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Correspondence

Seroepidemiology of avian influenza H5N1, H9N2 & Newcastle disease viruses during 1954 to 1981 in India

Sir,

Avian influenza (AI) viruses are some of the most important viruses prevalent in water birds[1]. The higher (~15%) AI infection rates have been reported in waterfowl, particularly in families *Anatidae*, *Gruidae*, *Phalacrocoracidae* and *Pelecanidae* as compared to terrestrial species (~2%)[2]. Influenza viruses belong to the family *Orthomyxoviridae* and are divided in 18 haemagglutinin (HA) and 11 neuraminidase (NA) subtypes based on genetic and antigenic properties. At least 103 of the possible 198 Type A influenza virus, HA-NA combinations have been found in wild birds[3]. Influenza pandemics occurred in 1918, 1957, 1968, 1977 and 2009 due to the major antigenic variation of the Type A influenza viruses[4]. AI viruses are broadly classified as low pathogenic AI and highly pathogenic AI (HPAI) viruses, based on their pathogenicity[5]. The H5N1 influenza virus was first isolated in 1996 from geese in the Guangdong province of China[6]. In India, the outbreaks of HPAI H5N1 virus were first reported in February 2006 in poultry at Navapur, Maharashtra and then 104 outbreaks have been reported in poultry and in wild and migratory birds in India from 2006 to 2016[7,8].

The National Institute of Virology (NIV), Pune, India, conducted avian surveys in India during the years 1954 to 1981 to study the role of wild and migratory birds in transmission of arboviruses in different States of India. During these surveys serum samples were collected from various bird species. These serum samples were stored at −20°C at the repository of NIV, for storage of archived samples. In view of the emergence of influenza viruses globally, the present exploratory study was undertaken at the NIV, Pune, during July 2013-March 2015, to study retrospective seroprevalence of AI H5N1 and H9N2 viruses in India using the archived avian serum samples collected earlier (1954-1981).

For determination of sample size, an assumption of less than five per cent antibody prevalence of H5N1 and H9N2 and Newcastle disease virus (NDV) was made. The sample size calculations were performed using online (OpenEpi) software, Centers for Disease Control and Prevention, USA[9]. The estimated sample size was 500 assuming five per cent prevalence, 95 per cent confidence interval and precision of 0.02 per cent by two-sided test with finite population correction with a population size of 5000 for the study area. A total of 557 representatives archived avian serum samples from 41 species from 15 avian families of wild, migratory and resident; water birds, water frequenting and terrestrial birds were selected for the study. These samples were from seven States, namely, Maharashtra, Karnataka, West Bengal, Jharkhand (formerly Bihar), Andhra Pradesh, Tamil Nadu and Rajasthan (Table).

These samples were tested against A/chicken/India/NIV33487/06-RG-2008 (H5N1), A/Turkey/Wisconsin/66 (H9N2) and NDV antigens obtained from the OIE/FAO National Reference Laboratory for AI and Newcastle disease obtained from Legnaro, Italy. The AI H5N1 and H9N2 viruses have been reported to be prevalent in avian species and poultry and resident birds in India[7,10].

These samples were tested by haemagglutination inhibition (HI) and microneutralization (MN) assays for detection of antibodies against AI H5N1 and H9N2 viruses[11,12]. Only HI assay was employed for the detection of antibodies against NDV. For HI assay, all serum samples were treated with receptor-destroying enzyme (RDE) (Denka Seiken, Japan) to remove non-specific inhibitors. Serum samples showing the presence of agglutinins were treated with horse and turkey red blood cells (RBCs) to remove non-specific agglutinins. One volume of packed RBCs was mixed with 20 volumes of RDE-treated serum and incubated

Table. Details of the archived avian serum samples from wild resident and migratory birds screened for avian influenza

Serial number	Family	Common name (scientific name)	State	Year of collection	Status
1	*Phasianidae*	Grey junglefowl (*Gallus sonneratii*)	Karnataka	1959	Resident
		Red spurfowl (*Galloperdix spadicea*)	Karnataka, Tamil Nadu	1967	Resident
2	*Anatidae*	Garganey or Blue-winged teal (*Anas querquedula*)	Andhra Pradesh	1974-1975	Wild migratory
		Common teal (*Anas crecca*)	Karnataka	1981	Wild migratory
		Cotton Pygmy-goose (*Nettapus coromadelianus*)	Andhra Pradesh	1957-1962	Resident, local migratory
		Bar-headed goose (*Anser indicus*)	Rajasthan	1969-1973	Wild migratory
		Northern shoveller (*Anas clypeata*)	Rajasthan	1969	Wild migratory
		Spot-billed duck (*Anas poecilorhyncha*)	Rajasthan	1969	Resident
		Lesser whistling duck (*Dendrocygna javanica*)	Karnataka	1959-1975	Resident, local migratory
		Domestic duck (*Anas platyrhynchos*)	Karnataka	1956-1980	Resident
		Duck	Andhra Pradesh	1975-1980	Not recorded
		Duckling juvenile	West Bengal	1973	
3	*Alcedinidae*	Common kingfisher (*Alcedo atthis*)	Karnataka	1960	Resident
4	*Alcedinidae*	White-throated kingfisher (*Halcyon smyrnensis*)	Karnataka	1981	Resident
5	*Rallidae*	Common coot (*Fulica atra*)	Andhra Pradesh	1961-1975	Resident, wild migratory
		Common moorhen (*Gallinula chloropus*)	Andhra Pradesh	1957-1975	Resident
		Purple swamphen (*Porphyrio porphyrio*)	Andhra Pradesh	1959-1975	Resident
		White-breasted water hen (*Amaurornis phoenicurus*)	Karnataka	1959-1982	Resident
6	*Scolopacidae*	Common sandpiper (*Actitis hypoleucos*)	Karnataka	1960	Wild migratory
		Green sandpiper (*Tringa ochropus*)	Karnataka	1981	Wild migratory
		Spotted sandpiper (*Tringa glareola*)	Karnataka	1981	Wild migratory
		Common greenshank (*Tringa nebularia*)	Andhra Pradesh and Karnataka	1957, 1981	Wild migratory
		Common or Fantail snipe (*Gallinago gallinago*)	Karnataka	1965, 1981	Wild migratory
		Ruff and Reeve (*Philomachus pugnax*)	Rajasthan	1975-1981	Wild migratory
7	*Rostratulidae*	Painted-Snipe (*Rostratula benghalensis*)	Andhra Pradesh	1975	Resident
8	*Jacanidae*	Bronze-winged jacana (*Metopidius indicus*)	Andhra Pradesh	1959-1961	Resident
		Pheasant-tailed jacana (*Hydrophasianus chirurgus*)	Andhra Pradesh	1974-1975	Resident
9	*Laridae*	Brown-headed gull (*Larus brunnicephalus*)	Karnataka	1956-1957	Wild migratory
10	*Recurvirostridae*	Black-winged stilt (*Himantopus himantopus*)	Karnataka	1981	Wild migratory

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Serial number	Family	Common name (scientific name)	State	Year of collection	Status
11	*Phalacrocoracidae*	Cormorant sp.	West Bengal	1973	Resident
12	*Ardeidae*	Grey heron (*Ardea cinerea*)	Andhra Pradesh and Karnataka	1956, 1981	Resident, local migratory, wild migratory
		Cattle egret (*Bubulcus ibis*)	Maharashtra	1954-1976	Resident, local migratory
		Large egret (*Casmerodius albus*)	Tamil Nadu	1957	Resident, local migratory
		Median egret (*Mesophoyx intermedia*)	Maharashtra	1955	Resident, local migratory
		Little egret (*Egretta garzetta*)	Karnataka, Andhra Pradesh and West Bengal	1956, 1973, 1981	Resident
		Black-crowned Night heron (*Nycticorax nycticorax*)	Andhra Pradesh	1956	Resident, local migratory
		Indian pond heron (*Ardeola grayii*)	Karnataka, Andhra Pradesh, Tamil Nadu	1955-1957, 1981	Resident, local migratory
13	*Ciconiidae*	Painted stork (*Mycteria leucocephala*)	Rajasthan	1972	Resident, local migratory, wild migratory
14	*Corvidae*	House crow (*Corvus splendens*)	Jharkhand	1954	Resident
		Jungle crow (*Corvus culminatus*)	Tamil Nadu, Karnataka, Maharashtra and West Bengal	1955-1957	Resident
15	*Hirundinidae*	Common swallow (*Hirundo rustica*)	Karnataka	1981	Wild migratory

at 37°C for one hour, centrifuged at 120x*g* for 10 min. Adsorbed serum was carefully removed without disturbing packed cells and used in the HI assay. HI assays were performed using 0.5 per cent turkey and one per cent horse red blood RBCs. The reference serum samples from OIE were used as positive control in both HI and MN assays.

The MN assay was used to detect the presence of neutralizing antibodies against AI viruses[13]. The MN assays were performed using Madin–Darby canine kidney (MDCK) cells maintained in Dulbecco's modified Eagles' medium (DMEM) containing 10 per cent foetal bovine serum (Gibco, USA), 2 mM L-glutamine and the antibiotics penicillin (100 U/ml) and streptomycin (100 µg/ml). The 50 per cent tissue culture infectious dose ($TCID_{50}$) of H5N1 and H9N2 viruses was determined. Half-log dilutions of virus were carried out in 96-well polystyrene immunoassay plates (Nunc, Denmark) and mixed with 100 µl of 1.5×10^5/ml MDCK cells and incubated for 18-22 h at 37°C and five per cent CO_2. Enzyme-linked immunosorbent assay (ELISA) was performed using influenza A-specific anti-nucleoprotein monoclonal antibodies (Millipore, USA). The $TCID_{50}$ was calculated as per Reed and Muench method[12]. For MN assay, RDE treated serum samples were serially two-fold diluted and mixed with an equal volume of influenza virus diluted at 100 $TCID_{50}$/50 µl. After incubating for one hour at 37°C with five per cent CO_2, it was mixed with 100 µl of MDCK cells at 1.5×10^5/ml. The plates were incubated for 18-22 h at 37°C and five per cent CO_2. The monolayers were washed with phosphate-buffered saline and fixed in cold 80 per cent acetone for 10 min. ELISA was performed as mentioned above. The titres of the antibodies by both HI and MN assays were expressed as reciprocals of the highest antibody dilution showing haemagglutination inhibition and virus neutralization, respectively.

All samples were negative for the presence of antibodies against AI H5N1 and H9N2 viruses. There are reports of AI H5N1 virus since 1959[14] and AI H9N2 virus was first reported in 1982 from domestic poultry in China[15]. AI H5N1 and H9N2 were first reported from India during 2006 and 2003, respectively[7,16]. The present study indicated that H5N1 and H9N2 AI viruses did not circulate in the studied avian population during the period 1954 to 1981. Thus, the emergence of these viruses in avian population in India may be recent.

Two samples isolated from Jungle crow (*Corvus macrorhynchos*) from Maharashtra and one sample from wild duck (species not identified) from Andhra Pradesh collected during 1955-1956 and 1975, respectively, were positive for the presence of antibodies against NDV. The antibody titres of three positive samples from crow and duck were 80, 40 and 20 by HI assay. NDV was first isolated during 1927 from England, but it is said to have been prevalent much earlier than that[17,18]. The seropositivity of NDV indicates the prevalence of this virus in the past. In a previous study from the NIV, conducted in 1980-1981, cloacal swabs from birds were negative for AI virus isolation and NDV was isolated from a chicken[19].

The HPAI H5N1 viruses cause high mortality in poultry. However, some species of wild birds survive HPAI H5N1 infection and show antibody response[20,21]. Therefore, the possibility of the absence of antibodies in the studied avian species due to quick mortality in infected birds by HPAI H5N1 viruses could be ruled out as the serum samples used in the study were from wild bird species. There have been retrospective studies on viral diseases, wherein archived serum samples have been tested for antibodies[22]. The samples used in the present study were stored at −20°C for 34-61 yr. The presence of antibodies against NDV in stored serum samples indicates that the long-term storage of samples did not affect the integrity of the antibodies. However, the protein degradation that might have taken place during the long storage could not be assessed in this study. The samples for virus isolation were not available. The findings of this study cannot be generalized, as the studied samples do not represent the complete geographical areas in India.

In conclusion, AI H5N1 and H9N2 viruses did not circulate in the studied avian population during 1954-1981, and emergence of these viruses in birds in India is probably recent. The seropositivity of NDV indicates the prevalence of this virus in the past.

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***Conflicts of Interest*:** None.

Shailesh D. Pawar*, Aniruddha V. Jamgaonkar, Umesh B. Umarani & Sadhana S. Kode
National Institute of Virology-Microbial Containment Complex Campus, Sus Road, Pashan, Pune 411 021, Maharashtra, India
**For correspondence:*
pawarshailesh@hotmail.com

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