Avian influenza surveillance in wild migratory, resident, domestic birds and in poultry in Maharashtra and Manipur, India, during avian migratory season 2006–07

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India reported outbreaks of Highly Pathogenic Avian Influenza (HPAI) H5N1 in poultry in the states of Maharashtra, Gujarat and Madhya Pradesh (February–April 2006); Manipur (July 2007); West Bengal (January 2008) and Tripura (April 2008). The role of migratory birds in the transmission of the HPAI H5N1 remains a subject of debate. Avian Influenza (AI) surveillance in wild migratory, wild resident, domestic birds and poultry was undertaken by National Institute of Virology (NIV) jointly with Ela Foundation, Pune, India during 2006–07. A total of 1968 faecal specimens (1369 droppings from wild migratory and wild resident birds; 474 droppings from poultry and 125 cloacal swabs from chickens and ducks) were collected. These samples representing 10 avian families of wild migratory birds, four families of wild resident birds totalling 36 species, were from eight districts of Maharashtra covering 20 water bodies and two districts of Manipur. The samples were screened for AI viruses by reverse transcriptase polymerase chain reaction (RT-PCR), real-time PCR and were processed for virus isolation in embryonated chicken eggs and cell culture. Two samples from wild ducks were positive for viruses other than AI, newcastle disease virus (NDV) and infectious bursal disease virus (IBDV). During the study period no sample was positive for Influenza A viruses, Influenza A (H5N1) or any other strain of HPAI by RT-PCR and virus isolation. In view of the recent HPAI H5N1 outbreaks in poultry in India, continued and more widespread AI surveillance is necessary to elucidate the role of wild migratory, resident, domestic birds and poultry in the transmission of AI viruses.

Keywords: Avian influenza surveillance, faecal samples, migratory birds.

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ZOONOTIC diseases like Avian Influenza (AI), Newcastle Disease (ND) and West Nile (WN) are some of the emerging viral diseases in water birds[1]. Due to large outbreaks in recent years and in some cases virus transmission from poultry to human with a high fatality rate, Avian Influenza A virus has currently aroused concern and received serious attention[2]. There may be an association between Highly Pathogenic Avian Influenza (HPAI) outbreaks and the presence of rapidly increasing poultry farms in several parts of the world[3]. It has been postulated that H5N1/97 virus for humans principally came from retail and live poultry markets in Hong Kong in 1997 and subsequently spread to Cambodia and South Korea[4]. It is believed that poor bio-security and poor hygiene were responsible for the spread of the virus and it is more likely that wild birds had no role in the spread[5].

Long-term screening and surveillance of migratory birds for the presence of AI virus is necessary as a part of wider range of preparedness to avert the future appearance of the virus in a pandemic form in humans[6]. Since 2003, HPAI H5N1 virus has spread to Europe and Africa and virus from birds in West Siberia, Europe and Africa is similar to that from Qinghai lake, China[7,8]. Importantly, East and Central Asian Flyways of migratory birds, which include India in their path, overlap extensively in West China (around Qinghai Lake), Mongolia and Central Siberia allowing interchange of diseases between these areas and particularly with India[9,10]. India reported AI H5N1 outbreaks in poultry[8,11–13]. The role of migratory birds in the movement of the HPAI H5N1 remains a subject of debate[14]. Therefore, in view of these recent AI H5N1 outbreaks in poultry in India, screening of wild migratory, wild resident and domestic birds as well as poultry was undertaken by National Institute of Virology (NIV) jointly with Ela Foundation to study the role of these birds in transmission of AI viruses. Migratory birds visit India during winter season (October–April) every year. There are no reports of AI surveillance in migratory/wild resident birds from India. This report presents the findings of AI surveillance during avian migratory season 2006–07.

Faecal samples (FS) of migratory birds were collected from several sites in Maharashtra, which are known for the arrival of migratory birds, during the avian winter migratory season 2006–07 (Table 1). The samples were collected and transported in viral transport medium (VTM) (Hank's balanced salt solution) with antibiotics (Penicillin, Streptomycin, Gentamycin, Amphotericin B) on wet ice/ice packs[15].

Samples of local birds were also collected during the same period. Poultry was sampled by site visits to commercial and backyard poultries. The samples consisted of faecal droppings in all birds, oral pellets and faecal droppings in case of gulls. Only fresh and wet samples were collected. When mixed flocks were encountered, names of all the species composing such flocks were entered for such samples.

Table 1. District-wise samples collected from December 2006 to April 2007

Location	No. of faecal samples		Total
	Wild migratory/ resident birds	Poultry and ducks	
Maharashtra			
Nandurbar	248	59	307
Raigad	58	–	58
Ahmednagar	–	20	20
Pune	706	365	1071
Nanded	3	30	33
Nagpur	51	–	51
Ratnagiri	167	–	167
Satara	136	–	136
Manipur			
West and East Imphal districts	–	125	125
Total	1369	599	1968

The various sites/water-bodies/dams visited were Vir, Ujani, Bhor, Naryangoan, Yedgoan, Kavdi, Khadakvasla, Panshet, Pashan Lake, Lonawala, Vadaj, Chaskaman, Revas, Akshi, Guhagar, Makar–Dhokla, Rangavali, Borpada, Bhaura and Khekada. These study sites included water-bodies from Navapur, where outbreaks of HPAI H5N1 have been previously reported in poultry.

All the avian species were correctly identified following standard field guides; FS were collected with sterile swabs or spoons in VTM. Sample tubes were immediately sealed with parafilm and stored in icebox. Aseptic precautions like wearing latex gloves, facemasks and correct disposal of used equipment were meticulously carried out. FS were characterized species-wise by performing measurements of liquid splash (urinary tract contribution) and solid pellet (digestive tract contribution) components, as well as noting their consistency, colour and pH. As far as possible, five droppings from single species from the same flock were pooled to make one sample. If pooling was not possible, single dropping was considered as one sample[16–19].

AI H5N1 outbreak occurred in poultry in Manipur[12] in July 2007. A total of 125 cloacal swabs from chickens and ducks were received from in and around 5 and 10 km distances from the H5N1-affected area, West and East Imphal districts, Manipur (received from the Director of Veterinary and Animal Husbandry Services, Government of Manipur, Manipur; Table 1)[12]. All the samples were processed for virus isolation in embryonated chicken eggs and tested by reverse transcriptase polymerase chain reaction (RT-PCR).

The contents of each collection vial were stirred and each vial was centrifuged at 2000 rpm for 5 min to remove debris. The supernatant was used for molecular diagnosis and for inoculation in specific pathogen free (SPF) embryonated chicken eggs obtained from Venkateshwara Hatcheries, Pune. Representative samples were also inoculated in Madin Darby Canine Kidney (MDCK) cell line.

All the 1968 samples were screened for the presence of influenza A and H5N1 viruses using standard one step RT-PCR method. Viral RNA was extracted using QIAamp viral RNA mini kit (Qiagen Inc, Germany). Qiagen One step RT-PCR kit (Qiagen Inc, Germany) was used to detect influenza-specific amplification of different genes according to manufacturer's instructions. WHO recommended influenza A-specific primer sets were used[15]. PCR protocols standardized at AI laboratory at NIV, using primers for detection of matrix (M) gene were also used for verification.

All 125 samples from Manipur and 300 representative samples collected from and around the outbreak locations were tested by real-time RT-PCR using the TaqMan influenza A/H5 detection kit cv1.0 (Applied Biosystems, USA). Analyses were carried out on Applied Biosystems 7300 real-time platform.

Ten-day-old SPF embryonated chicken eggs were used for inoculation. Each sample was inoculated in two eggs by allantoic route. These eggs were incubated at 37°C for 72 h, chilled at +4°C overnight, and allantoic fluid was harvested. The allantoic fluids were screened by haemagglutination (HA) test using 0.5% fowl and 1% horse erythrocytes (RBCs)[15]. Representative allantoic fluids were tested by RT-PCR for confirmation.

Each T-25 flask with confluent monolayer was infected with 500 µl of inoculum, allowed to adsorb for 30 min at 37°C followed by washing of monolayers with medium to remove un-adsorbed virus particles. Flasks containing 5 ml of Dulbecco's Modified Eagle Medium (DMEM) containing 2 µg/ml of TPCK trypsin without calf serum were then incubated at 37°C for 4–6 days. The flasks were observed daily for cytopathic effect (CPE). MDCK cell line infected with influenza viruses shows degeneration of cells which come out from the surface in super-

Table 2. Migratory and local wild birds screened for AI

Bird family/species	Bird family/species
Wild migratory birds	Little Stint *Calidris minuta*
Family: Ardeidae	Terek Sandpiper *Xenus cinereus*
Grey Heron *Ardea cinerea*	Temminck's Stint *Calidris temmincki*
Family: Ciconiidae	Common Greenshank *Tringa nebularia*
Asian Openbill *Anastomus oscitans*	Family: Laridae
White-necked Stork *Ciconia episcopus*	Black-headed Gull *Larus ridibundus*
Family: Threskiornithidae	Heuglin's Gull *Larus heuglini*
Glossy Ibis *Plegadis falcinellus*	Brown-headed Gull *Larus brunnicephalus*
Black-headed Ibis *Threskiornis melanocephalus*	Family: Sternidae
Eurasian Spoonbill *Platalea lecorodia*	Greater Crested Tern *Sterna bergii*
Family: Anatidae	Whiskered Tern *Childonia hybridus*
Bar-headed Goose *Anser indicus*	Gull-billed Tern *Gelochelidon nilotica*
Ruddy Shelduck *Tadorna ferruginea*	Sandwich Tern *Sterna sandvicensis*
Spotbilled Duck *Anas poecilorhyncha*	Lesser Crested Tern *Sterna bengalensis*
Family: Accipitridae	Family: Motacillidae
Steppe Eagle *Aquila nipalensis*	White Wagtail *Motacilla alba*
Family: Gruidae	Yellow Wagtail *Motacilla flava*
Demoiselle Crane *Grus virgo*	*Wild resident birds*
Family: Scolopacidae	Family: Podicipedidae
Lesser Sand Plover *Charadrius mongolus*	Little Grebe *Tachybaptus ruficollis*
Greater Sand Plover *Charadrius leschenaulti*	Family: Phalacrocoracidae
Kentish Plover *Charadrius alexandrinus*	Little Cormorant *Phalacrocorax niger*
Eurasian Curlew *Numenius arquata*	Family: Ardeidae
Curlew Sandpiper *Calidris ferruginea*	Cattle Egret *Bubulcus ibis*
Ruddy Turnstone *Arenaria interpres*	Family: Columbidae
Black-tailed Godwit *Limosa limosa*	Spotted Dove *Streptopelia chinensis*

natant. Cell cultures were harvested by day 6, if no CPE was observed. The tissue culture supernatants were tested by HA test using 0.5% fowl and 1% horse RBCs.

Allantoic fluids, which were positive in HA test with 0.5% fowl or 1% horse RBCs, but were negative for influenza A in RT-PCR, were tested with influenza A specific (QuikVue, USA), H5, NDV and IBDV rapid tests (Anigen, Korea). These are rapid qualitative antigen detection tests, which are based on the solid-phase immunochromatography.

A total of 1968 faecal samples comprising 1369 samples of wild migratory and resident birds, and 599 samples from poultry and ducks were collected from eight districts of Maharashtra and two districts of Manipur (July 2007) during the avian migratory season between December 2006 and April 2007 (Table 1). Samples representing 10 avian families of wild migratory birds, four families of wild resident birds totalling 36 species, were screened for AIV (Table 2).

All the 1968 samples were tested by RT-PCR. A total of 1219 samples (61.9% of the total sample size) were inoculated in SPF embryonated chicken eggs and 205 samples were inoculated in MDCK cell line. No sample was found positive for influenza A viruses, influenza A (H5N1) or any other strain of HPAI by RT-PCR and virus isolation, during the study period.

Two samples from wild ducks from Rangavali Dam, Navapur, Maharashtra were positive in HA test with 0.5% fowl and 1% horse RBCs. Mortality in SPF eggs was observed on day-2 post-infection after two passages. These allantoic fluids were observed under an electron microscope after negative staining, which revealed Reovirus-like particles. Further analysis of these samples is in progress.

The present study does not report any HPAI H5N1 or any other AI viruses from sampled birds during the study period. Although AIV has been reported earlier in the species/families of birds elsewhere, the screened population in the present study was free from any AI infection.

No convincing evidence has yet shown that infected, asymptomatic wild birds can or do carry influenza virus along established, seasonal long-distance migration routes. The hypothesis that migratory birds can transport HPAI H5N1 over long distances rests on the assumption that some infected, virus-shedding wild birds show no or only mild symptoms and migrate long distances unhampered. There has been no direct test of this assumption, but several findings from ecologic immunology and exercise physiology studies are not compatible with this conjecture[20].

An analysis by Feare and Yasué[20] supported the view that long-distance spread of virus by migratory birds is unlikely but short-distance spread is possible. They examined all known major outbreaks in wild birds and concluded that most occurrences reflect local acquisition from a contaminated source, followed by rapid death nearby. Outbreaks in Europe in 2006 indicate that infected wild birds can travel a limited distance before

dying of influenza and can pass the virus on to other wild or domestic birds. We have therefore included local wild, local migratory species like Little Cormorant, and bridge species like Cattle Egrets during AI surveillance[20].

Chen *et al.*[21] reported isolation of HPAI H5N1 viruses from six apparently healthy wild migratory birds at Poyang Lake, Jiangxi Province, China, in January and March 2005 and concluded that wild birds are able to disseminate the virus over long distances. Migratory birds and trade involving live poultry and poultry products have been suggested as the most likely causes of dispersal of the virus. Similarly, Lvov *et al.*[22] reported HPAI H5N1 in clinically healthy wild ducks (Mallard *Anas platyrhynchus* and Pochard *Aythya ferina*) and in another water bird, the great-crested grebe *Podiceps cristatus*, at Lake Chany, Novosibirsk, Russia, during an outbreak in poultry. Feare and Yasué[20] have reported that poor methodological description of the field sampling of wild birds, or poor methodology, in both of these reports cast doubt on the interpretation that these wild birds were carrying the virus asymptomatically. We have followed the guidelines suggested by Feare and Yasué, in order to avoid methodological errors.

During AI surveillance in migratory birds, lower virus isolation rates have been reported. Surveillance studies in China by Chen *et al.*[23] have reported no virus from the 1052 cloacal and oropharyngeal swabs except in dead bar-headed geese. They concluded that the influenza type A virus subtypes H2–H13 did not circulate at detectable levels within the sampled population. A low isolation rate of 0.34% from cloacal and faecal samples of migratory birds has been reported[21]. Krauss *et al.*[24] conducted AI surveillance in wild ducks in Canada and in shorebirds and gulls in the United States. They did not find HPAI in these birds and no serological evidence was recorded. Surveillance in migratory waterfowl in Southern France in 2005–06 did not find HPAI H5N1 virus but found 1.8% prevalence of other AI viruses[25]. Seven-year AI surveillance in waterfowls and shorebirds (1998–2004) in Alaska showed remarkably low infection rates (0.06%)[26]. Considering the lower rate of virus isolation, it is justified to screen larger sample sizes for AI surveillance, to continue the surveillance over a longer period of time and to cover more species.

About 1298 avian species have been recorded from the Indian subcontinent[27], of which about 1001 species are resident and 159 (12%) species are winter migrants. We tested 36 wild bird species, which represent 31% and 41.2% of the species and families respectively, found AI positive globally[28,29]. Generally, waterfowls have higher (~15%) AI infection rates, particularly in families Anatidae, Gruidae, Phalacrocoracidae and Pelecanidae as compared to terrestrial species (~2%)[30]. Other Charadriiformes waders are seldom infected as compared to families Lariidae and Sternidae, belonging to the same order[5]. Amongst these particularly vulnerable families, our samples are representative of families Anatidae, Gruidae, Phalacrocoracidae, Lariidae and Sternidae. However, we have screened a small fraction of migratory species that have tested positive for AI elsewhere outside India but which migrate to India, so also the area covered by us is limited. Accurate knowledge about the migratory grounds of wintering birds, the ability of correct identification of avian species and subspecies as migratory and resident, familiarity with study sites and approachability of such sites, limitations in large number of bird trapping for tracheal and cloacal swab screening, correct methodology of faecal sample collection, can be limitations in methods to obtain a meaningful design for a study on migratory birds to estimate their impact on epidemic spread of AI viruses.

To elucidate the role of wild migratory, resident, domestic birds and poultry in the transmission of AI viruses, continued AI surveillance is necessary in India where H5N1 virus outbreaks have been reported in poultry. Further collaborations of virologists, ornithologists, epidemiologists and ecologists are important to trace the actual role of migratory/wild birds in the geographical spread of AI viruses[31].

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Harmonizing soil organic carbon estimates in historical and current data

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Estimation of soil organic carbon (SOC) is indispensable in studies involving soils and global climate change. SOC retention in soil is a function of climate, vegetation and intrinsic soil properties. Historically, SOC estimates are based on wet digestion which gives low carbon recovery. This results in underestimation of its density and stock, however, most of the existing historical and current SOC data sets are based on wet digestion. Hence, we have compared the wet digestion with precise oxidative combustion method for SOC estimation, to develop factors for conversion of historical data into comparable values. It was found that the recovery percentage of SOC is lower than oxidative method and it further decreased with increase in clay content. In case of land use, the recovery percentage is higher in forest soils, followed by agricultural soils and the least in wasteland. A general correction factor of 1.42 and clay content specific correction factors of 1.35, 1.45 and 1.81 are recommended to convert historical data into current reliable SOC estimates.

Keywords: Clay, land use, oxidative combustion, soil organic carbon, Walkley and Black method.

INTEREST in soil organic carbon (SOC) has greatly increased in recent years because terrestrial organic carbon (OC) can be a key factor in understanding the effect of carbon (C) emission on global climate change. The increase of CO_2 from anthropogenic sources has especially been the focus of public concern. Emission of CO_2 from oxidation of soil organic matter or from respiration of the above-ground biomass is one of the largest sources of CO_2 in the atmosphere[1,2]. Researchers are interested in knowing the factors influencing soil as a source or a sink of atmospheric CO_2, apart from SOC content which is considered to be the key soil quality indicator.

Much of the current database on terrestrial C content has been gathered primarily from soil surveys, and the C content were commonly determined by wet digestion method[3–5]. Moreover, many researchers are interested not only in the total soil C content but also in specific components of soil C and the dynamics of its turnover. There could be methodological differences with the change of the analytical procedures and the instruments[6], leading to

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