Isolation and phylogenetic characterization of haemagglutinin and neuraminidase genes of H9N2 low pathogenicity avian influenza virus isolated from commercial layers in India

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Abstract Avian influenza is a highly infectious and dynamically evolving disease of birds causing high morbidity and mortality. It is caused by avian influenza virus (AIV) that belongs to the family Orthomyxoviridae. Two types of AIV have been described based on their pathogenicity viz. highly pathogenic avian influenza virus that causes severe disease with high mortality and low pathogenic avian influenza virus (LPAI) that generally causes asymptomatic infection or a mild disease. The H9N2 subtype is the widely circulated LPAI type in the world. The H9N2 subtype of was first reported from northern India in

March 2003. However, systematical surveillance information for the evolution of H9N2 viruses in poultry flocks of Southern India is lacking. The present study reports the isolation and characterization of H9N2 isolates from the southern parts of the country during the period between May 2010 and September 2011. Out of the 30 poultry flocks investigated, six were found to be positive for HA activity. Further, all the six samples conformed as AIV. Partial nucleotide sequencing of the HA and NA genes revealed that all were belonging to the H9N2 subtype. Phylogenetically, the HA and NA genes of the H9N2 viruses from India clustered with those isolated from Bangladesh, Pakistan and the Middle East, although we were not able to conclude on their exact geographic origin.

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Introduction

Among the respiratory diseases of poultry, avian influenza is the highly infectious and dynamically evolving disease of birds causing high morbidity and mortality. Avian influenza viruses (AIVs) are enveloped, negative stranded RNA viruses, capable of infecting a wide range of bird and animal species including human beings [1–3]. In poultry, two types of AIV have been described based on their pathogenicity, namely, a highly pathogenic type (HPAIV) that causes severe disease with high mortality and a low pathogenic type (LPAIV) that causes only asymptomatic infection or a mild disease [4]. Since the first detection from turkeys in Wisconsin in 1966 [5–7], the LPAI H9N2 virus subtype has

widely circulated. In Asia, H9N2 viruses were regularly isolated from ducks [8–10]. However, during the latter half of the last decade, H9N2 viruses had caused disease outbreaks in chicken in many parts of the world including Germany, Italy, Ireland, Iran, Pakistan, Saudi Arabia, South Korea, UAE, Israel, Jordan, China, South Africa and USA [11–17]. The LPAI H9N2 viruses generally cause decreased egg production and mild to moderate infections, but they can also cause heavy morbidity and mortality when the birds are co-infected with other agents [18, 19]. In India, the LPAI of H9N2 subtype was first detected in March 2003 in a private commercial farm in the Panchkula district of Haryana state [16]. Since then the virus has been isolated from 10 more States (www.hsadl.nic.in). [20] reported that 4 out of the 8 genes of Indian H9N2 isolates shared close phylogenetic relationship with Qa/HK/G1/97 isolate and rest of the genes were of unknown origin; six internal genes showed that the Indian H9N2 viruses isolated from northern and eastern India had undergone genetic reassortments [20]. Systematic surveillance information for the evolution of H9N2 viruses in poultry flocks of Southern India is not available, where the poultry population accounts for more than 50 % of the country's total poultry farming. In this context, the present study reports the isolation and molecular characterization of H9N2 LPAI viruses from the poultry flocks of the Southern India and presents the epidemiological data of genetic and phylogenetic analyses based on partitioned sequences of haemagglutinin (HA), and neuraminidase (NA) genes of the H9N2 LPAI viruses.

Materials and methods

Case history

A disease investigation was carried out in 30 commercial poultry layer flocks of Southern India including Tamil Nadu and Karnataka states with a history of generalized depression, respiratory complications, neurological disease and production drops during the period between May 2010 and September 2011. The poultry flocks consisted of growers and layers aged between 4 and 76 weeks, and reared under cage system. A random of ten moribund/dead birds per flock was brought to the laboratory, and necropsy examination was carried out. Cloacal swabs and tissue samples were collected for the virus isolation.

Sampling and virus isolation

The LPAI H9N2 viruses were isolated and identified by using standard laboratory procedures. Briefly, the cloacal swabs/clinical tissues were processed and inoculated into 11-days-old- embryonated chicken eggs via allantoic route.

The embryos were incubated at 37 °C till death or maximum of 5 days post inoculation. Amnioallantoic fluid (AAF) was harvested and subjected to haemagglutination assay (HA). Three blind passages were carried out before deciding the negativity of the samples.

RT-PCR and sequencing

Total RNA was extracted from allantoic fluid with the use of the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, California, USA) and reverse transcribed with Superscript First Strand Synthesis for RTPCR kit (Invitrogen, USA) and universal 12–base pair (bp) influenza A segments specific primer: Uni12(M) AGCRAAAGCAGG [21]. The presence of AIV was confirmed by RT-PCR targeting of Matrix (M) gene [22]. Subtype identification was then determined by PCR, with the use of HA and NA gene-specific primers [23, 24] and sequencing.

Sequence analysis

Partial nucleotide sequences (616 bp for HA and 570 bp for NA) were aligned with CLC 6.6.2 (CLC Bio, Aarhus, Denmark). Maximum-likelihood analyses were performed using the software PhyML 3.0 [25], with the general time reversible (GTR) evolutionary model, an estimation of the proportion of invariable sites (I) and of the nucleotide heterogeneity of substitution rates (α), as selected by ModelGenerator 0.85 [26]. Nodal supports were assessed with 1000 bootstrap replicates.

Results and discussion

Virological and pathological findings

Out of 30 poultry layer flocks screened, six were found positive for HA activity (Table 1). All HA positive samples also yielded positive amplification of the M, HA and NA genes. The birds from the affected farms showed depression, decreased feed intake and water consumption, watery diarrhea, drop in egg production and loss of egg quality etc. The postmortem lesions consisted of tracheitis, pulmonary congestion and edema, airsacculitis, perihepatitis, petechiae in serosal layers, haemorrhagic proventriculitis, necrotic pancreatitis, atrophy of spleen, egg peritonitis, oophoritis, and cloacal bursitis. The main histopathological lesions observed were haemorrhagic tracheitis, fibrinous pneumonia/fibrinous necrotizing bronchopneumonia, fibrino haemorrhagic airsacculitis, fibrinous pericarditis, haemorrhagic proventriculitis, catarrhal enteritis, necrotic pancreatitis, lymphoid atrophy of spleen, fibrinous serositis, atresia of ovarian follicles, tubular degeneration of kidneys.

Table 1 Epidemiological findings of layer flocks affected with low pathogenic avian influenza during 2010–2011

Flock code	Isolate name	GenBank accession no.		Farm size	Affected flock size	Age (weeks)	Mortality (%)
		HA	NA				
NKL-1	A/Chicken/India-TN/IVRI-0001/2010	CY099336.1	CY099337.1	3,50,000	1,00,000	76	4.9
NKL-3	A/Chicken/India-TN/IVRI-0002/2010	CY099338.1	CY099339.1	2,00,000	40,000	34	4.00
NKL-4	A/Chicken/India-TN/IVRI-0003/2010	CY099340.1	CY099341.1	25,000	6000	31	2.6
NKL-8	A/Chicken/India-TN/IVRI-0005/2010	CY099350.1	CY099351.1	3,00,000	50,000	8	22.02
NKL-9	A/Chicken/India-TN/IVRI-0006/2010	CY099352.1	CY099353.1	2,00,000	25,000	7	15.41
NKL-11	A/Chicken/India-TN/IVRI-0008/2010	CY099354.1	CY099355.1	17,500	3800	6	40.82

Fig. 1 Maximum likelihood consensus tree derived from 178 H9 Influenza A virus hemagglutinin nucleotide sequences. Computations were realized with the GTR + I + α evolutionary model ($I = 0.42$; $\alpha = 1.13$). *Blue branches* indicate viruses isolated in India. The *clade* including sequences derived from this study is highlighted in *red*. Main genetic lineages and their corresponding *bootstrap values* are indicated when higher than 80. A detailed phylogenetic tree including sequence strain names is available in the electronic supplementary figure S1 (color figure online)

Sequence and phylogenetic analysis

Sequence analysis of the HA gene cleavage site of the isolated six AIVs revealed that all were belonging to H9N2 LPAI viruses (RSSR*G in HA cleavage site) and shared 100 % identity with each other. The sequences were

deposited in the GenBank database (Table 1). Phylogenetic analyses of the partial nucleotides of the HA and NA genes revealed that multiple H9N2 genetic lineages have circulated in the poultry population of South Asia and in the Mideast during the last decade (Figs. 1 and 2; Supplementary Figs. 1 and 2). Analysis of the HA gene suggests

Fig. 2 Maximum likelihood consensus tree derived from 160 N2 Influenza A virus neuraminidase nucleotide sequences. Computations were realized with the GTR + I + α evolutionary model ($I = 0.29$; $\alpha = 0.72$). *Blue branches* indicate viruses isolated in India. The clade including sequences derived from this study is highlighted in *red*. Main genetic lineages and their corresponding *bootstrap values* are indicated when higher than 80. A detailed phylogenetic tree including sequence strain names is available in the electronic supplementary figure S2 (color figure online)

that these viruses may be closely related to AIVs that were isolated in India between 2003 and 2006, and also in Bangladesh, although low statistical support for internal nodes in the phylogenetic tree preclude definitive conclusion on their origin. The analysis performed on the NA gene did not provide more precise information on the geographic origin of the viruses isolated in the present study.

Although reports are available on incidences of LPAI in Northern/Eastern part of Indian subcontinent [16, 20, 27, 28], there is no information available for the incidence of LPAI in Southern States of India. The present study focused on the circulation of H9N2 LPAI viruses in layer population of Southern regions where larger population of poultry are reared but the status of LPAI viruses has not been known particularly regarding to phylogenetic and evolutionary analysis of virus isolates/strains affecting poultry in this region. In the present study, six H9N2 LPAI isolated were recovered from layer flocks during 2010 and 2011, confirmed for the presence of AIV by RT-PCR and sequencing. Comparative analysis of the sequences of HA and NA genes of the Indian strains with

Asian H9N2 subtype provides an overview of the relationships between these viruses over an extended period of time. Phylogenetically, the HA and NA genes of the H9N2 viruses from India clustered with those isolated from Bangladesh, Pakistan and the Middle East, although we were not able to conclude on their exact geographic origin. East and Central Asian flyways of migratory birds, comprise India in regular migratory route and extend beyond widely in West China, Mongolia and Central Siberia permitting possible interchange of AIVs between these areas and particularly with India [27]. It may reflect a geographical parameter for restriction of the viruses in the areas. While the HPAI situations have efficiently been brought under control, LPAI virus got increasingly circulating and regularly reported in India [16, 20, 27–30]. It is believed that during the early 2000s, H9N2 subtype viruses circulating in Pakistan and Middle East might have been introduced into India through regional poultry or migratory birds and subsequently in the intervening years of frequent antigenic drifts likely to have occurred in Indian LPAI-H9N2 viruses resulted in new drift variants which established themselves in domestic poultry population [31, 32]. Possible

risk factors for LPAI-H9N2 virus spread in India include poultry movements, contacts between wild/feral birds, backyard poultry and commercial poultry, poor husbandry practices, inadequate handling of sick and dead birds and backyard chickens by farmers, and poor awareness of control strategies among poultry farmers. The widespread circulation of H9N2 virus in the domestic poultry, co-distribution of different AIV subtypes and emergence of new antigenic variants emphasize the need for prevention of H9N2 virus in Indian poultry. Further molecular evolutionary studies on the circulating LPAI viruses are essential to develop better strategies for controlling AIV infections in the poultry flocks of the country. A comprehensive overview of the differences between the Asian and India H9N2 isolates will only be possible through complete genome sequencing. This is likely to shed light on the role of reassortment on the H9N2 virus population.

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