Trop Anim Health Prod (2017) 49:213–220
DOI 10.1007/s11250-016-1159-6

SHORT COMMUNICATIONS

Prospective study of avian influenza H9 infection in commercial poultry farms of Punjab Province and Islamabad Capital Territory, Pakistan

Mamoona Chaudhry[1] · **Maqbool Ahmad**[1] · **Hamad Bin Rashid**[2] · **Bakhat Sultan**[3] · **Haroon Rashid Chaudhry**[4] · **Aayesha Riaz**[5] · **Muhammad Shabir Shaheen**[6]

Received: 6 April 2016 / Accepted: 21 September 2016 / Published online: 20 October 2016

Abstract A prospective study was conducted from November 2013 to February 2014 to estimate the spatial clustering; cumulative incidence and risk factors associated with avian influenza (AI) subtype H9 infection on commercial poultry farms of Pakistan. A total of 400 farms were enrolled and followed during the study period. Among these, 109 farms submitted samples suspected for AI to the laboratory, and only 47 farms were confirmed positive by hemagglutinin inhibition (HI) test. Data was collected from these 109 farms about their demography, management, and biosecurity practices. The cumulative incidence of H9N2 was 11.75 % (95 % confidence interval (CI) 8.76–15.23). The highest number of cases (40.42 %) was reported in January. One most likely cluster ($p = 0.009$, radius = 4.61 km) occurred in the Kasur district. Multivariable logistic regression analyses showed that the presence of wild birds on the farms (odds ratio (OR) = 16.18; 95 % CI 3.94–66.45) was independently associated with H9N2 infection. Cleaning of cages before delivery on farm (OR = 0.16; 95 % CI = 0.06–0.47), presence of a footbath at the entrance of farm (OR = 0.24; 95 % CI 0.08–0.79), and changing of gloves (OR = 0.33; 95 % CI 0.11–0.99) were protective factors against H9N2 infection. Reducing the exposure to risk factors and adapting biosecurity measures may reduce the risk of AI H9N2 infection on commercial poultry farms in Pakistan.

Keywords Avian influenza · Attack rate · Prospective study · H9N2 serotype · Commercial poultry farms · Zoonosis

Abbreviations

AI	Avian influenza
AIVs	Avian influenza viruses
GIS	Geographic information system
HI	Hemagglutinin inhibition test
OR	Odds ratios
CI	Confidence intervals
RR	Relative risk
AR	Attack rate

Introduction

Influenza is a continuing threat to human and animal health. Every year, thousands of people are infected with seasonal influenza and may be exposed to subtypes of avian (H5, H6, H7, H9, and H10) and swine (H1 and H3) origin (García-Sastre and Schmolke 2014).

Avian influenza viruses (AIVs) of subtype H9N2 have spread widely since their first identification in turkeys in Wisconsin, USA, in 1966 (Homme and Easterday 1970).

✉ Mamoona Chaudhry
mamoona.chaudhry@gmail.com;
mamoona.chaudhry@uvas.edu.pk

[1] Department of Epidemiology and Public Health, University of Veterinary and Animal Sciences, Abdul Qadir Jilani Road, 54000 Lahore, Pakistan

[2] Department of Clinical Medicine and Surgery, University of Veterinary and Animal Sciences, Abdul Qadir Jilani Road, Lahore 54000, Pakistan

[3] Grand Parent Poultry Laboratory, 2 KM Bhobatia Chowk, Opposite to COMSAT University, 54000 Lahore, Pakistan

[4] College of Veterinary and Animal Sciences, Islamia University Bahawalpur, Hasilpur Road, Bahawalpur 63100, Pakistan

[5] Department of Pathobiology, Faculty of Veterinary and Animal Sciences, PMAS-Arid Agriculture University, Rawalpindi 46300, Pakistan

[6] Livestock and Dairy Development Department, Punjab, Pakistan

Table 1 Average mortality from AIV subtype H9 infection

S. no	Mortality on the farms (%)	No. of farms	Average (%) among total (47)
1	5–9 %	31	66.0
2	10–14 %	10	21.3
3	15 % and above	6	12.8

H9N2 viruses were isolated from pigs in 1998 and were subsequently isolated from humans with an influenza-like illness in both Hong Kong and Mainland China (Peiris et al. 1999). H9N2 are significantly important due to their extensive circulation in domestic poultry in different regions of world from the Far East to the Middle East (Fusaro et al. 2011). Genetic analysis of H9N2 viruses has showed extensive re-assortment of these viruses with many subtypes of AIVs including HPAI H5N1 and H7N3 viruses (Chaudhry et al. 2015; Fusaro et al. 2011).

In Pakistan, commercial poultry production has attained the shape of an industry in recent years with investment of billions of rupees. Since 1995, AIV subtypes H9, H7, and H5 are responsible for five massive epidemics in Pakistan affecting poultry and poultry products across the country (Naeem et al. 2007). Although H9N2 viruses are of low pathogenicity, the frequent heavy losses caused by them have raised serious concerns for the poultry industry in many countries.

Advancement has been made in disease investigations with new tools like geographic information system (GIS), which is used for spatiotemporal analysis of important emerging infections, e.g., severe respiratory syndrome (SARS), AIV H5N1, and influenza A (H1N1) (Tiensin et al. 2009; Martin et al. 2011; Lai et al. 2013). Disease clustering can be detected by using space-time scan statistics (Kulldorff et al. 2005).

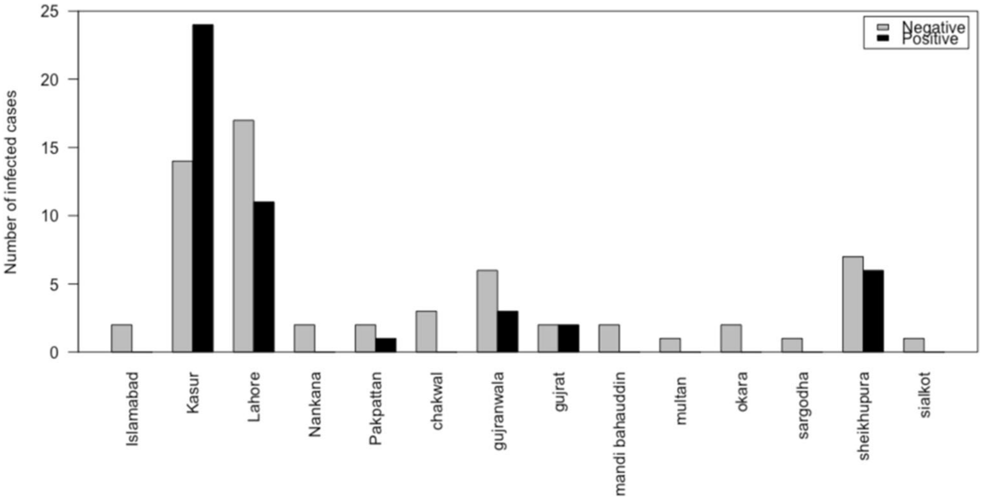
Few studies have examined the association of risk factors with AI on poultry farms in Pakistan (Abbas et al. 2012; Chaudhry et al. 2015). To date, very little information is available on spatial clustering of H9 infection in this region. Awareness about risk factors responsible for disease introduction and spatial clustering is critically important in developing risk-based surveillance strategies, policies, and timely recommendation for control. The primary objectives of this study were to calculate attack rate (AR) of H9N2 infection and to identify risk factors associated with this infection among poultry farms of Pakistan. The other objective was to identify any clustering of unusually high number of H9 cases than expected for early detection of any emerging outbreak of this disease in different areas of Pakistan when only the number of cases is available.

Materials and methods

A prospective study was conducted from November 2013 to February 2014. All commercial poultry farms of Pakistan raising domesticated poultry for sale were considered as the target population of study. The final study population was commercial poultry farms submitting samples for laboratory analysis to the collaborating private poultry laboratory for routine screening and suspected infections. Each commercial farm was taken as a sampling unit. All poultry farms, which were included in the study, were considered negative for H9 at the start of study due to the absence of any influenza or influenza-like illness in the flock. None of broiler flock was vaccinated against H9, H5, or H7. Breeder and layers were vaccinated against H9.

A total of 400 commercial poultry farms of different production categories (breeders, broiler, and layer farms) located in Punjab Province and Islamabad Capital Territory of Pakistan were enrolled in the study. Out of these 400 farms, only 109 farms submitted samples to laboratory for suspected infection with AIV, and a pretested questionnaire was filled from the owner/supervisor of these 109 farms in a face-to-face interview after explaining the objectives of study to the

Fig. 1 Attack rate of H9 infection in different districts of Pakistan



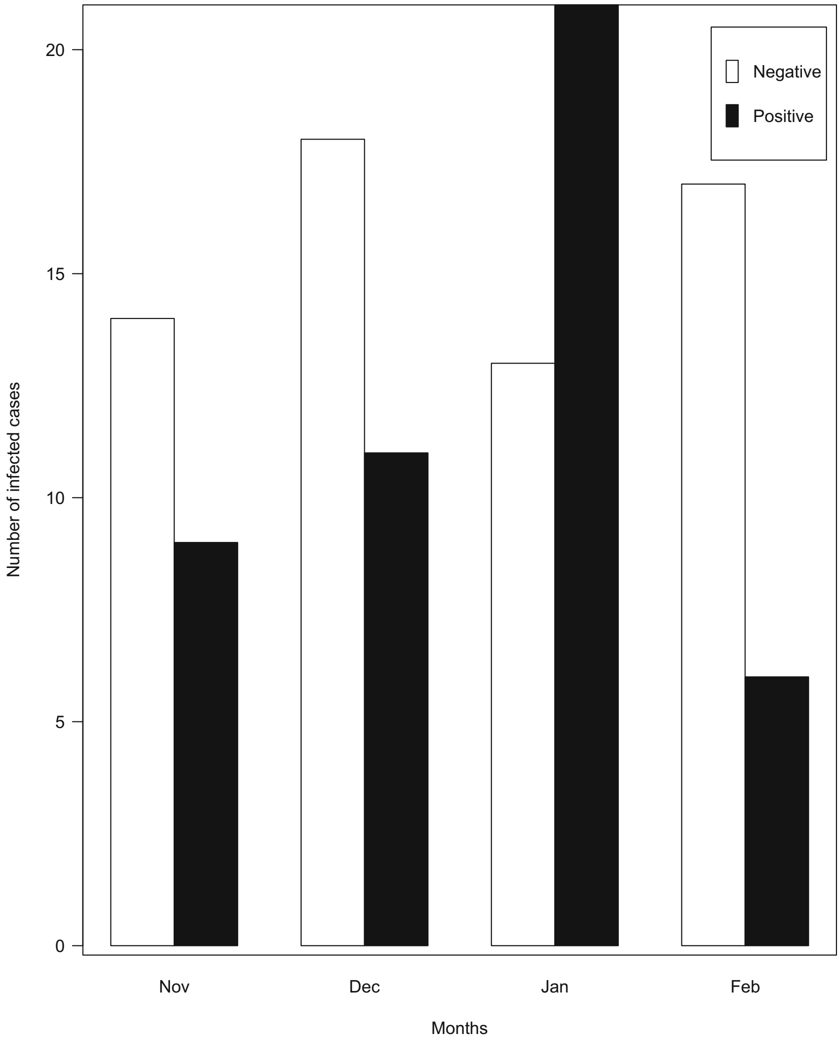


Fig. 2 Frequency of positive cases according to reporting month in the study

farmers. Prior to interview, written consent of the owner/attendant was obtained. The questionnaire contained 26 questions about risk factors, which were known to influence the disease occurrence and were selected after reviewing literature about AI (Nishiguchi et al. 1999; Ward et al. 2008; McQuiston et al. 2005; Fang et al. 2008; Woo and Park 2008; Abbas et al. 2012; Chaudhry et al. 2015; Nishiguchi et al. 2007) and from the observations of technical staff working on these farms.

The farmers were requested to provide five to ten dead birds from total mortality on farm, which were carefully examined by conducting postmortem examination for specific disease lesions. Typical pathological lesions in respiratory system, i.e., rhinitis, sinusitis, congestion, and inflammation in the trachea (Swayne 2008), were suspected for AIV. Confirmation was done by Anigen Rapid AIV Ag Detection Kit (BIONOTE Inc., Korea). The outcome of interest was H9 status, i.e., infected and non-infected farms. Samples confirmed by rapid test were further tested by virus isolation in embryonated chicken eggs, and subtyping was done by hemagglutinin inhibition (HI) test.

AR of H9N2 was calculated (Thrusfield 2007). All biologically plausible and relevant variables were screened in univariable analysis by using *glm* function of the *epicalc* package (version, 2.15.1.0) in R statistical software (available at http://www.R-project.org). A multivariable model was derived by forward stepwise selection procedure (Dohoo et al. 2003). Variables with significant univariable relationship at $P < 0.25$ were selected for inclusion in the final model. Odds ratios (ORs) and corresponding 95 % confidence intervals (CIs) were calculated (Hosmer and Lemeshow 2000).

Space-time scan statistic

All laboratory-confirmed cases of H9N2 between December 2013 to February 2014 were geocoded to street addresses. SatScan software version 9.1.1 developed by Martin Kulldorff, Havard Medical School (Boston, USA) and the Information Management Services Inc. (Maryland, USA) was used (available at http://www.satscan.org). The

Table 2 Univariable analysis of potential factors for AIV subtype H9 infection

Factors	Level of response	H9 +ve	H9 −ve	OR	95 % CI	*p* value
Wild birds on farm	Yes	44	36	10.59	2.96–37.86	0.000281[a]
	No	3	26			
Dropping removal	Before catching	5	47	1.0280	1.01–7.73	0.0476[a]
	After catching	42	15			
Farm fully fenced	Yes	3	17	0.18	0.05–0.66	0.0096[a]
	No	44	45			
Rubber boots channing	Yes	12	33	0.3	0.13–0.69	0.00432[b]
	No	35	29			
Clean cages before entering farm	Yes	9	31	0.24	0.1–0.57	0.00135[b]
	No	38	31			
Movement of workers within farms	No	34	55	3	1.09–8.28	0.0334[a]
	Yes	13	7			
Gloves changing	Yes	8	24	0.32	0.13–0.81	0.162
	No	39	38			
Vehicles entry into the farm	Yes	38	42	2.01	0.82–4.95	0.12[a]
	No	9	20			
Foot bath	Yes	40	44	0.43	0.16–1.13	0.014[b]
	No	7	18			
Share equipment	Yes	7	4	2.537	0.7–9.24	0.1580[a]
	No	40	58			
Waste disposal	Properly disposed	9	1	0.24	0.02–2.39	0.224[a]
	Not properly disposed	38	61			
Ventilation system	Fan	45	54	3.33	0.67–16.5	0.1401[a]
	Natural	2	8			

[a] Risk factors

[b] Protective factors

prospective space-time permutation scan statistic module was used to detect a local excess of events and to test if this excess could have occurred by chance (Hyder et al. 2011). This method consists of thousands of cylinders that move across space and/or time. Each cylinder has a base, which represents geographical area (in this study, a commercial farm), and height, which is time (in this study, a day). The base of each cylinder comprised a maximum of 50 % of the population, while height was a maximum of 50 % of the study time (60 days). The cylinder with more observed cases than expected, with respect to cases reported outside the cylinder, is called "most likely cluster." For each location and size of the cylinder, the number of observed and expected cases is counted. Among these, the most "unusual" excess of observed cases is noted. The statistical significance of this cluster is then evaluated taking into account the multiple testing stemming from the many potential cluster locations and sizes evaluated (Kulldorff et al. 2005). ArcGIS version 10 was used for the map display.

Results

During the study, we followed 400 commercial poultry farms of which 109 submitted samples. Among these 109 farms, 47

Table 3 Multivariable analysis of potential risk factors

S. no	Factors	Level of response	Positive	Negative	OR	95 % CI	*p* value
1	Wild birds entry into the farm	Yes	44	36	16.18	3.94–66.45	<0.001
		No	3	26			
2	Cleaning of cages before delivery	Yes	9	31	0.16	0.06–0.47	<0.001
		No	38	31			
3	Foot bath dipping area at the entrance	Yes	40	44	0.24	0.08–0.79	0.018
		No	7	18			
4	Worker change gloves	Yes	8	24	0.33	0.11–0.99	0.048
		No	39	38			

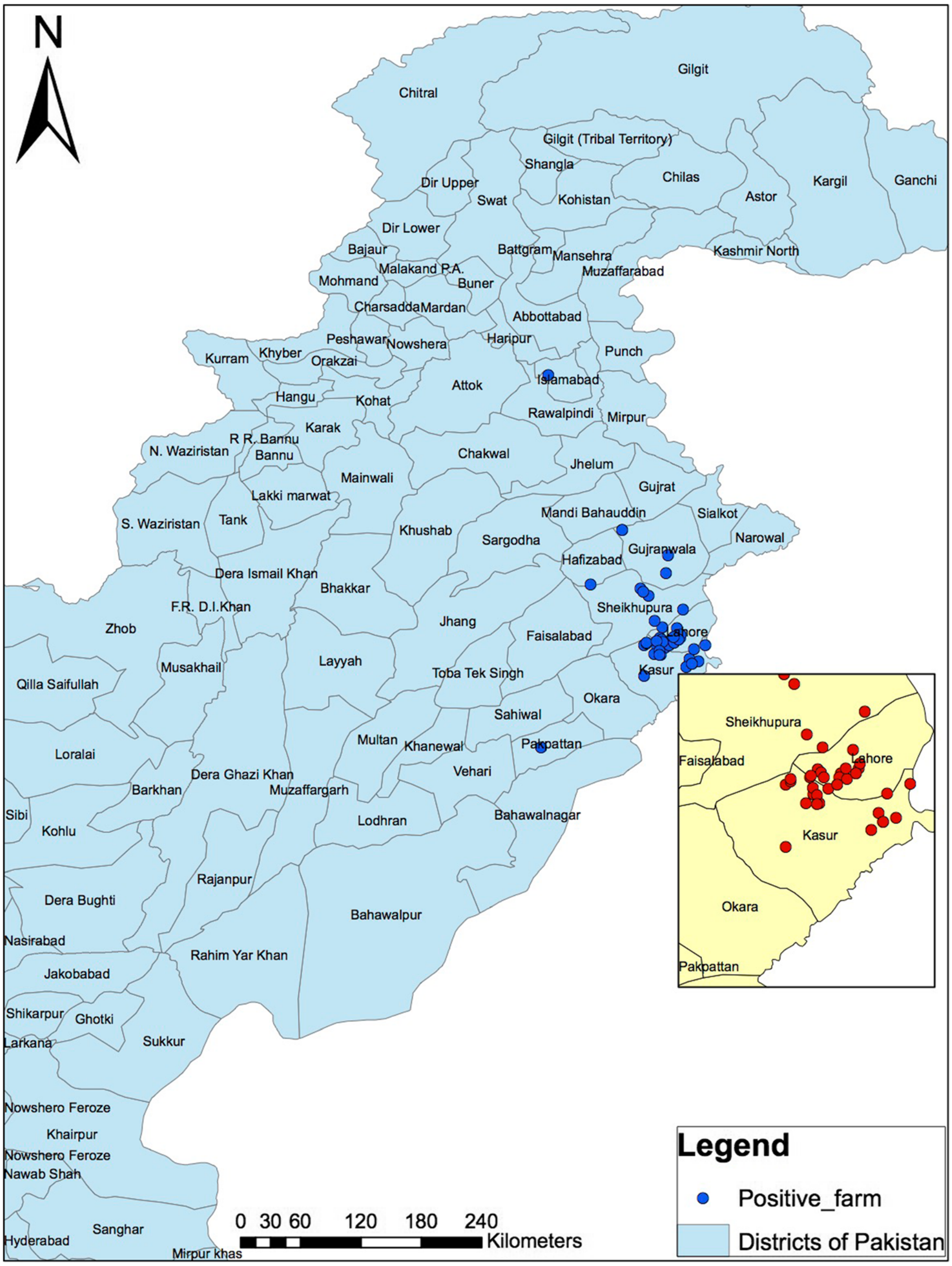


Fig. 3 Spatial distribution of positive poultry farms in different districts of Pakistan

poultry farms get infected with H9. The AR among total enrolled farms (47/400) was 11.75 % (95 % CI 8.76–15.23), while AR among total examined (47/109) was 43.10 % (95 % CI 34.20–52.50). Among the infected farms, majority (66 %) reported 5–9 % mortality due to H9 (Table 1).

The AR was highest in Lahore district (11/28) followed by Kasur (24/38) and Sheikhupura (6/13) districts (Fig. 1). No sample was positive for Newcastle disease virus, H5, and H7 AIVs.

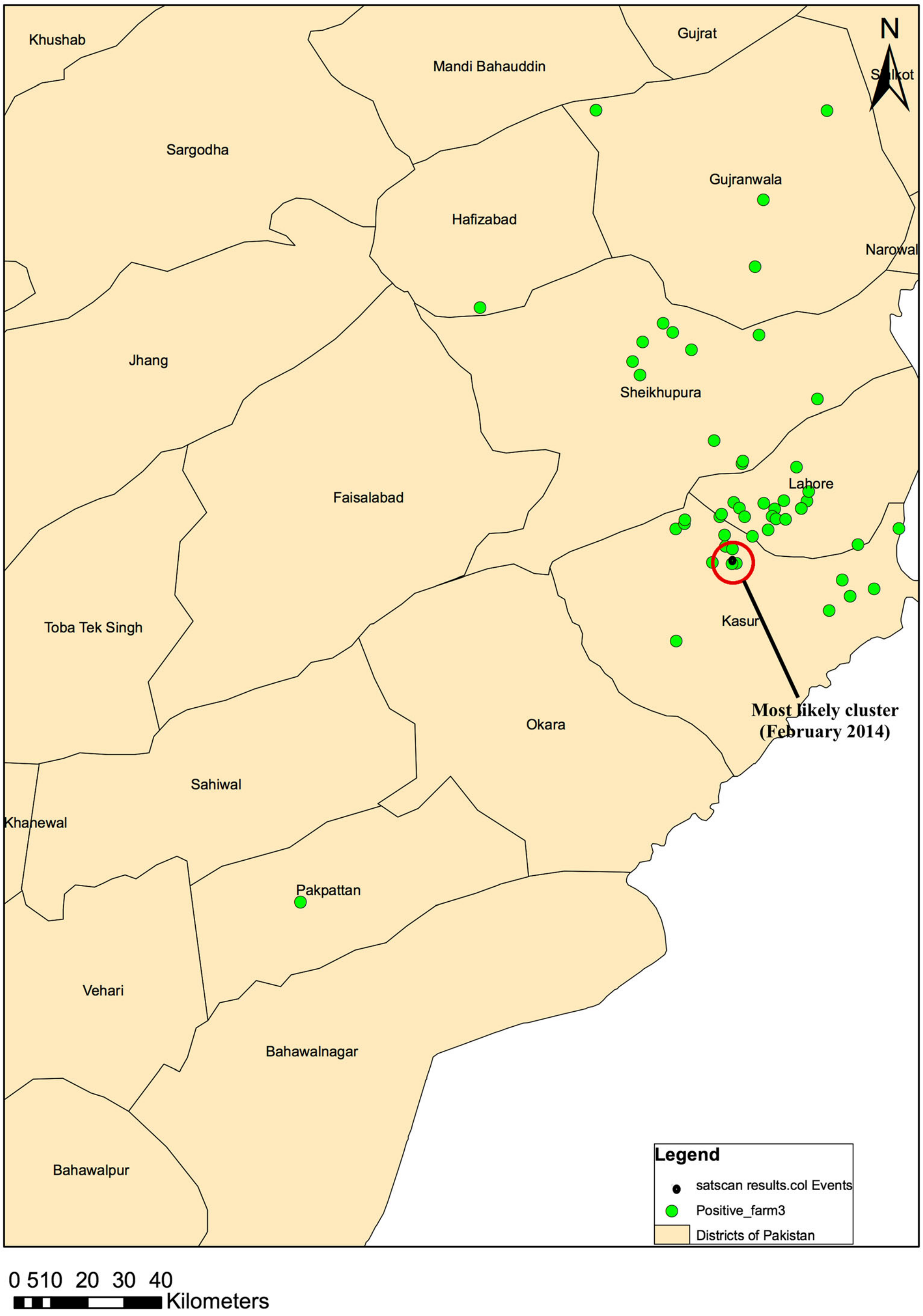
The study showed a high AR in the month of January (21/34) followed by December (11/29), November (9/23), and lowest incidence in February (6/23) (Fig. 2).

Risk factors identified during the study

Out of 26, 11 factors were selected for inclusion in final model of multivariable analysis (Table 2). Factors with $p > 0.25$ were excluded from further analysis.

In the final multivariable model, four factors were identified as significant (Table 3). Among those four factors, one factor was identified as risk factor (OR > 1), i.e., wild birds on the farm, and three factors were proved to be protective factor (OR < 1), namely cleaning of cages before entering the farm area, having foot bath/dipping area at the entrance of farm, and workers change gloves while entry into bird area.

Fig. 4 Spatiotemporal cluster of cases of H9N2 in Kasur district of Pakistan, November 2013 to February 2014



Spatiotemporal cluster analysis

Total examined farms in Punjab Province were 107, while 2 farms were examined from Islamabad Capital Territory (Fig. 3).

From November 1, 2013 to February 28, 2014, one most likely cluster ($p = 0.009$, radius = 4.61 km) occurred in the Kasur district of Punjab, Pakistan (Fig. 4). This signal had four cases observed over 25 days when 0.52 cases were expected [relative risk (RR) = 7.67], with a null occurrence rate of once every 111 days.

Discussion

Attack rate was highest in Lahore district (17/28) followed by Kasur (24/38) and Sheikhupura (6/13) districts. The reason for this high AR could be the high density of commercial poultry farms in these districts. Association of HPAI H5N1 and high and medium density of poultry farms has been studied and was identified as a risk factor in different countries (Henning et al. 2009; Abbas et al. 2012). Poultry farm densities in these regions ranged from 0.05 to 4 farms per square kilometer (Hamilton et al. 2009). As distance between the farms plays a significant role in the transmission of infection, poultry farms in these areas are exposed almost every year during the endemic (Abbas et al. 2012; Chaudhry et al. 2015). Furthermore, in densely populated poultry areas, movement of vehicles and people from farm to farm is considerably high, subsequently facilitating the spread of this virus through fomites. In the current study, the two districts (Lahore and Kasur) are on the main highway road (Grand Trunk road), which have a significant number of commercial poultry farms alongside. The vehicles over loaded with infected birds or mortality move on this road routinely. Previously, many studies have demonstrated that proximity to major roads was associated with avian influenza (Ward et al. 2008; Chaudhry et al. 2015). Movement of veterinarian and para-veterinary staff between different farms to implement control measures or to investigate mortalities can also contribute to the spread of virus among farms. In Pakistan, poultry farmers who apply strict biosecurity measures sometimes relax these rules for visitors, who enter the bird areas (Chaudhry et al. 2015).

Spatiotemporal analysis detected a most likely cluster in Kasur district ($p = 0.009$, radius = 4.61 km). Poultry farm in this cluster has a significantly higher risk of being infected as compared to poultry farms outside the cluster (RR = 7.67). These results represent the most important hotspot of expected outbreak and are valuable for improving knowledge and understanding of spatial pattern of H9N2 in specific areas. Targeted surveillance of these districts is needed for early detection of any future AI outbreak and their possible re-assortment. Spatial analyses have been used to study different outbreaks of human and avian influenza infections (Tiensin et al. 2009; Leveau et al. 2015).

Risk factor analyses showed that the presence of wild birds on farm could enhance the probability of infection (OR = 16.18; 95 % CI 3.94–66.45). Wild birds could serve as a potential source of propagation of AI virus especially when biosecurity measures are poorly implemented on farm. They can act both as mechanical and biological vectors (shedding the virus in droppings) and are important source of introducing virus to new areas (McQuiston et al. 2005; Henning et al. 2009; Chaudhry et al. 2015).

Cleaning of cages before delivery was strongly associated with decrease in risk of H9 (OR = 0.16, 95 % CI 0.06–0.47). Though statistically non-significant, cleaning of cages has been studied as a protective factor in lowering risk of H9 infection (Chaudhry et al. 2015). Poultry traders are well aware about the importance of cleaning cages and vehicles as an effective biosecurity measure (Kurscheid et al. 2015).

Presence of a footbath/dipping area at the entrance of farm and changing of gloves are also important as part of biosecurity measures on farm and have proved effective in decreasing risk of AI previously (Biswas et al. 2009; Chaudhry et al. 2015).

This study found evidence of clustering, in space and time, and identified some well-known factors mainly responsible for increasing risk of AIV infection. Enhancing good management practices and strict biosecurity can lower the risk of infection among poultry farms. Spatial clustering of disease provides information to health authorities to more effectively target and improve their surveillance and control strategies in affected areas.

Acknowledgments The authors are highly indebted to respondents of the commercial farms, who participated in study for data collection. We also acknowledge the support of staff of commercial poultry laboratory for providing us data from logbook about case farms, which were included in the study. Without their great cooperation and help, this study would not have been possible.

Compliance with ethical standards

Ethical approval This article does not contain any studies with animals performed by any of the authors.

Informed consent The manuscript does not contain clinical studies or patient data. The owners of the commercial farms were briefed about the objective of study, and informed consent was obtained from all individual participants included in the study to collect data.

Conflict of interest The authors declare that they have no conflict of interest.

References

Abbas, T., Wilking, H., Horeth-Bontgen, D., Conraths, F. J., 2012. Contact structure and potential risk factors for avian influenza transmission among open-sided chicken farms in Kamalia, an important poultry rearing area of Pakistan. Berliner und Münchener tierärztliche Wochenschrift, 125, 110–116

Biswas, P. K., Christensen, J. P., Ahmed, S., Barua, H., Das, A., Rahman, M., Giasuddin, M., Hannan, A., Habib, A., Debnath, N., 2009. Risk factors for infection with highly pathogenic influenza A virus (H5N1) in commercial chickens in Bangladesh. Veterinary Record, 164

Chaudhry, M., Rashid, H. B., Thrusfield, M., Welburn, S., Bronsvoort, M. B., 2015. A case-control study to identify risk factors associated with avian influenza subtype H9N2 on commercial poultry farms in Pakistan. PLoS ONE, 10(3), e0119019. doi:10.1371/journal.pone.0119019

Dohoo, I., Martin, W., Stryhn, H., 2003. Veterinary epidemiologic research (AVC Inc, Charlottetown, Prince Edward Island, Canada)

Fang, L., de Vlas, S.J., Liang, S., Looman, C.W.N., Gong, P., Xu, B., Yan, L., Yang, H., Richardus, J. H., Cao, W., 2008. Environmental factors contributing to the spread of H5N1 avian influenza in Mainland

China. PLoS One, 3, http://dx.doi.org/10.1371/journal.pone.0002268

Fusaro, A., Monne, I., Salviato, A., Valastro, V., Schivo, A., Amarin, N. M., Gonzalez, C., Ismail, M. M., Al-Ankari, A. R., Al-Blowi, M. H., Khan, O. A., Maken Ali, A. S., Hedayati, A., Garcia Garcia, J., Ziay, G. M., Shoushtari, A., Al Qahtani, K. N., Capua, I., Holmes, E. C., Cattoli, G., 2011. Phylogeography and evolutionary history of reassortant H9N2 viruses with potential human health implications. J Virol, 85(16), 8413-8421.

García-Sastre, A., Schmolke, M., 2014. Avian influenza A H10N8?a virus on the verge? Lancet 2014; 383: 676–677.

Hamilton, S. A., East, I. J., Toribio, J. A., Garner, M. G., 2009. Are the Australian poultry industries vulnerable to large outbreaks of highly pathogenic avian influenza? Australian Veterinary Journal, 87, 165–174

Henning, K. A., Henning, J., Morton, J., Long, N. T., Ha, N. T., Meers, J., 2009. Farm-and flock-level risk factors associated with highly pathogenic avian influenza outbreaks on small-holder duck and chicken farms in the Mekong Delta of Viet Nam. Preventive Veterinary Medicine, 91, 179-188

Homme, P. J., Easterday, B. C., 1970. Avian influenza virus infections: I. Characteristics of influenza A-turkey-Wisconsin-1966 virus. Avian Diseases, 14, 66–74

Hosmer, D. W., Lemeshow, S., 2000. Applied logistic regression (John Wiley & Sons, Inc, New York)

Hyder, K., Vidal-Diez, A., Lawes, J., Sayers, A. R., Milnes, A., Hoinville, L., Cook, A. J. C., 2011. Use of spatiotemporal analysis of laboratory submission data to identify potential outbreaks of new or emerging diseases in cattle in Great Britain. BMC Veterinary Research, 7, 14

Kulldorff, M., Heffernan, R., Hartman, J., Assunção, R., Mostashari, F., 2005. A space-time permutation scan statistics for disease outbreak detection. PloS Medicine, 2 (3), e59

Kurscheid, J., Millar, J., Abdurrahman, M., Ambarawati, I. G. A. A., Suadnya, W., Yusuf, R. P., Fenwick, S., Toribio, J. A., 2015. Knowledge and perceptions of highly pathogenic avian influenza (HPAI) among poultry traders in live bird markets in Bali and Lombok, Indonesia. PLoS ONE, 10(10), e0139917. doi:10.1371/journal.pone.0139917

Lai, P. C., Kwong, K. H., Wong, H. T., 2013. Spatio-temporal and stochastic modeling of the severe acute respiratory syndrome (SARS). Geospatial Health, 8(1),183-92

Leveau, C., Uez, O., Vacchino, M. N., 2015. Spatiotemporal trends of cases of pandemic influenza A (H1N1)pdm09 in Argentina, 2009-2012. Revista do Instituto de Medicina Tropical de São Paulo, 57(2), 133-138. doi:10.1590/S0036-46652015000200006

Martin, V., Pfeiffer, D. U., Zhou, X., Xiao, X., Prosser, D. J., Guo, F., Gilbert, M., 2011. Spatial distribution and risk factors of highly pathogenic avian influenza (HPAI) H5N1 in China. PLoS Pathogen, 7(3), e1001308

McQuiston, J. H., Garber, L. P., Porter-Spalding, B. A., Hahn, J. W., Pierson, F. W., Wainwright, S. H., Senne, D. A., Brignole, T. J., Akey, B. L., Holt, T. J., 2005. Evaluation of risk factors for the spread of low pathogenicity H7N2 avian influenza virus among commercial poultry farms. Journal of American Veterinary Medicine Association, 226, 767-772

Naeem, K., Siddique, N., Ayaz, M., Jalalee, M. A., 2007. Avian influenza in Pakistan: outbreaks of low- and high-pathogenicity avian influenza in Pakistan during 2003-2006. Avian diseases, 51, 189-193

Nishiguchi, A., Kobayashi, S., Yamamoto, T., Ouchi, Y., Sugizaki, T., Tsutsui, T., 2007. Risk factors for the introduction of avian influenza virus into commercial layer chicken farms during the outbreaks caused by a low-pathogenic H5N2 virus in Japan in 2005. Zoonoses Public Health, 54, 337–343, doi: 10.1111/j.1863-2378.2007.01074

Peiris, M., Yuen, K. Y., Leung, C. W., Chan, K. H., Ip, P. L., Lai, R. W., Orr, W. K., Shortridge, K. F., 1999. Human infection with influenza H9N2. Lancet, 354(9182), 916-917

Swayne, D. E. 2008. Avian influenza. In: Foreign animal diseases, (Boca Raton, FL: United States Animal Health Association), 137-146.

Thrusfield, M., 2007. Veterinary Epidemiology (Oxford, Blackwell Science. UK)

Tiensin, T., Ahmed, S.S., Rojanasthien, S., Songserm, T., Ratanakorn, P., Chaichoun, K., Kalpravidh, W., Wongkasemjit, S., Patchimasiri, T., Chanachai, K., Thanapongtham, W., Chotinan, S., Stegeman, A., Nielen, M., 2009. Ecologic risk factor investigation of clusters of avian influenza A (H5N1) virus infection in Thailand. Journal of Infectious Diseaes, 199, 1735–1743

Ward, M. P., Maftei, D., Apostu, C., Suru, A., 2008. Environmental and anthropogenic risk factors for highly pathogenic avian influenza subtype H5N1 outbreaks in Romania, 2005–2006. Veterinary Research Communication, 32, 627–634. doi: 10.1007/s11259-008-9064-8

Woo, J. T., Park, B. K., 2008. Seroprevalence of low pathogenic avian influenza (H9N2) and associated risk factors in the Gyeonggi-do of Korea during 2005–2006. Journal of Veterinary Science, 9, 161–168. doi: 10.4142/jvs.2008.9.2.161