

1 **Evaluation of transmission potential and pathobiological characteristics of Mallard**  
2 **originated *Avian orthoavulavirus 1* (sub-genotype VII.2) in commercial broilers**

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24 **Abstract**

25 Newcastle disease (ND), caused by *Avian orthoavulavirus 1* (AOAV-1), affects multiple avian  
26 species around the globe. Frequent disease outbreaks are not uncommon even in vaccinates  
27 despite routine vaccination and, in this regards, viruses of diverse genotypes originating from  
28 natural reservoirs (migratory waterfowls) play an important role in a disease endemic setting.  
29 Though genomic characterization of waterfowl originated viruses has been well-elucidated  
30 previously, there is a paucity of data on clinico-pathological assessment of mallard-originated  
31 sub-genotype VII.2 in commercial chickens. Hence, the current study was designed to evaluate  
32 its transmission potential, tissue tropism and micro- and macroscopic lesions in commercial  
33 broilers. Based on complete genome and complete *F* gene, phylogenetic analysis clustered the  
34 study isolate within genotype VII and sub-genotype VII.2 in close association with those  
35 reported previously from multiple avian species worldwide. The study strain was found to be  
36 velogenic on the basis for typical residue pattern in the F-protein cleavage site (<sup>112</sup>R-RQ-K-  
37 R↓F<sup>117</sup>), sever disease induction in chicken, tissue tropism and subsequent clinico-pathological  
38 characteristics. Giving a clear evidence of horizontal transmission, a 100% mortality was  
39 observed by 4<sup>th</sup> and 6<sup>th</sup> day post infection (dpi) in chickens challenged with the virus and those  
40 kept with the challenged birds (contact birds), respectively. The observed clinical signs,  
41 particularly the greenish diarrhea, and macroscopic lesions such as pinpoint hemorrhages in  
42 proventriculus and cecal tonsils were typical of the infection caused by an AOAV-1 in chickens.  
43 The virus exhibited a broad tissue tropism where genomic RNA corresponding to study virus  
44 was detected in all of the tissues collected from recently mortile and necropsied birds. The study  
45 concludes that mallard-originated *Avian orthoavulavirus 1* is highly velogenic to commercial  
46 chicken and, therefore, ascertain continuous vaccine monitoring in vaccinates and routine

47 surveillance of migratory/aquatic fowls to better elucidate infection epidemiology and  
48 subsequent potential impacts on commercial poultry.

49 **Key words:** *Avian orthoavulavirus 1*; water fowls; greenish diarrhoea; phylogenetic analysis;  
50 macroscopic changes; sub-genotype VII.2

## 51 **1. Introduction**

52 Newcastle disease (ND), caused by *Avian orthoavulavirus 1* (AOAV-1), is a contagious viral  
53 disease of a wide range of avian species worldwide [1]. First reported in Java (Indonesia,1926)  
54 [2] and England at Newcastle-upon-tyne [3, 4]. The disease is now endemic in six of the seven  
55 continents [5]. The virus is classified as a member of genus *Orthoavulavirus* within the  
56 family *Paramyxoviridae* [6]. It has a single-stranded, non-segmented pleomorphic RNA genome  
57 of 15186, 15192 or 15198 nucleotides in length that follow a rule of six in an order of 3'-NP-P-  
58 M-F-HN-L-5'. Depending upon pathogenicity in chickens, the virus exist as four pathotypes  
59 named velogenic, mesogenic, lentogenic and avirulent [7]. Two major classes, class I and II, of  
60 AOAV-1 are known. Class I viruses are isolated from waterfowls, comprised of a single  
61 genotype, carries a large genome (15198 bp) and are considered non-virulent for chickens. On  
62 the other hand, class II viruses are isolated from a wide range of avian species comprised of XXI  
63 genotypes [6], have relatively a short genome size (15186 bp and/or 15192 bp) and includes both  
64 virulent and non-virulent strains [8, 9].

65 *Avian orthoavulavirus1* has a potential to evolve with the passage of time and this has fairly been  
66 evidenced with the emergence of novel genotypes and sub-genotypes followed by outbreaks in  
67 multiple avian hosts across many parts of the world [10-12]. In this regards, migratory birds play  
68 a vital role that themselves remain asymptomatic (reservoir); however, while their movement  
69 from one part of the world to another [13], shed viruses of different genotypes and varying

70 pathogenicity resulting in clinical disease in their susceptible host, the poultry [14-16]. Isolation,  
71 identification and genomic characterization of sub-genotype VII.2 has previously been evidenced  
72 from migratory birds in Pakistan. Indeed, during the recent years, viruses of genotype VII have  
73 been reported from many poultry rearing settings worldwide and are known to have a panzootic  
74 potential [11, 17, 18]. Virulent strains of genotype VII causes the epidemics not only in Pakistan  
75 but also in China, Japan, and Korea [19-21] are co circulating into ducks and chickens [22].  
76 Vaccines routinely used in the country are based on genotype I and II strains such as LaSota and  
77 Mukteswer. Both the vaccine provide protective immunity but unable to block the virus shedding  
78 that lead to continuous presence of virulent strains in the environment. This is particularly more  
79 dangerous with genotype VII isolates, whose shedding after LaSota vaccinated birds is  
80 significantly higher than those of other genotypes [23]. Taken together, in a disease endemic  
81 setting worldwide such as Pakistan, it become utmost important not only to characterize biologic  
82 and genomic characteristics of waterfowl's-originated viruses in routine but also to evaluate their  
83 clinico-pathological assessment, transmission and tissue distribution pattern in susceptible hosts  
84 such as the commercial broiler. Genomic and biologic characteristic of study isolate has  
85 previously been documented [24] Here we report infection transmission potential, tissue tropism,  
86 macroscopic and microscopic lesions of duck-originated AOAV-1 in commercial broilers.

## 87 **2. Materials and Methods**

### 88 **2.1. Ethical approval**

89 All animal handling and sample processing procedures were carried out in strict accordance to  
90 institutional guidelines and regulations related to animal welfare and health. The used procedures  
91 were approved by the Ethical Review Committee for use of Laboratory Animals (ERCULA) of

92 the University of Veterinary and Animal Sciences, Lahore, Pakistan (Permit Number: ORIC/DR-  
93 70).

## 94 **2.2. Virus strain**

95 The study used virus strain was isolated from clinically healthy mallard (*Anas platyrhynchos*) in  
96 an influenza surveillance program at Chashma barrage during the period from June 2015 to  
97 September 2016. The barrage is built in the river Indus and is considered one of the major  
98 wildlife sanctuaries that accommodates a large variety of migratory and indigenous water birds  
99 each year [25]. Typical biologic (mean death time = 49.5-50hr and EID<sub>50</sub> = 10<sup>8.5</sup>/mL) and  
100 genomic characteristics (F protein cleavage site, <sup>112</sup>R-RQ-K-R↓F<sup>117</sup>) of the study virus  
101 (KY967612; Mallard-II/UVAS/Pak/2016) revealed the virulent nature of the isolate [24].

## 102 **2.3. Phylogenetic analysis**

103 Complete genome and complete *F* gene of study isolate and other representative strains of  
104 different genotypes of AOAVs reported from Pakistan and other parts of the world were  
105 retrieved from GenBank database and were aligned in BioEdit® version 5.0.6 [26]. A  
106 phylogenetic tree of both complete genome and *F* gene was constructed using neighbor-joining  
107 method with 1000 bootstrap replicates through MEGA® version 6.0 software [27]. Labeling and  
108 necessary interpretation of prevalent genotype was made as per newly proposed classification of  
109 AOAV-1 [6].

## 110 **2.4. Experimental design**

111 One-day-old chicks (n=25) were procured from a commercial hatchery and raised at the animal  
112 experiment unit in the Quality Operations Laboratory (QOL), University of veterinary and  
113 animal sciences, Lahore, Pakistan. Feed and water were provided *ad libitum*. All chickens  
114 included in the study were first screened for AOAV-1 and avian influenza virus specific

115 antibodies in blood through hemagglutination inhibition (HI) assay [28]. and antigen in oral and  
116 cloacal swabs through previously published RT-PCR assays targeting *F* gene for AOAV-1 [29]  
117 and *M* gene for AIV [30] and egg inoculation [31]. Briefly, for egg inoculation, the swab  
118 samples containing buffered saline were mixed with antimicrobials (Penicillin 2000 IU/mL,  
119 Gentamicin 200 µg/mL and Fungizone 1.5 µg/mL) and centrifuged at 3000g for 5min. About 1.0  
120 mL of each sample was filtered through 0.22µm syringe filter (EMD Millipore Millex™,  
121 Millipore Billerica MA, USA). A 0.2mL of filtrate was inoculated in 9 day-old chicken  
122 embryonated egg as described previously.

123 At the age of day 30, chickens were randomly divided into three groups. Group A (n =10) served  
124 as challenged birds, group B served as negative control (n=10), while birds in group C (n=5)  
125 served as contact birds. The group A was inoculated with  $10^8$  EID<sub>50</sub>/mL<sup>-1</sup> of Mallard-  
126 II/UVAS/Pak/2016 *via* intra-ocular and intranasal routes. The negative control group was  
127 inoculated with 1mL phosphate buffered saline. After 24hr of challenge, the contact birds (group  
128 C) were introduced with group A birds for an assessment of horizontal transmission of virus  
129 from infected (group A) to healthy birds (group C). Chickens of groups A, B and C were housed  
130 separately and monitored daily for clinical presentation. With the onset of generic and typical  
131 clinical signs suggestive of ND infection and/or sudden death, diseased birds were euthanized  
132 and tissue samples n=13 (brain, breast muscles, heart, tongue, trachea, hair follicles, caecal  
133 tonsils, gizzard, proventriculus, small intestine, liver and lungs) were collected for tissue tropism  
134 and histopathology.

#### 135 **2.4.1. Genomic identification of AOAV-1**

136 Tissue samples were ground completely in a pestle-mortar, centrifuged at 2000g for 15min and  
137 supernatant was collected and archived at -20 until used. Genomic RNA was extracted from the

138 cloacal and oral swabs, as well as tissue-extracted supernatant using a commercially available kit  
139 as per manufacturer's protocol (QIamp Viral RNA mini kit, Qiagen®, USA). Tissue distribution  
140 was determined using previously reported RT-PCR targeting *F* gene [29].

#### 141 **2.4.2. Histopathology**

142 Tissue samples (liver, lung, spleen, brain, cecal tonsils and intestine) from recently mortile birds  
143 were collected, stored in 10% buffered formalin and processed for hematoxylin and eosin  
144 staining [32]. Tissue section (5µm) were prepared and observed for microscopic changes under  
145 light microscope (10X and 40X).

### 146 **3. Results**

#### 147 **3.1. Phylogenetic analysis**

148 Phylogenetic analysis of complete genome clustered study isolate within genotype VII together  
149 with previously isolated strain of Pakistan-originated chicken (KX791187-88), duck  
150 (KU845252) and wild pigeon (KX496962) and vaccinated broilers (HQ697254) from Indonesia  
151 (Figure 1A). Based on complete *F* gene, phylogenetic analysis clustered study isolate within  
152 VII.2 together with AOAV-1 strains previously isolated from pheasant (JX854452) and backyard  
153 poultry (KX791187-88) from Pakistan (Figure 1B).

#### 154 **3.2. Infection potential, transmission and tissue tropism of mallard originated AOAV-1 in** 155 **commercial chickens**

156 All the birds in the challenged group died by 4<sup>th</sup> dpi while the contact birds died by 6<sup>th</sup> dpi clearly  
157 indicating velogenic nature of mallard-originated isolate. We observed generic as well as typical  
158 clinical signs that were suggestive of ND infection in both of the challenged and contact birds.  
159 Generic clinical signs included off-feed, general sickness (depression, isolation and lethargic),  
160 oculo-nasal discharge and coughing. On the other hand, typical signs included sudden death,

161 greenish diarrhea. Though macroscopic lesions such as pin point hemorrhages in proventriculus  
162 and cecal tonsil were evident in each of the recently mortile bird, all the birds were devoid of any  
163 nervous symptoms until their death. In challenged birds, the signs were appeared on 2<sup>nd</sup> dpi  
164 along with sudden death in three of the birds, become aggravated further by 3<sup>rd</sup> dpi and all of  
165 them get mortile by 4<sup>th</sup> dpi. Signs appeared in contact birds (group C) by 3<sup>rd</sup> dpi with no sudden  
166 death throughout the experiment period; however, severity of infection aggravated by 5<sup>th</sup> dpi and  
167 all the birds died by 6<sup>th</sup> dpi. Birds in group B (negative control) remained alive throughout with  
168 no evident clinical signs. A brief description of mortalities observed for each of the group is  
169 presented in Kaplan-Meier curve (Figure 2).

170 As for tissue tropism of AOAV-1 is concerned in diseased chicken originating from challenged  
171 and contact bird's group, viral RNA corresponding to *F* gene was detected in all of the select  
172 tissue samples (n =13) including brain, breast muscles, heart, tongue, trachea, hair follicles, cecal  
173 tonsils, gizzard, proventriculus, small intestine, liver and lungs.

### 174 **3.2.1. Macro- and microscopic changes**

175 Necropsy of recently mortile birds in each of the challenged and contact birds showed  
176 comparable macroscopic changes. These included swelling of inner lining of eyelid, enlargement  
177 of lymphoid organs (liver, spleen) hemorrhages in trachea, liver, lungs, proventriculus, and small  
178 intestine particularly cecal tonsils. Microscopic changes in liver included degeneration and  
179 necrosis of hepatic cords, severe congestion along with emphysema and degeneration (Figure  
180 3A). Congestion and hemorrhages with mononuclear inflammatory cells infiltration in sub-  
181 mucosa of lungs were observed (Figure 3 B). Similarly, congestion, degeneration and necrosis of  
182 lymphoid follicles were evident in cecal tonsils (Figure 3C). Microscopic lesion in small  
183 intestine included mononuclear cells infiltration; necrotic intestinal villi and sloughing of luminal

184 epithelial cells (Figure 3D). Hemorrhages and degenerative changes were evident both in the  
185 spleen (Figure 3E) and brain tissues (Figure 3F). However, no microscopic changes were  
186 observed in control group.

#### 187 **4. Discussion**

188 The study revealed disease causing potential of mallard-originated AOAV-1 in its susceptible  
189 host (chicken) and, therefore highlight potential role of such natural reservoirs in disease  
190 epizootology, particularly in a setting where ND is endemic and frequent occurrence of  
191 outbreaks is not unusual. Phylogenetic analysis of independent complete genome and *F* gene  
192 revealed clustering of study isolate with previously reported viruses from different host (duck,  
193 backyard poultry and pigeon) suggesting its interspecies potential. A close association with an  
194 Indonesian isolate is interesting that not only is a clear indicator of its potential ancestral link but  
195 also it does provide an evidence on potential role of wild birds in dissemination of pathogens  
196 across the borders. This is not exclusively limited to transboundary transmission of AOAV-1  
197 through their natural reservoirs but also has been evidenced previously for other respiratory  
198 pathogens of birds in Pakistan such as highly pathogenic avian influenza strain H5N1 [33, 34].

199 As per recently proposed classification, the study isolate classified as VII.2. Indeed, AOAV-1  
200 within genotype VII are considered to be genetically diverse group of viruses and are found  
201 associated with recurrent outbreaks in their susceptible hosts, particularly the commercial  
202 poultry, in Middle East, Asia, Africa and South America [5, 35-38]. Among genotype VII  
203 viruses, sub-genotype VII.2 has demonstrated an intercontinental spread and, therefore has a  
204 global significance in the perspective of potential fifth panzootic [11]. Hence, an extensive  
205 surveillance of wild birds for AOAV-1 is very much necessary to monitor circulating genotypes  
206 in the country and/or region.

207 We used oculonasal route for virus exposure in birds simply because it is considered a natural  
208 route of infection and the same has previously been employed in various other experimental  
209 infection of AOAV-1 under captivity [39, 40]. Clinico-pathological findings of duck originated  
210 AOAV-1 revealed a highly pathogenic nature of virus to their susceptible host, the chicken.  
211 Given a broad range of host susceptibility to AOAV- 1 across many part of the globe [41-44],  
212 particularly sub-genotype VII.2, clinical implications such as observed in this study were not  
213 very much unexpected. However, it were disease progression, clinical signs and their severity  
214 that may be taken as unusual particularly in birds representing a disease-endemic region. An  
215 occurrence of disease in contact birds was found to be a clear indication of horizontal  
216 transmission of virus from birds in challenged group to contact birds. A lack of nervous sign in  
217 both group of challenged and contact birds indicated a viscerotropic nature of study pathotype [5,  
218 37]. Sudden death observed exclusively in challenged birds is another typical feature of ND  
219 infection [45]. A high mortality rate (100%) observed in both challenged and contact birds was  
220 found to be in agreement to observation made previously where, post infection with velogenic  
221 virus, all the challenged birds died within a week [37]. The clinical infection was found to be  
222 more pronounced and severe between 2<sup>nd</sup> to 4<sup>th</sup> dpi with an exhibition of ocular and nasal  
223 discharge, conjunctivitis, ruffled feathers, greenish diarrhea and sudden death. Consistent to this  
224 observation, a similar pattern of clinical sign progression has previously been documented with  
225 an exposure of wild-bird originated velogenic strain in chicken [39, 46]. Similarly, macroscopic  
226 lesion upon necropsy as well as microscopic changes observed under microscope were found  
227 suggestive of virulent AOAV-1 in a pattern similar to those reported previously during  
228 experimentally infected commercial chickens with wild bird originated velogenic isolates [37,  
229 39].

230 **5. Conclusion**

231 We determined the infection potential of migratory duck originating AOAV-1 in commercial  
232 chickens. Clustered within the viruses of genotype VII.2, the study isolate was found to be highly  
233 pathogenic to chicken and had a broad tissue tropism. Hence, in a disease endemic setting  
234 worldwide including Pakistan, continuous surveillance of migratory birds coupled with genomic,  
235 biologic and clinico-pathologic assessment is ascertained for its effective control and  
236 management.

237 **Competing interest statement**

238 All authors declared no competing interest with data presented in this manuscript

239 **Contributors**

240 MH and MZS conceived and designed the work. MH, TY and AR performed laboratory  
241 procedures and relevant methods. MH, AR and MZS involved in data analysis. MZS, WS, MS  
242 and TY provided necessary laboratory resources and consumables. MH, AR, MM and MZS  
243 wrote the draft and edited.

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246 **Figure legends**

247 **Figure 1:** A phylogenetic tree based on complete genome (1A) and complete F *gene* (1B) of  
248 study isolate, duck origin and other representatives of *Avian orthoavulavirus 1*. The tree was  
249 constructed in MEGA 6.0 software using neighbor joining method with 1000 bootstraps. The  
250 study isolate is marked with black circle.

251 **Figure 2:** Percent survival of infected chicken with duck isolate along with control (non-  
252 infected) and contact group. All birds in infected and contact group were died at 4 and 6 dpi  
253 respectively. No mortality was observed in control group.

254 **Figure 3:** Microscopic examination of histopathological changes at different resolutions (10 &  
255 40X) in different tissues of experimentally infected broiler with Mallard I/UVAS/Pak/2016  
256 isolate. Arrows indicate pathological lesions in liver (A), lung (B), caecal tonsil (C), villi (D),  
257 spleen (E) and brain (F).

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