Suitability of specimen types for isolation of avian influenza viruses from poultry

Sandeep D. Kale · Akhilesh C. Mishra ·
Shailesh D. Pawar

Received: 22 February 2013 / Accepted: 25 June 2013 / Published online: 20 August 2013
© Indian Virological Society 2013

Abstract In view of the outbreaks of the highly pathogenic avian influenza (HPAI) H5N1 virus in poultry in India, its impact on global public health and growing concerns of avian influenza (AI) viruses, surveys in wet poultry markets were conducted in the states of Maharashtra, West Bengal and Jharkhand in India during the period 2009–2012. During these surveys various types of samples from poultry were collected. During outbreaks and surveys in poultry, tracheal swabs (TS), cloacal swabs (CS), poultry drinking water (PDW) samples and fecal samples (FS) are preferred samples for AI diagnosis. The suitability of various types of poultry samples for AI virus isolation was analyzed. The parameters such as availability of specimen, ease of collection, quality of the specimen for the presence of contaminants such as organic debris or solid matter were considered for the analysis. A total of 2,405 samples were collected, which included 1,297 TS, 1,012 CS, 79 PDW, and 17 FS. Out of 2,309 TS and CS samples 1,752 samples were paired samples, collected from 876 birds. All samples were processed for virus isolation and identification. Of the 2,405 samples AI H9N2 was isolated from 199 samples (8.27 %). The virus isolation rate was significantly higher in PDW samples (21.5 %) ($P < 0.05$) and TS samples (12.1 %), in comparison with CS (2.3 %) ($P < 0.001$). Other viruses isolated were AI H4N6 and HPAI H5N1 viruses; however the number of isolates of AI H4N6 and H5N1 were not sufficient for comparison. In conclusion, the PDW and TS samples were suitable for AI H9N2 virus isolation from poultry.

Keywords Avian influenza virus · Tracheal swab · Cloacal swab · H9N2 · India

In view of the outbreaks of highly pathogenic avian influenza (HPAI) H5N1 in poultry, its impact on global public health and growing concerns of avian influenza (AI) viruses, surveys were conducted in wet poultry markets in the states of Maharashtra, West Bengal and Jharkhand in India during the period 2009–2012 [1, 6] (<http://www.fao.org/docrep/015/an388e/an388e.pdf>). The AI surveillance was performed in Maharashtra, West Bengal and Jharkhand states because these states reported outbreaks of HPAI H5N1 viruses and in the current scenario of emerging AI viruses in South-East Asia, AI surveillance studies are necessary to monitor AI viruses. During these surveys, tracheal swabs (TS), cloacal swabs (CS), fecal samples (FS) and poultry drinking water (PDW) samples were collected for diagnosis of AI viruses. AI viruses are transmitted primarily by the fecal–oral route. During AI surveillance, TS, CS, FS and PDW samples are collected for AI diagnosis (http://whqlibdoc.who.int/hq/2002/WHO_CDS_CSR_NCS_2002.5.pdf). Collection and processing of all types of samples is labour intensive, time consuming and expensive. Therefore, it is necessary to determine suitability of samples for diagnosis of AI viruses. With this background, the present study was undertaken to determine the suitability of various types of poultry samples for AI virus isolation. The parameters such as availability of the specimen, ease of collection, quality of specimen for the presence of contaminants such as organic debris or solid matter were considered for the analysis.

A total of 2,405 samples were collected from poultry, which included 1,297 TS, 1,012 CS, 79 PDW, and 17 FS. Out of 2,309 TS and CS samples 1,752 samples were paired samples, collected from 876 birds. PDW samples were

S. D. Kale · A. C. Mishra · S. D. Pawar (✉)
National Institute of Virology, Microbial Containment Complex,
130/1, Sus Road, Pashan, Pune 411021, India
e-mail: pawarshailesh@hotmail.com; pawar.sd@niv.co.in

collected from the drinking water containers of birds kept inside the cages and only fresh and wet FS were collected. Samples were collected using sterile swabs (HiMedia Labs) in 1 ml of viral transport medium (VTM) containing antibiotics, penicillin G (2×10^4 U/ml), gentamicin (2.5 mg/ml), streptomycin (2 mg/ml) and nystatin (0.5×10^3 U/ml). Samples collected were sealed, packed and transported immediately to the laboratory while maintaining the cold chain and stored at -80°C until testing.

The samples were processed in Biosafety level -2 and -3 (BSL-2 and BSL-3) laboratories as per biosafety guidelines for handling AI viruses. All samples were processed for virus isolation in 10-day-old embryonated chicken clean eggs from antibody negative flocks (Venkateshwara Hatcheries Pvt. Limited, Pune, India). Processing of the samples was carried out in BSL-2 and -3 laboratory as required. Hemagglutination (HA) positive samples were identified by hemagglutination inhibition (HI) assay using a panel of reference antisera (H1–H16) (OIE/FAO National Reference Laboratory for AI and Newcastle disease, Legnaro, Italy). Neuraminidase (NA) subtyping was done by partial sequencing of NA gene [6] (http://whqlibdoc.who.int/hq/2002/WHO_CDS_CSR_NCS_2002.5.pdf).

Of the 2,405 samples, AI H9N2 was isolated from 199 samples (8.27 %). Virus isolation rate was significantly higher in PDW samples (21.5 %) ($P < 0.05$) and TS samples (12.1 %), in comparison with CS (2.3 %) ($P < 0.001$) (Table 1). The number of FS was not adequate for comparison. AI H4N6 and HPAI H5N1 viruses were also isolated from different types of samples. AI H4N6 virus (number of isolates = 01) was isolated from only CS; whereas HPAI H5N1 (number of isolates = 10) virus isolates were obtained from TS, CS and PDW samples. The numbers of isolates of AI H4N6 and HPAI H5N1 viruses were low for sample wise comparison.

Collection of environmental samples (PDW and FS) is useful to study the presence of the virus in birds in groups. The higher rates of AI virus isolation were found in PDW samples. These findings are similar to the findings by Leung et al. [5]. The PDW is a convenient, non-invasive sample in wet poultry markets. Advantage of PDW sample is that one need not screen individual bird from the flock as the water

sample represents the whole flock. Persistence of AI viruses in water under different conditions of temperature and pH has been studied. AI virus viability in the environment depends on the cumulative effects of chemical and physical factors, such as humidity, temperature, pH, salinity, and organic compounds, as well as differences in the virus itself [4, 7]. It has been shown that AI viruses remain viable for an extended period of time in water [8]. However the PDW sample limits its use to understand the virus distribution in individual bird. Therefore sampling of TS or CS has been shown to be useful to understand the extent of distribution of AI viruses in individual birds, which is also helpful to understand the viral load in the poultry.

During this study, very few FS were collected because the present study was conducted in poultry, where individual birds were available for sampling. FS are acidic in pH due to presence of uric acid and viability of AI virus has been shown to be reduced with acidic pH [2, 7]. In case of TS and CS, advantage of use of TS over CS was that TS did not contain large amounts of contaminants like organic debris or solid matter which were present in CS. This could be one of the possible reasons of better virus isolation rates of AI H9N2 virus in TS than CS samples. Krauss et al. [3] suggested that AI H9N2 viruses are shed primarily through the respiratory tract and may not be detected in CS. The findings of the present study are in agreement with this study showing higher rates of AI H9N2 virus isolation in TS than CS.

The predominant virus isolated was AI H9N2. The other AI viruses including HPAI H5N1 may have different shedding patterns, which need further studies. In conclusion PDW and TS were suitable over CS and FS for AI H9N2 virus isolation from poultry.

Acknowledgments The authors thank MR Khude, SK Waghmare, JPN Babu and P. Shinde for their support in field and laboratory work. We thank the Dept. of Animal Husbandry, Ministry of Agriculture, and Government of India for support, the Indian Council of Medical Research, New Delhi, India for intramural funds.

References

1. Chakrabarti AK, Pawar SD, Cherian SS, Koratkar SS, Jadhav SM, Pal B, Raut S, Thite V, Kode SS, Keng SS, Payyapilly BJ, Mullick J, Mishra AC. Characterization of the Influenza A H5N1 Viruses of the 2008–09 outbreaks in India reveals a third introduction and possible endemicity. *PLoS ONE*. 2009;4:e7846.
2. Davidson I, Nagar S, Haddas R, Ben-Shabat M, Golender N, Lapin E, Altory A, Simanov L, Ribshtein I, Panshin A, Perk S. Avian influenza virus H9N2 survival at different temperatures and pHs. *Avian Dis*. 2010;54:725–8.
3. Krauss S, Pryor SP, Raven G, Danner A, Kayali G, Webby RJ, Webster RG. Respiratory tract versus cloacal sampling of migratory ducks for influenza A viruses: are both ends relevant? *Influenza Other Respir Viruses*. 2013;7(1):93–6.

Table 1 Number of AI H9N2 virus isolates obtained and percent positivity in different types of specimens from poultry

Sr. No.	Specimen types	No. of samples	No. of virus isolates (%)
1	Tracheal swabs	1,297	157 (12.1)
2	Cloacal swabs	1,012	23 (2.3)
3	Poultry drinking water	79	17 (21.5)
4	Fecal samples	17	02 (11.8)
	Total	2,405	199 (8.27)

4. Lebarbenchon C, Yang M, Keeler SP, Ramakrishnan MA, Brown JD, Stallknecht DE, Sreevatsan S. Viral replication, persistence in water and genetic characterization of two influenza viruses isolated from surface lake water. *PLoS ONE*. 2011;6(10):e26566.
5. Leung YH, Zhang LJ, Chow CK, Tsang CL, Ng CF, Wong CK, Guan Y, Peiris JS. Poultry drinking water used for avian influenza surveillance. *Emerg Infect Dis*. 2007;9:1380–2.
6. Pawar SD, Kale SD, Rawankar AS, Koratkar SS, Raut CG, Pande SA, Mullick J, Mishra AC. Avian influenza surveillance reveals presence of low pathogenic avian influenza viruses in poultry during 2009–2011 in the West Bengal State. *India Virol J*. 2012;9:151.
7. Shahid MA, Abubakar M, Hameed S, Hassan S. Avian influenza virus (H5N1); effects of physico-chemical factors on its survival. *Virol J*. 2009;6:38.
8. Stallknecht DE, Shane SM, Kearney MT, Zwank PJ. Persistence of avian influenza viruses in water. *Avian Dis*. 1990;34(2):406–11.