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SHORT
COMMUNICATION



Isolation and phylogenetic analysis of *Avian orthoavulavirus* 1 sub-genotypes VII.2 and XXI.1.2 from caged birds in the Lahore district, Pakistan – Short communication

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ABSTRACT

In this study, the prevalence of *Avian orthoavulavirus-1* (AOAV-1) (also commonly known as Newcastle disease virus) was investigated in caged birds kept in bird markets in the Lahore district of Pakistan. A total of 354 swab samples were obtained from 14 different species of clinically healthy birds. The overall virus prevalence was 12.7% in 9 out of the 14 species. Phylogenetic analysis of the complete fusion protein (F) gene showed that 23 isolates from different avian species belonged to sub-genotype VII.2 while three isolates of pigeon origin clustered with sub-genotype XXI.1.2. The VII.2 viruses isolated had a high nucleotide identity to viruses repeatedly isolated from poultry in Pakistan from 2011 to 2018. To date, sub-genotype XXI.1.2 viruses have only been identified in Pakistan. These findings suggest that the Newcastle disease (ND) outbreaks occurring in Pakistan involve multiple hosts and environments. The study emphasises the importance of continuing to monitor multiple avian species for the presence of AOAV-1s and implementing effective ND control strategies.

KEYWORDS

Avian paramyxovirus-1, caged birds, prevalence, phylogeny, Lahore district, Pakistan

Newcastle disease (ND) is widely distributed globally and has the potential to cause huge economic losses to the poultry trade. The disease is caused by virulent strains of *Avian orthoavulavirus-1* (AOAV-1) [also known as Newcastle disease virus (NDV)], which is the cause of a highly contagious disease affecting multiple poultry and non-poultry avian species and can cause 100% mortality in naïve or poorly vaccinated chickens (Wajid et al., 2017). The virus belongs to the genus *Orthoavulavirus* of the family *Paramyxoviridae*. The viral genome is non-segmented and contains negative sense, single-stranded RNA, which encodes six structural proteins (3'-NP-P-M-F-HN-L-5') (Hussain et al., 2020). AOAV-1 isolates are classified into at least 21 genotypes, separated into class I and class II. ND is a serious problem in many countries despite intensive vaccination (Mousa et al., 2020). Although ND is enzootic in Pakistan, and has been reported in ducks, geese, exotic parakeets, pigeons, backyard, and commercial poultry, the mechanism of maintenance and the evolution of APMV-1s in the country are still not completely understood (Miller et al., 2015; Wajid et al., 2017, 2018). The persistence of the viruses in Pakistan may be due to climate conditions, poor

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farming practices, low biosecurity, transport of infected poultry and their products, contaminated equipment, and migratory bird routes.

Multiple avian species of various ages are commonly kept in cages for selling purposes in bird markets in Pakistan. These markets mostly sell ducks, geese, Australian parakeets, partridges, sparrows, pheasants, peacocks, mynahs, macaw parrots, African grey parrots, guinea fowl, quail, pigeons, and backyard birds. These birds are commonly kept either mixed in the same cage or in cages close to other species. This study was undertaken to investigate the prevalence of AOAV-1s in multiple avian species kept in captivity in bird markets and to determine the genetic relationship between isolated viruses and those that circulate (or have circulated) in poultry and non-poultry avian species in Pakistan.

In this study, a total of 354 swab samples were obtained from 14 different species of caged birds (Table 1) kept in captivity in bird markets in the Lahore district. The swab samples were immediately placed in 500 µl transport medium (phosphate-buffered saline, PBS, pH 7.0 with 200 mg of streptomycin/ml, 2000 U of penicillin/ml, 2.5 mg of amphotericin B ml⁻¹ and 250 mg of gentamicin/ml). The swabs were inoculated into 9–10 day-old embryonated chicken eggs and incubated at 37 °C for 72–96 h. The harvested allantoic fluids were tested by haemagglutination assay using 10% chicken red blood cells. All positive samples were further characterised by amplification by reverse transcriptase polymerase chain reaction (RT-PCR) followed by the sequencing of the complete fusion gene. Briefly, viral RNA was extracted from the allantoic fluids using TriZol reagents (Invitrogen) as recommended by the manufacturer. The cDNA was synthesised using an RT-PCR kit with random hexamer (Thermo-Scientific) according to the manufacturer's recommendations. The full F gene was amplified using primers previously described by Wajid et al. (2017). The complete F gene (1,662 bp) of the isolates was sequenced with an ABI-3130 automated sequencer (Applied Biosystem), and the sequences were included in a

phylogenetic analysis using MEGA v6 software (Tamura et al., 2013) to infer the evolutionary history of the AOAV-1s with other representatives of class II AOAV-1s using the recently updated classification system (Dimitrov et al., 2019).

Many Asian countries including Pakistan have mixed poultry production systems with large numbers of birds that are raised in villages with minimal biosecurity and an abundance of markets selling birds of various species and age. Bird markets have been considered a hub for the spread and maintenance of multiple avian viruses of different pathogenicity (Miller et al., 2015). Different species or breeds of birds originating from various sources are intermingled in a single place encouraging viral transmission through contact with food, faeces, and water. Here we describe the role and potential of different species of caged birds to act as a reservoir for virulent strains of AOAV-1s. For this purpose samples were randomly collected from 14 different species of unvaccinated or partially vaccinated caged birds kept in bird markets between June 2018 and February 2019 (Table 1).

The overall virus prevalence was 12.7% by RT-PCR. The highest individual species-level prevalence was recorded in ducks (31.25%) and the lowest in quails (4.65%). APMV-1 was not detected in partridge, sparrow, mynah, macaw parrot, or guinea fowl. A total of twenty-six ($n = 26$) AOAV-1 isolates were selected for complete F gene sequencing and genotyping. The samples selected for sequencing were from ducks ($n = 5$), geese ($n = 4$), ring-necked parakeets ($n = 2$), African grey parrot ($n = 1$), exotic parakeets ($n = 3$), quails ($n = 2$), pheasants ($n = 3$), peacock ($n = 3$) and pigeon ($n = 3$). The F gene sequences (1,662 bp) obtained were submitted to the GenBank and are available under accession numbers MT920189–MT920214.

The three isolates of pigeon origin had an F gene cleavage site motif of 113-RKKRF-117 while the remaining 23 isolates had a 113-RQKRF-117 motif. Both motifs are characteristic of virulent viruses. Phylogenetic analysis

Table 1. Number and host origin of the samples tested for avian orthoavulavirus 1 (AOAV-1) in this study

Common name	Scientific taxon name	Number of samples	HA	RT-PCR	Total prevalence by RT-PCR (%)
African grey parrot	<i>Psittacus erithacus</i>	12	1	1	8.3
Budgerigar	<i>Melopsittacus undulatus</i>	43	5	5	11.6
Duck	<i>Anas platyrhynchos</i>	32	11	10	31.2
Goose	<i>Anser</i> spp.	29	8	8	27.5
Guinea fowl	<i>Numididae</i>	8	0	0	0
Macaw parrot	<i>Ara ararauna</i>	11	0	0	0
Indian mynah	<i>Acridotheres tristis</i>	16	0	0	0
Partridge	<i>Perdix perdix</i>	7	0	0	0
Peacock	<i>Pavo cristatus</i>	25	6	5	20.0
Pheasant	<i>Phasianus colchicus</i>	31	6	6	19.3
Pigeon	<i>Columba</i> spp.	50	7	6	12.0
Quail	<i>Coturnix coturnix</i>	43	2	2	4.6
Ring-necked parakeet	<i>Psittacula krameri</i>	21	2	2	9.5
Sparrow	<i>Passeridae</i>	26	0	0	0
Total (14 species tested)		354	48	45	12.7



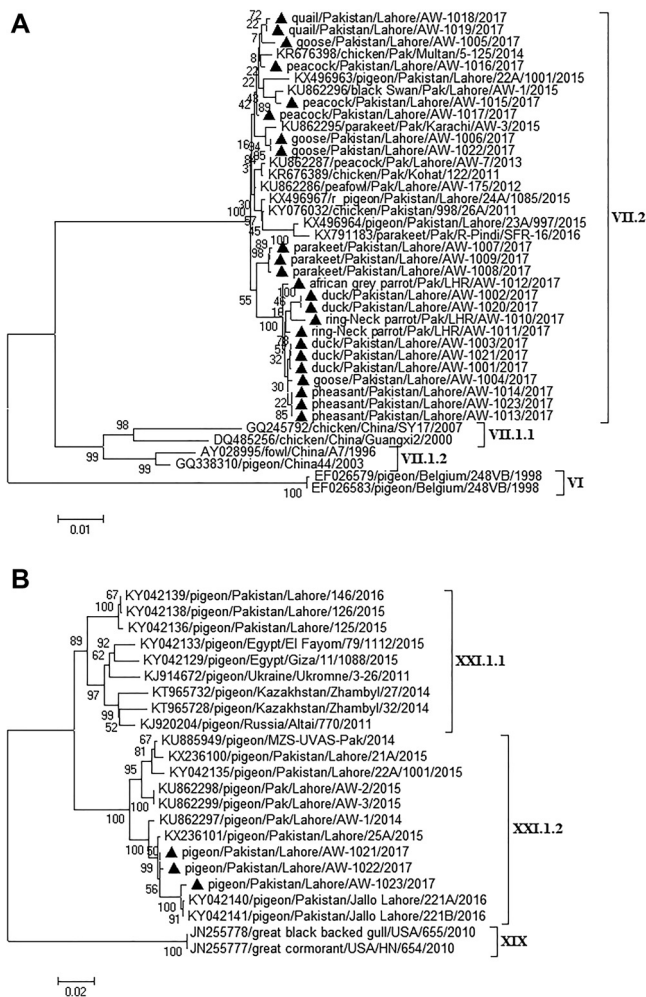


Fig. 1. Phylogenetic analysis of the avian orthoavulavirus 1 isolates based on the full-length nucleotide sequence of the gene of the fusion protein. The evolutionary history was inferred by using the neighbour-joining method with 1,000 bootstrap replicates as implemented in the MEGA v6. The isolates sequenced in this study are marked by black triangles. A: The tree showing the novel isolates that belongs to genotype VII.2. The alignment included 56 sequences with a length of 1,653 nucleotide positions. B: The tree presenting the positions of the isolates that were found to belong to genotype XXI.1.2. The alignment involved 42 nucleotide sequences with 1,662 positions in the final dataset

showed that 23 of the 26 sequences belonged to VII.2 (previously classified as VIIi), and were closely related to each other (98.8–99.9%) and to other AOAV-1s isolated from multiple avian species during a period of nine years in Pakistan, the Middle East and Indonesia (Fig. 1A). Similar viruses have been detected in multiple avian species in other countries including Iran (Ghalyanchilangeroudi et al., 2018), Jordan (Ababneh et al., 2018), Oman, and Turkey (Alsahami et al., 2018). Genotype VII.2 contains viruses recently isolated from poultry, duck, geese, pigeon, peacock, pheasant, parakeet, and black swan in Pakistan. The isolation of virulent strains of AOAV-1 indicates that the sub-genotype VII.2 viruses continue to be maintained and evolve in multiple non-poultry avian species. The virulent strains of

sub-genotype VII.2 have caused several outbreaks in non-poultry avian species in Pakistan and other countries (Wajid et al., 2017, 2021).

The remaining three AOAV-1s clustered in sub-genotype XXI.1.2 (previously classified as sub-genotype VIIm of genotype VI) (Fig. 1B). Viruses of sub-genotype XXI.1.2 have been isolated from pigeons and have been found only in Pakistan (Sabra et al., 2017). Pigeon paramyxovirus-1s (PPMV-1s) are frequently isolated from pigeons worldwide, but pigeons are also reported to carry other AOAV-1 genotypes (Wajid et al., 2017). Among other pet/captive birds, pigeons are frequently kept for hobbies (pigeon racing and shows), so the isolation of other AOAV-1 genotypes from pigeons should always warn the poultry industry that pigeons may facilitate the transfer of multiple viruses.

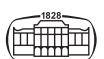
This study highlights the potential role of caged birds sold at bird markets in the circulation of AOAV-1 in Pakistan. It is suspected that these cases correspond to a specific spill-over incident from poultry farms or from other unknown sources. The presence of virulent strains of AOAV-1s in caged birds in Pakistan is of great concern, as they could be involved in the transmission of these viruses to other susceptible birds, particularly poultry. Therefore, continuous monitoring of the presence of AOAV-1 in non-poultry avian species is recommended as part of the prevention strategies for the interspecies transmission of Newcastle disease.

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