.5 turkeys within a susceptible farm, although R_0 varied greatly among farms. Furthermore it resulted in final estimates for the mean latent and infectious periods of 2.9 and 8.1 days, respectively, and provided a mathematical model to simulate within-farm outbreaks of LPAI.

The objective of the present study was to evaluate different surveillance strategies in terms of their capacity to act as early warning systems for LPAI epidemics in an area with a high risk of virus exposure. To this end, we carried out simulations with a model parameterized with data from the Italian LPAI epidemics [10]. Although future outbreaks will probably be with different strains and in different areas, this is the model that combines most available field data and therefore represents the best available knowledge. The strategies differed in terms of the number of samples collected, sampling frequency, the type of laboratory tests performed, whether or not active surveillance was performed, and when active surveillance was begun. Our simulations were based on actual data and were performed considering turkeys, which has been the most affected species in Italy.

METHODS

We compared surveillance strategies by simulating within-farm outbreaks. As output measures, we used the proportion of infected farms (i.e. outbreaks) that are detected and the farm reproduction number (R_h) (i.e., the average number of outbreaks caused by one infectious farm) of an infected farm at detection, assuming that the farm is not infectious to other farms after detection (e.g., because of culling or quarantine). The proportion of infected farms that are detected provides an indication of the sensitivity of the surveillance system to detect within-farm infections. It is a result of surveillance that is easier observed in the field but has no direct relation to R_h, which, in turn, reflects the effectiveness of outbreak detection: an R_h of <1 at detection (i.e., the threshold value to prevent an epidemic) means that the infection is detected (and stopped) at an early stage, so that epidemics can be brought under control.

Within-farm surveillance model

Within-farm outbreaks were simulated by means of the stochastic susceptible-exposed-infectious-recovered (SEIR) model (baseline model) of Comin *et al.* [10]. The parameters are given in Table 1. Farms were assumed to consist of a single flock. Outbreaks started at a uniform random flock age with one latently infected bird. We assumed that the

latent and infectious periods had a gamma distribution, with average durations of 2.9 and 8.1 days, respectively [10]. During their infectious period, birds transmitted the virus to susceptible birds within the farm at rate β, which was chosen such that the mean number of secondary cases generated by one infectious bird (i.e., R_0) is equal to 5.5 [10]. Simulations were carried out using *R* [11].

Detection may take place by passive or active surveillance. For passive surveillance, we assumed that detection occurs when the proportion of infected birds was sufficient for allowing clinical disease to be detected in the farm, which occurred when the within-farm prevalence reached the detection threshold, D (see below for estimation of D). For active surveillance, detection could occur on specific sampling days, depending on the surveillance strategy (e.g., 60, 90, and 120 days after the onset of the production cycle in the reference strategy). On a given sampling day, a random sample of birds was taken, and if at least one of them was infectious (i.e., positive virological test) or had recovered from infection (i.e., positive serological test), then the sampling day was defined as detection day.

Table 1. Parameters used in the within-farm transmission model (from Comin *et al.* [10])

	value in the baseline model	parameters' value
time step	0.02 days	-
simulation period	130 days	-
farm size	10,000 turkeys	-
day of virus introduction	~ uniform(a; b)	a=0; b=130
farm-specific basic reproduction number	~ gamma(s; r)[§]	s=2.73; r=0.49
bird-specific duration of latent period	~ gamma(s_L; r_L)	s_L=17.41; r_L=5.95
bird-specific duration of infectious period	~ gamma(s_I; r_I)	s_I=4.64; r_I=0.57

[§]s = shape parameter; r = rate parameter

Once an infected farm was detected, the expected number of farms infected secondarily prior to detection was calculated by multiplying the bird-to-farm transmission rate (β_h, the average number of farms infected by one infected bird per unit of time) by the cumulative number of infectious birds (area under the curve, AUC) up to the detection day. If an outbreak was still not detected on the last sampling day, the AUC at the time of slaughter was used (Figure 1.b). By repeating this for many simulated outbreaks, the mean of β_h*AUC was computed, which is interpreted as the between-farm reproduction number R_h (i.e., the mean number of farms infected by a single infected farm).

Estimation of D, the detection threshold for passive surveillance

To estimate D, we used the field data provided by the intensive surveillance system in place in Italy during the LPAI epidemics, which is described in detail elsewhere [9]. During and around the time of the epidemics, a total of 6,102 poultry farms were routinely visited; there were more than 13,000 sampling events, and 497 outbreaks (i.e., infected farms) were detected. For the calculation of D, we used data from only unvaccinated infected turkey farms with multiple samplings and available information on farm size, mean sampling interval, mean sample size and whether the outbreak had been detected by active or passive surveillance. Because many records were incomplete, we also included farms consisting of multiple flocks to increase the number of data points. As outbreaks on farms generally start in one flock and will be detected in that flock, we believe that inclusion of these multiple-flock farms is valid. Based on these criteria, 96 turkeys farms were selected: 72 (75%) were detected by active surveillance and 24 (25%) by passive surveillance. Unfortunately, we could not directly use the data from the 24 flocks detected by passive surveillance, because they did not contain any information about the prevalence at the time of clinical suspicion, since sample collections are always performed some days later, after farmer's notification. As an alternative, we assumed that passive detection is related to the prevalence of infectious birds, as that probably best reflects the clinical picture on the farm. The prevalence threshold for passive detection, D, was thus determined by first simulating outbreaks with flock sizes and active surveillance scheme as in the dataset, and then looking what threshold prevalence would have resulted in 24 farms being detected by passive rather than active surveillance (as in the dataset).

Following this assumption, to estimate D we simulated 100 sets of 96 outbreaks, using the average flock sizes reported in the aforesaid dataset and the corresponding 96 farm-specific sampling intervals and sample sizes (Table 2) . This resulted in 96 detection times for each set of simulations and 96 peak prevalences at or before the day of active detection. In order to have 24/96 outbreaks detected by passive rather than active surveillance (as in the data), the minimum detectable prevalence was selected as the 24th highest peak prevalence.In brief, we performed the following steps:

1. simulate 96 outbreaks, with flock size, sampling interval, and sample size being the same as in the data;
2. determine the maximum of all prevalences until the day of detection for each outbreak;
3. arrange the maximum prevalences in order and select the 24th highest as the minimum detectable prevalence;
4. repeat 1-3 100 times to have 100 minimum detectable prevalences;
5. determine mean and 95% confidence interval.

Table 2. Descriptive statistics of the farm characteristics and surveillance schemes in the 96 turkey farms selected to estimate the detection threshold for passive surveillance

	mean	SD	5th percentile	95th percentile
number of birds per farm	14 000	7856	5075	26 825
number of flocks per farm	2.2	1.5	1	5
number of birds per flock	8139	6496	2317	18 050
duration of the production cycle (days)	138	22	98	163
day of first sampling	65.1	22.6	29.0	103.0
day of detection	109	23	65	141
number of sampling events per farm	3.2	1.2	2.0	5.3
average sampling interval per farm	23.5	12.1	10.2	49.5
average number of samples collected per flock	6.2	3.3	2.3	10

Estimation of β_h

The bird-to-farm transmission rate β_h represents the number of newly infected farms generated by a single infectious bird housed in an infected farm per unit of time. For estimation of β_h, the relation $R_h = \beta_h$*AUC can be reversed for a known value R_h' during an epidemic, and a mean AUC' of actual flocks during that same epidemic: $\beta_h = R_h'/ AUC'$. For this calculation, we used a published value of R_h' of 2.15, which was the farm reproduction number at the beginning of the Italian 2000-2001 LPAI epidemic, when no control measures were in place yet [12]. The value of AUC' was estimated as the average AUC of 1000 simulated outbreaks in 1000 single-flock farms with different flock sizes and gender of birds (randomly selected from a list of single-flock turkey farms available at the Regional poultry farm registry), assuming the absence of any control measure. For each outbreak, we simulated the day of virus introduction (from day 0 to 100 or 130 of the production cycle, depending on the gender of the turkeys) and the infection dynamics, using the above-described SEIR model and parameters. We assumed that the infection process in a farm continued until it reached its end or the production cycle was stopped (i.e., because control measures were not in place) (Figure 1.b). After repeating the simulation 1000 times, we calculated the mean and 95% confidence interval of the AUC in absence of control measures.

Simulation of surveillance strategies

To simulate different surveillance strategies, we combined the information on within- and between-farm disease dynamics, the number of samples, the frequency of sampling, and the type of surveillance (passive, active, or both, and whether virological or serological testing was performed), and the detection ability of passive surveillance, assuming that a farm stopped being infective immediately after detection.

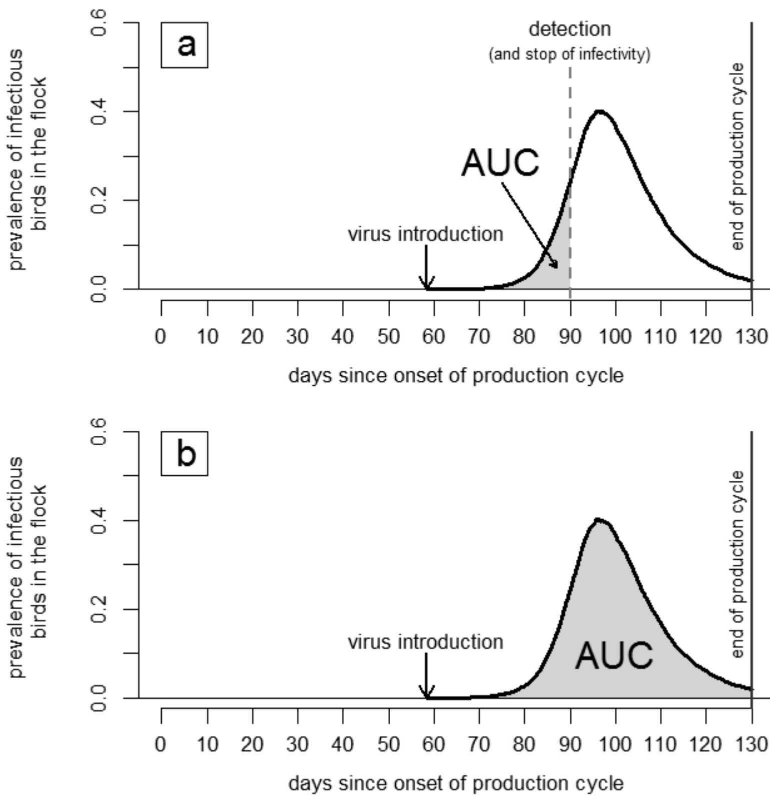

Figure 1. Area under the prevalence curve (AUC, shaded area). (a) AUC assuming that the infectivity is efficiently stopped at day of outbreak detection. (b) AUC in the whole infectious period.

In the reference surveillance strategy, we assumed a combination of both active and passive surveillance applied to a single-flock farm of 10,000 male turkeys. Sampling for active surveillance was assumed to start at day 60 of the production cycle (based on the results of Comin *et al.* [9]) and to be performed on a monthly basis (as in Mulatti *et al*. [12]). At each sampling, we assumed that 10 birds were tested by means of both serological and virological assays (assuming perfect accuracy for both tests, which was relaxed in sensitivity analyses, see below). If the sample included at least one positive bird, the farm was considered to be infected and the corresponding sampling day was defined as the detection day. However, if the prevalence of infectious birds had exceeded the threshold D before positive samples were found, we assumed that the infection had been found by passive surveillance and the first day on which the prevalence exceeded the threshold was considered as the detection day.

The reference strategy was compared to six alternatives, namely: starting the active surveillance at day 30 of the production cycle, increasing the sampling frequency to once

every 15 days, decreasing the sampling frequency to once every 60 days, collecting 30 samples per sampling, performing only serological testing, and applying only passive surveillance (Table 3). For each surveillance strategy we calculated the AUC at detection day (i.e., the area under the prevalence curve until detection day) (Figure 1.a) and at different time points after detection day (i.e., from 1 to 10 days) , given that in reality culling is not performed on detection day. The farm reproduction number of an infected farm at the different time points was calculated by multiplying the AUCs by β_h.

Table 3. Summary of the investigated surveillance strategies

Surveillance strategy:	sampling days	surveillance components[a]	# samples per sampling	tests[b]
Reference	60 - 90 - 120	AS + PS	10	ST + VT
start day 30	30 - 60 - 90 - 120	AS + PS	10	ST + VT
freq 15 days	60 - 75 - 90 - 105 - 120	AS + PS	10	ST + VT
freq 60 days	60 - 120	AS + PS	10	ST + VT
30 samples	60 - 90 - 120	AS + PS	30	ST + VT
only serology	60 - 90 - 120	AS + PS	10	ST only
only PS	–	only PS	–	–

[a] AS = active surveillance; PS = passive surveillance
[b] ST= serological testing; VT = virological testing

Sensitivity analysis

The simulated surveillance strategies referred to the Italian situation at the beginning of the 2000-2001 LPAI epidemic, when the first outbreaks appeared and no control measures were in place. To investigate how the surveillance strategies would perform under different conditions, we simulated some alternative scenarios (Table 4), exploring adjustment of the strategy by varying parameters one by one. In particular we assumed:

a) imperfect sensitivity (Se) of serological testing;
b) imperfect sensitivity of virological testing;
c) larger farm size;
d) smaller farm size;
e) earlier virus introduction;
f) later virus introduction;
g) longer mean generation time (i.e., generation time is defined as the mean time interval between infection of a primary case and infection of secondary cases caused by the primary case);
h) shorter mean generation time;
i) higher mean basic reproduction number;
j) lower mean basic reproduction number.

Table 4. Summary of the alternative scenarios investigated in the sensitivity analysis.

Reference settings	mean R_0	mean generation time (days)	farm size (turkeys)	serological test Se	virological test Se	mean virus introduction (days)
	5.55*	**7.90***	**10000**	**100%**	**100%**	**54.71**
Lower serological test sensitivity	Sensitivity of serological test = **90%**§					
Lower virological test sensitivity	Sensitivity of virological test = **85%**§					
Larger farm size	**20000** turkeys in the farm					
Smaller farm size	**5000** turkeys in the farm					
Earlier virus introduction	Virus introduction: on average **27.18** days after the onset of the production cycle [introduction day ~ uniform(a; 54.71)]					
Later virus introduction	Virus introduction: on average **80.94** days after the onset of the production cycle [introduction day ~ uniform(54.71; b)]					
Longer generation time	Mean generation time: **11.85** days [LP ~ gamma(s_L; r_L/1.5) ; IP ~ gamma(s_I; r_I /1.5)]					
Shorter generation time	Mean generation time: **5.27** days [LP ~ gamma(s_L; r_L*1.5) ; IP ~ gamma(s_I; r_I*1.5)]					
Higher R_0	Mean basic reproduction number: **11.19** [R_0 ~ gamma(s; r/2)]					
Lower R_0	Mean basic reproduction number: **3.11** [R_0 ~ gamma(s; r*2)]					

* values derived from Comin *et al*. [10]

§ values based on van der Goot *et al*. [13]

Note: values of parameters a, b, s_L, r_L, s_I, r_I, s and r are those previously reported in Table 1.

RESULTS

The average threshold prevalence for outbreak detection by passive surveillance was 56.0% (95%CI: 55.5 – 56.5%), meaning that on average roughly half of the birds in the farm would have to be simultaneously infected (i.e., virus-positive) for LPAI infection to be clinically suspected. The estimated mean AUC at the end of the outbreaks was 57,061 birds (95%CI: 54,162 – 59,960), which, when divided into R_h in the absence of control measures [12], yields the number of farms that one infectious bird can infect per day: $\beta_h = 3.768 \cdot 10^{-5}$ (95%CI: $3.586 \cdot 10^{-5} – 3.970 \cdot 10^{-5}$).

Table 5 and Figure 2.a summarize the results of the surveillance strategies under the reference epidemiological scenario (i.e., early incursions of LPAI viruses in turkey farms in the absence of control measures). When considering the reference surveillance strategy, 73% of the infected farms were detected, on average 35 days after virus introduction and mainly by active surveillance (54% of infected farms, 74% of detected outbreaks). The simulation of alternative surveillance strategies suggested that decreasing the sampling frequency from once a month to once every two months leads to detection on average 43 days after virus introduction, and performing only passive surveillance reduces the detection rate to 26% of infected farms, thus making it impossible to detect the infection soon enough for avoiding the spread of the virus to other farms (i.e. $R_h > 1$).

Table 5. Detection ability towards LPAI infections in absence of control measures (reference scenario). Results of 1000 simulated outbreaks applying different surveillance strategies.

Surveillance model	proportion of outbreaks that are detected	proportion of outbreaks that are detected by active surveillance	mean detection time since virus introduction (days)	mean prevalence in the farm at detection	R_h at detection [95%CI]
eference	73.4%	53.6%	34.89	31.3%	0.80 [0.75 - 0.85]
start day 30	73.5%	55.4%	34.47	30.6%	0.77 [0.72 - 0.82]
freq 15 days	74.0%	58.5%	30.79	27.7%	0.53 [0.49 - 0.57]
freq 60 days	65.2%	48.2%	43.22	27.6%	1.21 [1.14 - 1.28]
30 samples	81.3%	63.5%	34.94	26.5%	0.62 [0.57 - 0.66]
only serology	68.3%	44.9%	36.50	34.5%	1.05 [0.99 - 1.11]
only PS	26.1%	–	22.35	59.2%	1.48 [1.41 - 1.55]

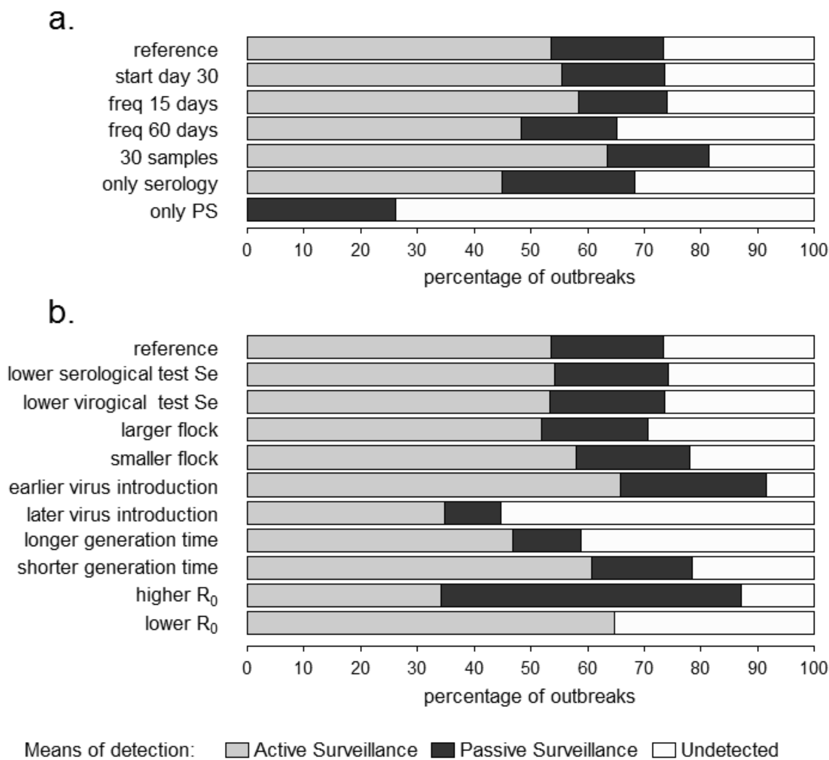

Figure 2. Percentages of outbreaks detected by active and passive surveillance. (a) Various surveillance schemes under the reference scenario. (b) Reference surveillance scheme under different scenarios (i.e., sensitivity analysis).

The detection rate of the reference surveillance strategy under different scenarios (sensitivity analysis) is shown in Figure 2.b. The use of imperfect diagnostic tests (90% and 85% sensitivity for serological and virological testing, respectively) did not result in a significant decrease in the percentage of detected outbreaks. Furthermore, surveillance was more sensitive in smaller farms. The reference scenario refers to the LPAI virus that was introduced in Italian farms at the beginning of the LPAI epidemic in 2000; if a strain with a higher R_0 was introduced, the role of passive surveillance would seemingly be enhanced.

To better understand the effectiveness of the surveillance strategies under different scenarios, we determined the maximum number of days after detection day at which R_h was still below 1, which indicates the time available to prevent an epidemic (Figure 3).

SURVEILLANCE STRATEGIES

SCENARIOS	Reference	start day 30	freq 15 days	freq 60 days	30 samples	only serology	Only PS
Baseline	2	2	5	–	4	–	–
Lower serological test Se	2	2	5	–	4	–	–
Lower virological test Se	2	2	4	–	4	–	–
Larger flock size	–	–	0	–	–	–	–
Smaller flock size	10	10	10	10	10	10	8
Earlier virus introduction	–	–	1	–	0	–	–
Later virus introduction	10	10	10	8	10	9	5
Longer generation time	4	5	9	0	8	0	–
Shorter generation time	0	1	3	–	2	–	–
Higher R_0	1	1	2	–	1	–	–
Lower R_0	8	8	10	–	10	3	–

Figure 3. Effectiveness of outbreak detection. Grey-scale plot indicating the maximum number of days after positive sampling to efficiently stop the infection (i.e. last day at which $R_h<1$) for alternative surveillance strategies under different scenarios.

Legend: The number reported in each cell represents the last day at which Rh<1 for that specific combination of surveillance and scenario (light=better, dark=worse).

When considering the reference (or "baseline") scenario, to prevent an epidemic, between-farm transmission would need to be stopped by control measures within 2 days of the positive sampling. The use of imperfect diagnostic tests (i.e., lower test sensitivity) did not result in a significant worsening of the effectiveness of the surveillance strategies.

When increasing the sampling frequency (once every 15 days), the maximum number of days allowed for preventing an epidemic increased to 5 (i.e., providing 3 more days to stop between-farm transmission). Increasing the sample size (30 birds) also increased the maximum number of days for preventing an epidemic (i.e., 4 days). By contrast, performing only passive surveillance made it impossible to detect infection in time to sufficiently reduce transmission to other farms (i.e., $R_h > 1$), except in the case of small farms or late virus introduction. Similarly, the use of only serological testing did not allow for extra time to set up control measures except in case of lower R_0 or late virus introduction.

DISCUSSION

In this study, we evaluated diverse surveillance strategies for the purpose of early warning of LPAI epidemics, considering the actual epidemiological conditions during the Italian LPAI surveillance programme in 2000. Surveillance was only successful in preventing an epidemic if actions were taken within two days of sampling, which is rather unfeasible, given the waiting time to have the results from the laboratory. The sensitivity analysis showed that the surveillance strategies are not effective for larger farms or if the LPAI virus is introduced in the farm earlier or has a shorter generation time or a higher R_0. In the last three scenarios, a high infectivity level is reached before detection (i.e., high AUC at detection), which results in an $R_h > 1$; on the other hand, under these circumstances the detection rate is higher (i.e., ~ 80−90%, Figure 2.b) mostly due to the increase of the detection by passive surveillance. Furthermore, the surveillance strategies do not seem to be affected by the quality of the diagnostic test within the range examined (range based on van der Goot *et al.* [13]), although performing both serological and virological assays is important for increasing the sensitivity of active surveillance strategies. Given that preventive action must be taken as soon as possible, imperfect diagnostic tests which provide results quickly may be preferable to a higher-quality assay that takes longer.

The comparison of different sampling strategies showed that increasing the sampling frequency is the most effective means of improving the timeliness of detection (i.e., R_h is minimized) but that increasing the sample size increases the likelihood of detection (i.e. smaller outbreaks have a higher chance to be detected). This has also reported for the surveillance of other viral and bacterial infectious diseases, in particular, bovine herpesvirus I [14], *Mycobacterium bovis* [15], and *Salmonella* enteritidis [16]. However, it has been demonstrated that there is a limit to optimization by increasing the frequency and decreasing the sample size, given that if the size of the sample is too small, then the specificity of the surveillance decreases [16].

The reference scenario was the epidemiological situation at the beginning of the first Italian LPAI epidemic, when no compulsory biosecurity measures were in place. At present, commercial turkey farms must be managed according to strict biosecurity measures [17], which consist of physical and temporal barriers, cleaning, and disinfection. Given that such measures reduce both the risk of incursion of AI viruses in individual production units (i.e., bioexclusion) and the risk of outward transmission (i.e., biocontainment) [18], the current between-farm transmission may be lower than that in 2000, which could imply that current LPAI surveillance in Italy is more effective than indicated by our results.

We simulated passive surveillance using a threshold value, yet in reality passive surveillance depends on many factors, such as the virus strain, the infectious dose, the individual susceptibility of the farm (which may be enhanced, for instance, by concurrent infections with other pathogens), and the awareness of the farmer. However, our intention was to simulate a situation in which a certain percentage of outbreaks would be detected by passive surveillance, which is related to the efficiency with which infection spreads in the farm. Of the outbreaks simulated, about 26% were detected by passive surveillance (Table 5), which is consistent with the data from the 96 actual outbreaks (25%). However, in simulated outbreaks based on the 96 real farms (Table 2), 37% would have reached the threshold (i.e., if they had not been detected beforehand by active surveillance or had not terminated too early because of the end of the production cycle) (data not shown). This result is consistent with the estimated sensitivity of passive surveillance for LPAI in chickens reported by Alba et al. (2010) [8] using a scenario-tree approach (i.e., 36%, assuming a design prevalence at holding level of 5%).

Although we chose to focus our evaluation on LPAI in turkeys, we can speculate on how the surveillance strategies would perform in chickens. Chickens are known to be less susceptible to LPAI infection than turkeys [19,20] and laboratory experiments have shown that bird-to-bird transmission of LPAI viruses in chickens can be low to moderate, depending on the virus strain [21,22]. Furthermore, in densely populated poultry areas with both turkey and chicken farms, LPAI outbreaks have often occurred only on turkey farms, for example in Virginia in 2002 [23], in Italy in 2004 and 2005 [24], and in Germany in 2008 [25]. We can thus assume that both within- and between-farm transmission of LPAI infections are less efficient in chickens than in turkeys, as supported by the fact that, to date, no massive LPAI epidemics have been reported in chickens [26,27]. However, a distinction should be made between broilers and layers, which have different rearing systems and lengths of production life. Although for layers passive surveillance based on decreases in egg production and feed intake has in some cases contributed to the prompt recognition of LPAI [28], the early detection of LPAI can be difficult to achieve in broilers unless many samples are tested very frequently.

Current EU legislation on the control of avian influenza focuses on early detection and

prompt reaction in the event of an outbreak [3]; however, the primary goal of EU surveillance programmes for AI in poultry is to detect the annual presence of infections caused by the subtypes H5 and H7 of HPAI and LPAI [6]. Active surveillance programmes that include only one sampling event per production cycle probably result in missed or late detection of LPAI virus incursions in turkeys.

In conclusion, in this study, we tested a number of surveillance strategies that can be used for the early detection of LPAI infections, thus preventing major epidemics and the possibility of a virulence shift to HPAI. Early detection of LPAI outbreaks in turkeys can be achieved through the combination of passive and active surveillance. Passive surveillance may be quite effective when clear clinical signs are present, but for strains similar to those on which our model parameters were based (circulating in Italy in 2000-2005), active surveillance is needed as well. Concerning the sampling strategies for active surveillance, increasing the sampling frequency to once every 15 days leads to prompt detection, providing 5 days to react after taking a positive sample to prevent a major epidemic ($R_h < 1$). However, taking samples this frequently may not be sustainable in the long term, for both economical and practical reasons. Nonetheless, we deem the above mentioned strategy specifically suitable for cases in which either an LPAI virus has been recently introduced in a previously unaffected area or when the surveillance activities are performed in an area with a high risk of virus exposure. When no LPAI virus is circulating and there is no immediate risk of virus introduction, a less frequent sampling approach might be admitted, provided that the surveillance is intensified as soon as the first outbreak is detected. It would be useful to address such risk-based optimization of surveillance in a future study.

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CHAPTER 7

General discussion

INTRODUCTION

Infection with low pathogenicity avian influenza (LPAI) virus is widespread and has led to outbreaks in domestic birds in many countries [1,4]. Although infection does not pose a serious concern for animal health, LPAI virus subtypes H5 and H7 can mutate into the highly pathogenic form (HPAI), which can cause massive epidemics and threaten human health [33]. However, the knowledge of the underlying mechanisms of mutation from LPAI to HPAI is insufficient for predicting these mutations, especially because the molecular changes necessary for the virulence shift seem to occur at random. Hence to reduce the likelihood of HPAI emergence, LPAI circulation in general should be reduced. To this end, early detection of LPAI infections in domestic poultry is fundamental.

To promptly detect the introduction of LPAI virus in poultry populations, surveillance should be carried out. Although current European Union (EU) legislation on the control of avian influenza (AI) focuses on the early detection of outbreaks and on prompt reaction [13], the primary goal of EU surveillance programmes for AI in poultry is to record the annual presence of infections caused by the subtypes H5 and H7 of HPAI and LPAI [11].

The main objective of this thesis was to develop a framework of surveillance strategies for the early detection of LPAI infections in poultry. In the EU, Italy has had the greatest number of AI outbreaks, which have affected mainly the country's only densely populated poultry area. The studies described in this thesis were based on surveillance data collected during the LPAI epidemics in Italy in 2000-2009, which involved both the industrial and rural sectors. In CHAPTER 2, the Italian monitoring system, which is the longest running system in the EU, was evaluated in terms of its capacity to detect outbreaks of LPAI infection in poultry, with particular regard to fattening turkeys, which has been the most affected species. It was shown that the majority of outbreaks were detected by active surveillance, which was especially successful in vaccinated flocks, given the vaccine-induced decrease in the severity of clinical signs and the consequent reduction in the sensitivity of passive surveillance. In CHAPTER 3, the involvement of the rural poultry sector in LPAI outbreaks was investigated. Grower and dealer holdings probably play a major role in perpetuating the infection, due to the peculiar husbandry and trade practices adopted, which facilitate the circulation and persistence of LPAI viruses in the rural sector. In CHAPTER 4, the transmission dynamics of LPAI infections in industrial turkey flocks was modelled based on field data, and it was shown that these dynamics varied greatly among flocks. In CHAPTER 5, the sensitivity and specificity of diagnostic assays for detecting LPAI antibodies were estimated without considering any assay as a gold standard. The estimates confirmed that the hemagglutination inhibition (HI) test, which is generally accepted as the gold standard, has a near perfect accuracy and may be considered as a reference test; however, to perform LPAI surveillance, for which large numbers of samples have to be processed, valid alternatives exist. Finally, in

CHAPTER 6, different surveillance strategies were evaluated in terms of their performance for early warning; it was shown that the prompt detection of LPAI outbreaks in turkeys can be achieved through active surveillance by increasing the sampling frequency to once every 15 days, though very frequent sampling may not be sustainable in the long term.

In the current chapter, the main findings of the previous chapters are integrated and critically discussed; the possible differences in the effectiveness of surveillance for different host species are explored; and the results of the studies presented in the previous chapters are extrapolated to come up with a framework of surveillance strategies for the early detection of LPAI infections in industrial poultry.

EFFECTIVENESS OF COMPONENTS OF SURVEILLANCE IN DETECTING LPAI INFECTIONS

In the EU, the main objective of LPAI surveillance in poultry is to detect infection with virus subtypes H5 and H7 by testing birds. A specific farm or farms may be targeted for testing either because clinical signs were observed (i.e., passive surveillance) or based on the monitoring activity that each Member State must implement (i.e., active surveillance) [11,13]. Active surveillance is more effective than passive surveillance in detecting LPAI outbreaks in fattening turkeys, as shown by the evaluation of field data (CHAPTER 2) and the outbreak simulations (CHAPTER 6), as well as by previous studies [9,27]. However, the timeliness with which the virus is detected through active surveillance greatly depends on the specific monitoring program (i.e., sampling frequency and sample size). Although increasing the sampling frequency and/or sample size would allow for earlier detection, in general terms, the earlier testing is performed, the lower the probability of detection, so that later samplings are more likely to detect LPAI infections (CHAPTER 2). This is the reason for which testing is performed at the slaughterhouse: although such sampling would be ineffective in terms of early detection, it has the highest chance of detecting infections with LPAI viruses, which is the current goal of EU surveillance.

Passive surveillance seems to be less sensitive than active surveillance in detecting LPAI infections in turkeys, probably because the disease may induce only mild and unspecific symptoms [23,27] and the sensitivity of passive surveillance depends on several factors, including the farmers' awareness of clinical signs and the severity of the disease. In fact, according to the analysis of the Italian LPAI epidemics in 2000-2005 (CHAPTER 2), passive surveillance detected 114 LPAI outbreaks in turkeys, compared to the 222 outbreaks detected through active surveillance. However, during the 2000-2005 outbreaks, of the samplings (i.e., visits to a turkey farm to collect samples) performed for passive surveillance, 40% resulted in outbreak detection when using serological testing and 20% when using virological testing (Table 1), indicating that the reporting of suspected infections by farmers has a high specificity.

In addition to the sampling performed for active or passive surveillance, current EU provisions require supplemental sampling when a farm is epidemiologically linked with an infected premise or located within the restriction zone surrounding a confirmed outbreak [13]. In CHAPTER 2, this supplementary surveillance component is referred to as "targeted surveillance" because it was aimed at farms located near infected premises, which were believed to be at higher risk of becoming infected. This belief derived from the fact that in the Italian and Dutch HPAI epidemics of 1999-2000 and 2003, local transmission played an important role in the spread of the disease [3,21]. However, as shown in CHAPTER 2, for LPAI infection, the additional sampling performed on farms that were located in restriction zones or were epidemiologically linked with infected premises (herein referred to as "contact surveillance") was much less effective than expected. In fact, during the four Italian LPAI epidemics in this period, the detection rate (i.e., efficiency) of contact surveillance was only about 7% for the serological samplings and 9% for the virological samplings (Table 1), which is only slightly higher than the rates for active surveillance.

Table 1. Efficiency of detection by surveillance component and type of testing during the 2000-2005 epidemics in Italy

Testing	surveillance component	number of samplings	number of detections (outbreaks)	efficiency (relative detection rate)	Exact binomial 95% CI of efficiency
serological	passive surveillance	168*	67	39.9%	32.4 - 47.7%
	active surveillance	4716	150	3.2%	2.9 - 4.0%
	contact surveillance	311	21	6.8%	4.2 - 10.1%
Virological	passive surveillance	232*	47	20.3%	15.3 - 26.1%
	active surveillance	2471	72	2.9%	2.3 - 3.6%
	contact surveillance	64	6	9.4%	3.5 - 19.3%

* the number of samplings reported for passive surveillance does not refer to the entire component but only to a part of it; in fact, flocks are assumed to be regularly inspected, but samples are collected only in case the farmers suspect infection. Therefore the efficiency reported in this table for passive surveillance refers to the farmers' awareness of clinical signs rather than the entire passive surveillance strategy.

The low efficiency of contact surveillance in detecting LPAI infections may have several explanations. Based on the dynamics of disease transmission, the farms that are epidemiologically linked to an infected premise are expected to have a higher probability of being infected: the low detection rate on these farms suggests that contact tracing simply may have not functioned properly. Regarding the farms located in proximity to LPAI-infected premises, Mulatti et al. (2010) [22] showed that LPAI viruses are more likely to spread to medium and long-distances (i.e., within 60 km), compared to short distances (i.e., within 1.5 km), as occurs with HPAI infections [3,21], due to the transmission of LPAI viruses through the contact structure of the poultry production sector (i.e., direct or

indirect contact with infected birds via live poultry, staff, vehicles, equipment or contaminated materials); thus the low detection rate found on these farms could support the findings that neighbourhood spread plays a minor role. Another possible explanation for the low detection rate is that the biosecurity and control measures implemented during the Italian LPAI epidemics progressively decreased the between-flock transmission [22], which would have reduced the probability of detecting infection through contact surveillance.

DIAGNOSTIC TOOLS TO DETECT LPAI INFECTIONS

Diagnostic testing for the surveillance of LPAI infection can be aimed at identifying the causative agent (i.e., the replicating virus or viral RNA) or the antibodies against viral proteins. Generally speaking, the LPAI virus and its genome can be detected in an individual bird for only a few days, depending on the virus strain, the bird species, the infectious dose and the method of detection, whereas antibodies are often present for the entire production life of the infected poultry [27]. In CHAPTER 6, the combination of virological and serological testing, which target antigens and antibodies, respectively, was shown to increase the sensitivity of surveillance systems in terms of the early detection of LPAI infection. This depends on the fact that parallel testing increases the overall sensitivity of the testing regime itself [10]. It also depends on the fact that the targets of the virological and serological tests show up sequentially during infection (i.e., at the onset of infection only antigens are detectable, then both antigens and antibodies are present, and later only antibodies persist); thus looking for both antigens and antibodies enhances the chance of detecting at least one of the two. However, additional simulations (based on the models implemented in CHAPTER 6) showed that if sampling is very frequent, then performing only serological testing could suffice for detecting the outbreak in time to avoid a major epidemic (i.e., R_h<1) (Table 2).

Table 2. Effectiveness in detecting LPAI infections in the absence of control measures (baseline scenario), by surveillance strategy; results of 1000 simulated outbreaks.

Surveillance strategy	proportion of outbreaks that are detected	proportion of detected outbreaks identified by active surveillance	R_h at detection
serological + virological tests at days 60, 90, 120	73.4%	73.0%	0.80 [0.75 - 0.85]*
serological test at days 60, 90, 120	68.3%	65.7%	1.05 [0.99 - 1.11]*
serological test at days 30 - 45 - 60 - 75 - 90 - 105 – 120	79.5%	61.2%	0.77 [0.72 - 0.82]

* Results also shown in CHAPTER 6.

Several serological tests for LPAI detection are available, and they can be classified into two main categories: type A specific tests, which can identify antibodies against any type A influenza virus of avian and mammalian origin, and subtype-specific tests, which can only detect antibodies to a specific virus subtype [25]. The most commonly applied subtype-specific tests are those for detecting the H5 and H7 subtypes, given their potential to become highly pathogenic in gallinaceous birds. The selection of diagnostic tests depends on the purpose of testing, as well as on factors such as cost, sensitivity, specificity, rapidity, ease of use, and the availability of personnel, equipment, and test reagents. In CHAPTER 5, some serological tests were evaluated for their ability to detect LPAI antibodies, and the HI test, which is currently considered the gold standard for subtype-specific LPAI antibody detection, was confirmed to be very sensitive and specific. However, this test is difficult to automate, and its performance is influenced by the homology between the reference viral antigen and the strain being tested [14]. For this reason, for the purposes of surveillance, in which large numbers of samples have to be processed and the circulating LPAI virus may not be known, a simple-to-perform assay that can be easily automated may be preferable. Based on the results of CHAPTER 5, a valid alternative could be subtype-specific blocking ELISA, which during epidemic periods would be even more valuable if it could be performed directly on the farm. In fact, on-site rapid testing would improve the timeliness of detection, thus allowing preventive actions to be taken earlier.

ROLE OF THE RURAL POULTRY SECTOR AND IMPLICATIONS FOR SURVEILLANCE

The non-industrial breeding of birds is widespread throughout Europe. In 2010, more than one million holdings rearing backyard and rural poultry, game birds and exotic birds were reported in the 19 EU countries that conducted surveillance activities for avian influenza in such birds [12]. In Italy, in particular, the non-industrial (i.e., rural) poultry sector has considerable economic importance and constitutes 20% of national poultry meat production. Furthermore, the rural sector has composite relationships and may, in some cases, come into contact with the industrial sector through legal and illegal connections. In CHAPTER 3, the involvement of the rural poultry sector in the Italian 2007 and 2009 H7N7 LPAI epidemics was discussed, showing that it can represent an important human-made reservoir of avian influenza virus. In fact, the husbandry and trading system of the Italian grower/dealer holdings and the constant introduction of naïve birds allow LPAI viruses, once they enter the rural sector, to become established, as occurred in the United States (US) within the live-bird market circuit [24,29].

From the perspective of surveillance for early detection, although rural poultry holdings are at high risk of LPAI virus introduction, in industrial holdings the high density

confinement can greatly facilitate the spread of the virus [19]. For this reason, to prevent an epidemic, early detection and prompt reaction are crucial in the industrial poultry sector. However, surveillance in the rural poultry sector is nonetheless important, especially in densely populated poultry areas. In particular, given that industrial and rural poultry holdings can coexist in these areas (e.g., in Italy, Figure 1-A.), surveillance in large grower/dealer holdings, which are at higher risk of LPAI virus introduction and amplification because they raise and trade numerous birds of multiple species and ages, may act as a warning system for the nearby industrial holdings, inducing them to implement additional biosecurity measures and/or intensify monitoring activities (Figure 1-B.).

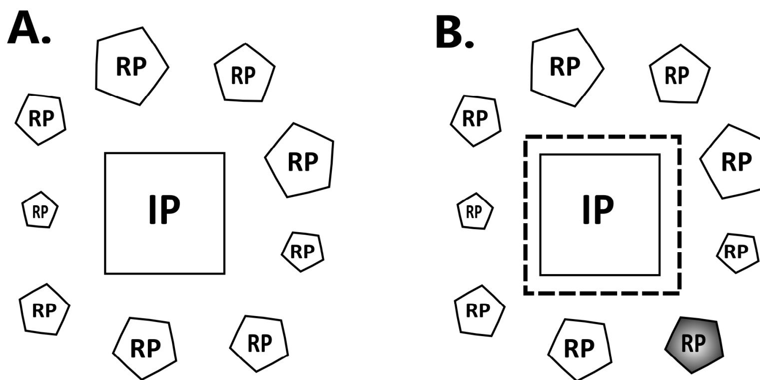

Figure 1. Schematic representation of the alert system based on the surveillance of rural premises. **A.** Coexistence of industrial (IP) and rural (RP) premises in the same area; **B.** Enforcement of monitoring and control measures in industrial premises (dashed square) in case of recognition of LPAI infections in rural premises (shaded shape).

SURVEILLANCE IN DIFFERENT POULTRY SPECIES

The effectiveness of surveillance in terms of its capacity for early detection of LPAI infections may vary depending on the dynamics of within- and between-flock transmission, which in turn depend on the specific characteristics of the LPAI virus, the susceptibility of the poultry species, the production system and the biosecurity measures applied. However, predicting the impact of a specific virus on the success of early detection is difficult because the H5 and H7 LPAI virus subtypes have different transmission dynamics, even within the same host species [e.g., 16,31]. Bird-to-bird transmission can also vary considerably within the same H subtype (e.g., H7N1 vs. H7N7) [17]. Furthermore, even within the same H-N subtype (e.g., H7N3) the virus strains

isolated at various times during the same epidemic can differ because AI viruses mutate rapidly and continuously, occasionally undergoing virulence shift [28]. For this reason, the outbreak simulation model for fattening turkeys described in CHAPTER 4 was developed to take into account the different transmission dynamics of LPAI viruses by letting the within-flock transmission vary from flock to flock; this model was then used as background for the various surveillance strategies under different scenarios investigated in CHAPTER 6, so that the conclusions could be valid for several LPAI viruses infecting turkeys.

Throughout this thesis, surveillance strategies are investigated in relation to fattening turkeys, which as mentioned was the most affected species in the 2000-2005 Italian epidemics. However, some speculation can be made as to the performance of the same strategies in other species and production types. Although turkeys and chickens belong to the same taxonomic order (*Galliformes*), chickens are less susceptible to LPAI infections than turkeys [26,30]. Furthermore, laboratory experiments have shown that bird-to-bird transmission of LPAI viruses in chickens ranges from low to moderate, depending on the virus strain [16,17,31]. We can thus assume that the within-flock transmission of LPAI infections is less efficient in chickens than in turkeys, which suggests that the average prevalence of infectious chickens at any given time in the epidemic will be lower than the prevalence of infectious turkeys. To this regard, Gonzales et al. (2011b) [17] estimated that the maximum prevalence of infectious birds in chickens experimentally infected with H7N7 LPAI was lower than 10% and that it would take more than two months after infection for the seroprevalence of infected birds to reach 30%.

Among chickens, a further distinction should be made between broilers and layers, which have different rearing systems and durations of production life. There are approximately 10 times more broilers raised in the EU (5.5 billion) than layers, turkeys, and breeders combined [15], and in the US there are up to 20 times more broilers than other types of poultry [19]. Nonetheless, there have been very few reports of large LPAI outbreaks among broilers in the EU and US, and all major epidemics of LPAI have mainly, if not exclusively, involved industrial turkeys [2,5,7,18] or industrial layers [5,6,32]. Whereas for turkeys this could be explained by the high susceptibility to LPAI viruses, species-specific susceptibility cannot explain why layer flocks are much more frequently infected than broiler flocks. It can thus be reasonably assumed that the greater involvement of layer flocks is related to the rearing system itself, in particular, weaknesses in both structural biosecurity (e.g., moving birds from site to site, using shared equipment, and multiple age farms) and operational biosecurity (e.g., use of hired labour and multiple entries to the farm to collect eggs), as well as the greater longevity of layers. We can thus hypothesize that the between-flock transmission of LPAI in broilers is lower than in layers.

In light of these considerations, passive surveillance in broilers would probably be inefficient because the clinical manifestation of the disease is barely evident [26] and the within-flock prevalence is too low to notice symptoms. Active surveillance based on monthly sampling of 10 birds would also probably be inefficient because seroprevalence increases slowly (e.g., it would require more than two months after infection to reach 30% of seroconversions in H7N7 infected chickens [17]) and thus the infection would seldomly be detected before slaughtering (i.e., at 5-7 weeks of age). In layers, clinical symptoms are just as mild, yet decreases in egg production and feed intake may be reliable warning signs, leading to the suspicion of LPAI infections [8,20] and efficiently acting as passive surveillance. Since within-flock transmission is lower in layers than in turkeys, the performance in layers of the reference surveillance strategy in CHAPTER 6 would be presumably similar to that for the scenarios with a low R0 or long generation time (CHAPTER 6, Figures 2b and 3), provided that the two species have the same between-flock transmission. However, since LPAI outbreaks have often affected only turkey premises in densely populated poultry areas with both layer and turkey farms (e.g., Virginia 2003 [2], Italy 2004 and 2005 [5], Germany 2008 [7]), it is plausible that the between-flock transmission of LPAI among layer farms is less efficient than in turkey farms. We can thus hypothesize that surveillance in turkeys represents the worst case scenario, so that early detection would probably be easier to achieve in layers even with less frequent testing, and turkeys may act as sentinels for infected layer flocks.

FRAMEWORK OF SURVEILLANCE STRATEGIES FOR EARLY DETECTION OF LPAI INFECTIONS

The research developed in this thesis is based on the integration of epidemic data, experimental data and modelling, which, together with the information from the literature discussed in the present chapter, allows the most important aspects of surveillance for the early detection of LPAI in industrial poultry holdings in densely populated poultry areas to be outlined. Based on the issues and results presented in this thesis, the following activities for LPAI surveillance geared towards early detection in gallinaceous birds can be proposed:

- perform frequent sampling (i.e., every 20-30 days) for active surveillance in densely populated poultry areas, especially in long-living highly susceptible species, such as fattening turkeys, or in production types at higher risk of exposure, such as free-range layers;
- exploit sampling performed for other purposes (e.g., national surveillance programmes for salmonella or voluntary programmes for monitoring infectious diseases in poultry), which can minimize both the costs and the number of

interventions on the farm (which also represent a risk for disease introduction during epidemic periods);

- sample short-living poultry (i.e., broilers) only at the slaughterhouse, which, though not allowing for early detection, will provide information on the annual presence of infection, as prescribed by EU legislation;
- perform both serological and virological testing, unless sampling is very frequent (i.e., every 15 days), in which case active surveillance based solely on serological testing is effective;
- develop and validate H5 and H7 subtype-specific blocking ELISA tests, in order to increase the timeliness and reduce the costs of testing (valuable especially in case of outbreaks). As an alternative, during peace time or when the circulating virus is unknown, initial screening using commercial type A specific ELISA tests can be performed, followed by confirmation and subtyping with the HI test;
- implement passive surveillance based on the detection of symptoms and changes in production data as an essential supplement to active surveillance.
- implement surveillance activities also in the rural sector in case of a high density of grower/dealer poultry holdings and industrial poultry holdings coexisting in the same area; this can serve as an additional warning system for the increased risk of LPAI virus introduction and spread within the industrial sector.

These activities are specifically addressed to situations in which either an LPAI virus has been recently introduced or the surveillance activities are performed in an area at high risk of virus exposure. However, when no LPAI virus is circulating and there is no immediate risk of virus introduction, it might be difficult to sustain an intensive sampling protocol. To this regard, further calculations could be made in order to determine to what extent surveillance could be relaxed, taking into account the maximum number of infected farms at first detection that would be acceptable. In such a situation, sampling could be performed less frequently and only using serological testing, provided that the surveillance is intensified (i.e., increasing sampling frequency and performing both serological and virological tests) as soon as the first outbreak is detected or there is a high risk of virus introduction.

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ADDENDUM

Summary
Samenvatting
Curriculum vitae
List of publications
Acknowledgements

SUMMARY

Infection with low pathogenicity avian influenza (LPAI) virus is widespread and has led to outbreaks in domestic birds in many countries. Although infection does not pose a serious concern for animal health, LPAI virus subtypes H5 and H7 can mutate into the highly pathogenic form (HPAI), which can cause massive epidemics in poultry and threaten human health. However, the knowledge of the underlying mechanisms of mutation from LPAI to HPAI is insufficient for predicting these mutations, especially because the molecular changes necessary for the virulence shift seem to occur at random. Hence to reduce the likelihood of HPAI emergence, LPAI circulation in general should be reduced. To this end, early detection of LPAI infections in domestic poultry is fundamental.

To promptly detect the introduction of LPAI virus in poultry populations, surveillance should be carried out. The specific strategy adopted varies from country to country, however, it generally includes a combination of the passive and active surveillance of wild and domestic bird populations. In poultry, passive surveillance relies on the observation at the flock level of signs that could be indicative of infection (e.g., increased mortality, reduced feed and water consumption, reduced egg production, and respiratory symptoms). Active surveillance entails visiting farms and sampling animals for diagnostic tests, which can be performed either for detecting the disease by testing for viral antigens (virological surveillance) or for detecting the infection by looking for antibodies (serological surveillance). European Union (EU) legislation on the control of avian influenza (AI) put particular emphasis on the early detection and prompt reaction in the event of an outbreak. Nevertheless, current EU surveillance programmes for AI in poultry aim at determining yearly the presence of infections with subtypes H5 and H7 in different bird species, rather than at the early detection of new incursions (**CHAPTER 1**).

Since the timeliness, effectiveness, and cost of AI control during an epidemic greatly depend on how soon the first cases are diagnosed and how quickly control strategies can be implemented, the main objective of this thesis was to develop a framework of surveillance strategies for the early detection of LPAI infections in poultry.

The studies described in this thesis were based on surveillance data collected during the past LPAI epidemics occurred in Italy, which is the country with the longest experience in surveillance and outbreaks in the EU. Since the surveillance system was made operational, four major LPAI epidemics have occurred: in 2000-2001, 2002-2003, 2004, and 2005, in addition to two small epidemics in 2007 and 2009, which mainly involved non-industrial flocks. Given that this is the longest running surveillance system in Europe, a large quantity of data has been collected, which, together with the experience gained, could be useful in evaluating and improving surveillance programmes.

In **CHAPTER 2**, the components of the Italian surveillance system and its resources were evaluated in terms of the capacity to detect LPAI infected premises (i.e., outbreaks) of vaccinated and unvaccinated poultry, with particular regard to fattening turkeys, which has been the most affected species. It was shown that the majority of outbreaks occurred in turkeys in 2000-2005 were detected by active surveillance (i.e. 61% of the outbreaks), which was especially successful in vaccinated flocks, given the vaccine-induced decrease in the severity of clinical signs and the consequent reduction in the sensitivity of passive surveillance. In addition to the sampling performed for active or passive surveillance, EU provisions require supplemental sampling when a farm is epidemiologically linked with an infected premise or located within the restriction zone surrounding a confirmed outbreak. This supplementary surveillance component was believed to be targeted at farms at higher risk of becoming infected (based on previous experience with HPAI epidemics). However, for LPAI infection, such additional sampling was much less effective than expected, probably because the contact tracing did not functioned properly and/or the implemented biosecurity measures progressively reduced the between flock-transmission.

Although the concern for AI infection is greatest for industrial poultry, the potential role of the non-industrial (i.e., rural) poultry sector in the maintenance and spread of LPAI viruses should not be overlooked, as demonstrated by the circulation of H5 and H7 LPAI viruses for more than 10 years in the rural poultry premises belonging to the live bird market circuit in the New York City area. In Italy, the rural poultry sector has considerable economic importance and constitutes 20% of national poultry meat production. Furthermore, it has composite relationships and may, in some cases, come into contact with the industrial sector through legal and illegal connections. In **CHAPTER 3**, the involvement of the rural poultry sector in the Italian 2007 and 2009 H7N7 LPAI epidemics was discussed, showing that it can represent an important human-made reservoir of avian influenza virus. In fact, the husbandry and trading system of the Italian grower/dealer holdings and the constant introduction of naïve birds allow LPAI viruses, once they enter the rural sector, to become established.

CHAPTER 4 investigated the transmission dynamics of LPAI within fattening turkey farms using serosurveillance data from the Italian LPAI epidemics in 2000-2005 and experimental infection data. The study showed that the basic reproduction number (R_0) within farms was on average 5.5, meaning that an infectious turkey infects on average 5.5 turkeys within a susceptible farm, although R_0 varied greatly among farms. Furthermore it resulted in final estimates for the mean latent and infectious periods of 2.9 and 8.1 days, respectively, and provided a mathematical model to simulate within-farm outbreaks of LPAI. The simulated outbreaks indicated that the presence of seropositive birds does not necessarily mean that the virus has already been cleared and the flock is no longer infective. This implies that seropositive flocks may still pose a risk for other flocks; thus

the enforcement of appropriate restrictions, the culling of seropositive flocks or pre-emptive slaughtering may be useful in preventing farm-to-farm transmission.

LPAI surveillance involves the detection of either the disease (virological surveillance) the infection (serological surveillance) based on the results of diagnostic tests. It follows that the accuracy of the diagnostic assays applied may have an impact on the conclusions derived from surveillance itself. Serological tests coupled with clinical inspection of poultry holdings are among the methods currently applied for LPAI surveillance. Serological diagnosis of LPAI can be performed using different methods, yet the hemagglutination inhibition (HI) test is considered as the ''gold standard'' for AI antibody subtyping. Although alternative diagnostic assays have been developed, in most cases their accuracy has been evaluated in comparison to HI test results, whose performance for poultry has not been properly evaluated. In **CHAPTER 5**, the sensitivity (Se) and specificity (Sp) of diagnostic assays for detecting LPAI antibodies were estimated without considering any assay as a gold standard. The results confirmed that the HI test has a near perfect accuracy (98.8% Se and 99.5% Sp) and may be considered as a reference test. However, HI test is laborious and its performance is influenced by the homology between the reference viral antigen and the strain being tested. In the framework of LPAI surveillance, where large numbers of samples have to be processed and the circulating LPAI virus strain may not be known, a subtype-specific blocking ELISA could be a valid alternative to the HI test, in that it is almost as accurate as the HI test yet quicker and easier to automate.

In **CHAPTER 6**, information on baseline surveillance activities, within- and between-flock transmission of LPAI, and accuracy of diagnostic tools was entered into a simulation model to evaluate different surveillance strategies in terms of their performance as an early warning system. The strategies differed in terms of sample size, sampling frequency, diagnostic tests, whether or not active surveillance was performed, and were also tested under different epidemiological scenarios. Surveillance strategies were compared by simulating within-farm outbreaks and the output measures were the proportion of infected farms that are detected and the farm reproduction number (R_h). The first one provides an indication of the sensitivity of the surveillance system to detect within-farm infections, whereas R_h reflects the effectiveness of outbreak detection (i.e. if detection occurs soon enough to bring an epidemic under control). Increasing the sampling *frequency* was the most effective means of improving the timeliness of detection (i.e., it occurs earlier), whereas increasing the sample *size* increased the likelihood of detection. Surveillance was only effective in preventing an epidemic if actions were taken within two days of sampling. The strategies were not affected by the quality of the diagnostic test, although performing both serological and virological assays increased the sensitivity of active surveillance. The results showed that early detection of LPAI outbreaks in turkeys can be achieved by increasing the sampling frequency for active surveillance to once

every 15 days, though very frequent sampling may not be sustainable in the long term.

The research developed in this thesis is based on the integration of epidemic data, experimental data, modelling, and information from the literature (discussed in **CHAPTER 7**), allows the most important aspects of surveillance for the early detection of LPAI in industrial poultry holdings in densely populated poultry areas (DPPAs) to be outlined. Based on the issues and results presented in this thesis, the following activities for LPAI surveillance geared towards early detection in gallinaceous birds can be proposed:

- perform frequent sampling (i.e., every 20-30 days) for active surveillance in DPPAs, especially in long-living highly susceptible species, such as fattening turkeys, or in production types at higher risk of exposure, such as free-range layers;
- exploit sampling performed for other purposes (e.g., national or voluntary programmes for monitoring infectious diseases in poultry), which can minimize both the costs and the number of interventions on the farm;
- sample short-living poultry (i.e., broilers) only at the slaughterhouse, which, though not allowing for early detection, will provide information on the annual presence of infection, as prescribed by EU legislation;
- perform both serological and virological testing, unless sampling is very frequent;
- develop and validate H5 and H7 subtype-specific blocking ELISA tests, in order to increase the timeliness and reduce the costs of testing (valuable especially in case of outbreaks);
- implement passive surveillance based on the detection of symptoms and changes in production data as an essential supplement to active surveillance.
- implement surveillance activities also in the rural sector in case of a high density of grower/dealer poultry holdings and industrial poultry holdings coexisting in the same area;

These activities are specifically suitable for cases in which either an LPAI virus has been recently introduced in a previously unaffected area or when the surveillance activities are performed in an area with a high risk of virus exposure. When no LPAI virus is circulating and there is no immediate risk of virus introduction, a less frequent sampling approach might be admitted, provided that the surveillance is intensified as soon as the first outbreak is detected. It would be useful to address such risk-based optimization of surveillance in a future study.

SAMENVATTING

Infecties met laagpathogene aviaire influenza (LPAI)-virus leiden geregeld tot uitbraken bij pluimvee. Hoewel infectie niet tot ernstige ziekte leidt, kan LPAI-virus van de subtypes H5 en H7 muteren tot hoogpathogeen (HPAI) virus dat door selectievoordeel in pluimveepopulaties grote epidemieën kan veroorzaken en tevens een bedreiging vormt voor de volksgezondheid. Het is nog niet mogelijk om te kunnen voorspellen wanneer zo'n mutatie op zal treden, maar omdat de moleculaire veranderingen die nodig zijn voor de virulentie verschuiving willekeurig lijken op te treden, ligt het voor de hand dat de kans op het ontstaan van HPAI-virus wordt verkleind als circulatie van LPAI-virus vroeg wordt opgespoord en gestopt.

Om de introductie van LPAI-virus vroeg op te sporen is surveillance nodig. Passieve surveillance bij pluimvee is gebaseerd op het waarnemen van verschijnselen die kunnen wijzen op infectie zoals verhoogde mortaliteit, verminderd voer- en waterverbruik, verminderde eiproductie en respiratoire symptomen. Actieve surveillance houdt in dat het pluimveebedrijf wordt bezocht en monsters worden afgenomen voor onderzoek naar het virus of naar afweerstoffen tegen het virus.

In de wetgeving van de Europese Unie (EU) betreffende de bestrijding van AI wordt bijzondere nadruk gelegd op tijdige opsporing en snelle actie in geval van een uitbraak. Niettemin, zijn de huidige surveillanceprogramma's voor AI bij pluimvee in de EU gericht op het jaarlijks bepalen van de aanwezigheid van infecties met de subtypes H5 en H7, niet op de vroege opsporing van nieuwe uitbraken.

De belangrijkste doelstelling van dit proefschrift was om een kader te ontwikkelen voor de vroege detectie van LPAI-infecties bij pluimvee. De studies beschreven in dit proefschrift zijn gebaseerd op surveillance gegevens die zijn verzameld tijdens vier grote LPAI epidemieën in Italië in 2000-2001, 2002-2003, 2004, en 2005 en twee kleine epidemieën in 2007 en 2009 bij niet intensief gehouden pluimvee.

Als eerste werden de onderdelen van het Italiaanse surveillancesysteem geëvalueerd met betrekking tot hun capaciteit om LPAI uitbraken op te sporen onder gevaccineerde en niet-gevaccineerde koppels pluimvee, in het bijzonder vleeskalkoenen. 61% van de uitbraken in de periode 2000-2005 werd opgespoord met actieve surveillance. Actieve surveillance was vooral succesvol in gevaccineerde koppels, wat verklaard kan worden door de vaccin-geïnduceerde afname van klinische verschijnselen. Opmerkelijk genoeg werd maar een beperkte proportie besmette bedrijven opgespoord op grond van een epidemiologische link met een eerder besmet bedrijf. Mogelijke verklaringen hiervoor zijn een inadequate tracering of een verminderde buurtinfectie door betere hygiënische maatregelen.

Hoewel de bedreiging door AI-infecties het grootst is voor intensief gehouden pluimvee, vertegenwoordigt in Italië het niet intensief gehouden pluimvee ook een groot

economisch belang met ca 20% van de nationale pluimveevleesproductie. Daarnaast bestaan er relaties tussen deze sector en het intensief gehouden pluimvee. In hoofdstuk 3 werd de betrokkenheid van de niet intensieve pluimveesector in de Italiaanse 2007 en 2009 H7N7 LPAI epidemieën beschreven, waaruit blijkt dat deze sector een belangrijk reservoir van AI virus kan vormen. In feite maakt deze vorm van pluimveehouderij met haar frequente introducties en veel handel door dealers het makkelijk voor de virussen zich te handhaven. De overdracht van virussen naar de intensieve pluimveehouderij is echter beperkt.

In hoofdstuk 4 wordt de transmissie van LPAI binnen koppels vleeskalkoenen beschreven zoals kon worden afgeleid van de serologische gegevens van de LPAI epidemieën en experimentele infectie data. De studie toonde aan dat de reproductie ratio (R_0) gemiddeld 5,5 was, oftewel een besmettelijke kalkoen infecteert gemiddeld 5,5 kalkoenen in een gevoelig koppel. R_0 varieerde echter sterk tussen bedrijven. In kalkoenen bleek de gemiddelde latente en de gemiddelde infectieuze periode respectievelijk 2,9 en 8,1 dagen. Gesimuleerde uitbraken gaven aan dat de aanwezigheid van seropositieve dieren niet hoeft te betekenen dat het virus niet meer aanwezig is en de koppel niet meer besmettelijk is; seropositieve koppels kunnen nog steeds een risico voor niet besmette koppels vormen.

Momenteel worden serologische tests in combinatie met klinische controle toegepast voor LPAI surveillance. De serologische diagnose van LPAI kan worden uitgevoerd met behulp van verschillende methoden, maar de hemagglutinatie remmingstest (HAR) wordt beschouwd als de'' gouden standaard''. Hoewel alternatieve diagnostische testen zijn ontwikkeld wordt in de meeste gevallen de juistheid ervan geëvalueerd ten opzichte van de HAR, waarvan de prestaties voor pluimvee niet goed is geëvalueerd. In de studie beschreven in hoofdstuk 5 werden de gevoeligheid (Se) en specificiteit (Sp) van diagnostische tests voor de detectie van LPAI antilichamen geschat zonder een gouden standaard te definiëren. De resultaten toonden dat de HAR-test accuraat is (98,8% Se en 99,5% Sp). De HAR-test is echter omslachtig en de prestaties worden beïnvloed door de homologie tussen het test antigeen en het circulerend virus. In het kader van LPAI-surveillance, waar grote aantallen monsters moeten worden verwerkt en de circulerende LPAI-virus stam vaak niet bekend is, is een subtype-specifieke blokkerings-ELISA een goed alternatief voor de HI test en bijna net zo accuraat als de HI-test, maar sneller en gemakkelijker te automatiseren.

In hoofdstuk 6 wordt een simulatiemodel beschreven waarmee het effect van aanpassingen van het basissurveillance programma (steekproeffrequentie en –omvang) en de accuraatheid van de diagnostische test op het vermogen tot vroeg detectie kan worden getoetst. Surveillance strategieën werden vergeleken op basis van het aantal gedetecteerde besmette bedrijven en de koppel reproductie ratio (R_h). De eerste geeft een indicatie van de gevoeligheid van de surveillance om binnen een bedrijf infecties te

detecteren, terwijl R_h de effectiviteit van de uitbraakdetectie weergeeft om een epidemie onder controle te krijgen. Het verhogen van de steekproeffrequentie was het meest effectieve middel om de tijdigheid van de detectie te verbeteren, terwijl het vergroten van de steekproefomvang de kans op detectie op een geïnfecteerd bedrijf vergroot. Surveillance bleek alleen effectief bij het voorkomen van een epidemie als de erop volgende bestrijdingsmaatregelen werden genomen binnen twee dagen na de bemonstering. De kwaliteit van de diagnostische test zelf bleek van beperkte invloed, hoewel het uitvoeren van zowel serologische als virologische tests de gevoeligheid van surveillance verhoogde. De resultaten toonden aan dat het tijdig opsporen van uitbraken van LPAI bij kalkoenen kan worden bereikt door het verhogen van de bemonsteringsfrequentie bij actieve bewaking tot eens per 15 dagen. Echter, zeer frequente bemonstering zal op de lange termijn moeilijk vol te houden zijn.

Het onderzoek beschreven in dit proefschrift is gebaseerd op de integratie van de epidemiologische gegevens, experimentele gegevens, modellering en informatie uit de literatuur. Op basis van de resultaten gepresenteerd in dit proefschrift, worden de volgende activiteiten voor LPAI surveillance gericht op vroege opsporing bij pluimvee voorgesteld:

- Frequent (dat wil zeggen, elke 20-30 dagen) onderzoek van koppels pluimvee voor actieve surveillance in pluimveedichte gebieden, met name in langlevende soorten, zoals vleeskalkoenen en vrije uitloop kippen;
- bemonster kortlevend pluimvee (zoals vleeskuikens) alleen in het slachthuis, om informatie over de jaarlijkse aanwezigheid van een infectie, zoals voorgeschreven door de EU-wetgeving, te verstrekken;
- Voer zowel serologische als virologische testen uit bij frequente bemonstering;
- ontwikkel en valideer H5 en H7 subtype-specifieke blokkerings-ELISA testen voor direct gebruik op de boerderij om tijdige detectie te bevorderen en de kosten te beperken;
- implementeer passieve surveillance gebaseerd op verschijnselen en veranderingen in de productie als een essentiële aanvulling op actieve bewaking;
- surveillance bij niet intensief gehouden pluimvee in het geval van een hoge dichtheid van dergelijke bedrijven en intensieve pluimveehouderijen in hetzelfde gebied.

Deze activiteiten zijn speciaal geschikt voor gevallen waarin ofwel een LPAI-virus recent is geïntroduceerd in een eerder onaangetast gebied of in gebieden met een hoog risico op introductie. Als er geen LPAI-virus aanwezig is en het risico op insleep beperkt kan een minder frequente steekproefbenadering worden toegelaten, op voorwaarde dat het toezicht wordt aangescherpt zodra de eerste uitbraak wordt gedetecteerd. Het zou nuttig zijn om dergelijke risico's gebaseerde optimalisatie van het toezicht aan te pakken in een toekomstige studie.

CURRICULUM VITAE

Arianna Comin was born on October 27th 1978 in Camposampiero (Padua), Italy. In 2003 she received the degree of Doctor of Veterinary Medicine (D.V.M.) from Padua University, Italy. Then, she joined a post-graduate programme at the Department of Animal Science of Padua University, studying the heritability of quantitative traits related to milk coagulation properties in dairy cows. In 2006 she went to Finland for six months on a Socrates/Erasmus exchange study at the University of Helsinki, where she studied the effect of β- and κ-casein genotypes on milk coagulation properties and milk production in dairy cows. In 2007 she obtained the degree of Doctor of Philosophy (Ph.D.) in Animal Breeding and Quantitative Genetics from Padua University, defending a thesis entitled "Analysis of individual genetic features influencing the milk coagulation ability in Italian Holstein cows". Then she was granted a scholarship from the *Istituto Zooprofilattico Sperimentale delle Venezie* (IZSVe) to perform a retrospective analysis on prevalence of main zoonoses in Italy and a survey on zoonotic gastroenteritis among children. In 2008 she joined the European project FLUTEST and started her PhD programme at Utrecht University, The Netherlands. Whilst she has been doing her (second) PhD she also worked at the Department of Veterinary Epidemiology of IZSVe as junior researcher, studying the epidemiology and control of several animal diseases (i.e., avian influenza, Q fever, paratubercolosis, bluetongue, rabies, hepatitis E). In 2010, she obtained the post-graduate Diploma of Specialization in Animal Health, Farm and Animal Production Hygiene from the University of Bologna, Italy. Since 2011 she is working as junior epidemiologist at the Department of Animal Health Management of IZSVe.

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Scientific journals

COMIN A., STEGEMAN A., MARANGON S., KLINKENBERG D. Evaluating surveillance strategies for the early detection of low pathogenicity avian influenza infections. PLoS ONE 2012: 7(4) e35956. doi: 10.1371/journal.pone.0035956

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Factum est illud: fieri infectum non potest

(PLAUTUS)