Infectivity of wild bird-origin avian paramyxovirus serotype 1 and vaccine effectiveness in chickens

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Newcastle disease virus, a prototype avian paramyxovirus serotype 1 (APMV-1), causes economically devastating disease in avian species around the world. Newcastle disease is enzootic in Pakistan and recurrent outbreaks are frequent in multiple avian species even after continuous and extensive use of vaccines. A number of APMV-1 and pigeon paramyxovirus serotype 1 (PPMV-1) strains have been isolated and genetically characterized in recent years. However, the impact of recently characterized wild bird-origin APMVs in domestic poultry, and the potency of routinely used vaccines against these novel and genetically diverse viruses remain unknown. Here, we applied next-generation sequencing for unbiased complete genome characterization of APMV-1 and PPMV-1 strains isolated from clinically diseased peacocks (Pavocristatus) and pigeons (Columbalivia), respectively. Global phylodynamics and evolutionary analysis demonstrates Pigeon/MZS-UVAS-Pak/2014 is clustered into lineage 4 (or genotype VI) and Peacock/MZS-UVAS-Pak/2014 into lineage 5 (or genotype VII). The genomes of both isolates encoded for polybasic residues (112RRQKR117) at the fusion protein cleavage motif along with a number of important substitutions in the surface glycoproteins compared with the vaccine strains. Clinicopathological and immunological investigations in domesticated chickens indicate that these isolates can potentially transmit between tested avian species, can cause systemic infections, and can induce antibodies that are unable to prevent virus shedding. Collectively, the data from these genomic and biological assessments highlight the potential of wild birds in transmitting APMVs to domesticated chickens. The study also demonstrates that the current vaccine regimens are incapable of providing complete protection against wild bird-origin APMVs and PPMVs.

INTRODUCTION

Newcastle disease (ND) is one of the most important and highly contagious viral diseases of domestic poultry and wild birds (Alexander et al., 1998), and is caused by the ND virus (NDV). NDV, the type-species of avian paramyxovirus serotype 1 (APMV-1), is an enveloped, negative-sense, non-segmented ssRNA virus of the genus Avulavirus within the family Paramyxoviridae (Alexander et al., 1998; Kolakofsky et al., 2005). The genome of all APMVs is approximately 15.2 kb in length and encodes the nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase (HN) and large polymerase (L) genes, in addition to the V protein that is expressed by the RNA editing process of the P gene (Kolakofsky et al., 2005).

Based on the analysis of F gene sequences, all APMV-1 strains can be divided into either lineages or genotypes (Aldous et al., 2003; Cattoli et al., 2010; Czeglédi et al., 2006; Diel et al., 2012; Kim et al., 2007; Munir et al., 2012b; Perozo et al., 2008; Snoeck et al., 2009). In the lineage-based classification system, at least six lineages and 13 sub-lineages have been proposed (Aldous et al., 2003), whereas in the genotype-based classification, all strains of APMVs can be divided into two classes (class I and class II). Class I has been further divided into nine genotypes (1–9), and class II comprises at least 15 genotypes (XV) (Czeglédi et al., 2006; Kim et al., 2007; Miller et al., 2010; Tsai et al., 2004). Strains of class I have been isolated mainly from waterfowl as well as from shorebirds, and are...
considered avirulent to chickens. However, class II strains are categorized into lentogenic, mesogenic or velogenic based upon their pathogenicity to the host. Given the number of recent outbreaks around the globe particularly in Asia, Africa, the Middle East and South America, it appears that the strains belonging to lineage 4 (genotype VI) and lineage 5 (genotype VII) are highly virulent and are the most frequent cause of clinical infections (Diel et al., 2012). A distinct cluster of APMV-1 viruses within lineage 4 (genotype VI) that have been predominantly isolated from pigeons, are referred to as pigeon paramyxovirus type 1 (PPMV-1) (Collins et al., 1994).

Owing to the high pathogenicity of NDV, and associated major economic losses in domestic commercial poultry, strict biosecurity and vaccination programmes are being used in NDV-endemic countries with varying schedules and successes. Under high vaccine-induced immunological pressures and continuous virus evolution, the emergence of novel genotypes/lineages with potential failure of vaccines and diagnostics is inevitable (Cattoli et al., 2010; Diel et al., 2012; Munir et al., 2012b; Shabbir et al., 2013b). Besides the persistence of NDV in backyard poultry and wild birds (Jindal et al., 2009; Munir et al., 2012b; c; Zhang et al., 2011), the genetic divergence between vaccine strains and the dominant field genotypes is increasing exponentially (Miller et al., 2007, 2009, 2010; Munir et al., 2012b). All these factors not only contribute to the complexity of the ND pathobiology but also pose constraints in the control of ND.

Since its first identification, ND has remained endemic in Pakistan, and despite extensive use of indigenous and imported vaccines, occurrence of the disease has been frequently reported (Khan et al., 2010; Munir et al., 2012a, b; c; Rehmani et al., 2015; Shabbir et al., 2012, 2013b; Siddique et al., 2013). In our previous studies, spanning over a period of 7 years of disease monitoring and virus characterization in Pakistan, we have isolated virulent NDV strains from wild birds such as peacocks and pheasants, and from commercial and backyard poultry (Munir et al., 2012a, b; c; Shabbir et al., 2012, 2013a). Genetic comparison of the genotype VIIa strains isolated from peacocks (Munir et al., 2012c) and pheasants (Shabbir et al., 2012), and genotypes VIIc, VIIb and VIIi strains reported from domesticated poultry (Munir et al., 2012b; c; Shabbir et al., 2012, 2013a) depict a high level of genetic diversity and highlight the distribution of multiple strains of NDV in commercial, backyard and wild bird populations in the country. Currently, we have also characterized a velogenic strain of NDV from clinically asymptomatic birds, adding further complexity to the pathobiology of NDVs in different avian species (Munir et al., 2012b). These studies, mainly focused on virus genetics, prompted us to determine and evaluate the transmissibility, clinical impact and to assess the potential threats of APMVs isolated from wild birds, in domestic chickens. From the data presented here, we conclude that the APMV-1 and PPMV-1 viruses isolated from wild birds are fully infectious and pathogenic for the domesticated chicken, and that these strains can potentially be transmitted between infected and healthy birds. Moreover, commonly applied vaccines are unable to fully protect the vaccinated birds from the clinical disease induced by wild bird-origin APMV-1 and PPMV-1, since virus shedding was not contained despite the evidence of seroconversion. Based on the viral bioinformatics analysis, we highlight the circulation of diverse genetic clusters of APMV-1 and PPMV-1 in Pakistan.

RESULTS

Wild bird-origin APMV-1 strains are genetically divergent compared with vaccine strains

The consensus complete genome of both pigeon- and peacock-origin APMV-1 were submitted to GenBank under the accession numbers KU885948 (Peacock/MZS-UVAS-Pak/2014) and KU885949 (Pigeon/MZS-UVAS-Pak/2014), respectively. The genome lengths of pigeon and peacock isolates followed the ‘rule-of-six’, the characteristic essential feature for efficient replication of paramyxoviruses (Kolakofsky et al., 2005). As expected for AMPV-1 and PPMV-1, the characterized strains showed the order of six ORF: 3'-N-P-M-F-HN-L-5'.

The nucleotide identity between the studied isolates was 86.6%, and these isolates shared a varying degree of genetic diversity with representative genotypes/lineages of APMVs. Comparison of nucleotide and deduced amino acid sequences for each coding region of APMVs corresponding to representatives of major genotypes including the vaccine strains is given in Table S1 (available in the online Supplementary Material).

Comparison of the full-length deduced amino acid sequence of the F protein of pigeon and peacock isolates showed a divergence of 11.6 and 11.8 to genotype II (LaSota) and 9.8 and 9.3 to genotype III (Muketeswar), respectively. Both isolates had predicted multiple basic amino acid residues at proteolytic cleavage site (F0) that are considered typical of mesogenic/velogenic strains (Kolakofsky et al., 2005). The corresponding residues at the F1 protein and the NH2-terminus of the F1 protein were found to be 112R-R-Q-K-R116 and phenylalanine (116F117), respectively. Invariable cysteine residues were observed for both isolates: 12 in the pigeon and 13 in the peacock isolate. In contrast to well-known conserved sites among different APMV-1 strains, both isolates had a cysteine residue at position 27, while in the peacock isolate, a C25Y substitution was observed. Similar to previously reported genotypes, six potential glycosylation sites [Asp(N)-X-Ser(S)/Thr(T), X could be any residue except proline (P) and aspartic acid (D)] were identified in the studied isolates. The sites 85N-R-T87, 191N-T-T193, 366N-T-S368, 471N-N-S473 and 541N-N-T543 were common, while a substitution distinct to pigeon (I448V) was observed for 447N-I-S449.

Differences in amino acid length of the HN protein have been reported previously from different strains of APMV-1 (Römer-Oberdörfer et al., 2006). However, the isolates in the current study had an ORF (6418–8133, 1716 bp) encoding 571 residues; a feature common to most of the virulent
Fig. 1. Phylogenetic analysis of isolates. (a) Complete genome-based phylogenetic analysis of pigeon and peacock isolates with the representative strains of each lineage/genotype in class I and class II reported elsewhere in the world. (b) Partial F gene (374 nt)-based phylogenetic analysis of pigeon and peacock isolates with the representative strains of each lineage/genotype. Same colours in both (a) and (b) represent identical lineages. Reported isolates clustered in the lineage 4 and lineage 5, and are coloured blue and green, respectively. Position of clustering is marked with red arrows for both isolates. Vaccine Genetics of APMVs and infection in chickens
strain (LaSota) is marked by a black star for comparison of genetic distances between vaccine and field strains. (c) A higher resolution tree representing lineage 5/genotype VII. The pigeon isolate of lineage 4 was selected as outgroup. (d) A higher resolution tree representing lineage 5/genotype VI. The peacock isolate of lineage 5 was selected as outgroup. Bayesian trees were constructed in the MrBayes package as described in Methods, and the consensus tree was shown. Bars indicate nucleotide substitutions per site.


**Pigeon and peacock isolates belong to genotype VI and genotype VII**

To determine epidemiological clustering of isolates in the current study with the APMV-1 and PPMV-1 reported in the public domain, all available complete genomes were downloaded from National Center for Biotechnology Information databases and used for the phylogenetic analysis and comparative genomics. APMV-1 isolated from the peacock shared maximum genetic similarity with an isolate originating from lineage 5 (genotype VII), while the isolate from the pigeon shared genetic resemblance to isolates originating from lineage 4 (genotype VI) (Fig. 1a). Since most of the reported strains of APMVs and PPMVs are characterized based on the 3' hypervariable region of F gene (374 bp), we next analysed the entire datasets of F gene (~3000) sequences. The Bayesian consensus phylogenetic analysis, verified by the neighbour-joining method, clearly divided the APMV-1 strains into six lineages, and Peacock/MZS-UVAS-Pak/2014 isolate clustered with isolates of lineage 5 in association with strains reported previously from Pakistan, whereas the Pigeon/MZS-UVAS-Pak/2014 isolate resembled lineage 4 along with isolates reported from Russia (Fig. 1b). This isolate shared highest genetic identity with the NDV/Altai/pigeon/770/2011, recently reported from Russian pigeons (Yurchenko et al., 2015). The clustering pattern at a higher resolution was performed with selected isolates within lineage 4/genotype VI (Fig. 1d) and lineage 5/genotype VII (Fig. 1c), showing their close association within their sub-genotypes/sub-lineages. Based on sequence comparison, both isolates showed more than 15% nucleotide divergence compared with routinely used vaccines in the country [LaSota (genotype II) and Muketeswar (genotype III)]. Taken together, distinct grouping and level of genetic divergence of these isolates are in agreement with our studies on previously characterized isolates from wild birds, commercial and backyard poultry in Pakistan. These differences warrant future studies to evaluate the contribution of genetic differences on the protective efficacy of vaccines.

**Routinely used attenuated and killed vaccines provide partial protection against field strains of APMV-1**

The protective efficacy of commonly used vaccine type (LaSota and indigenously produced killed vaccines) and routinely used vaccine schedule were assessed individually against pigeon- and peacock-originated APMVs, as outlined in Fig. 2. The birds used in the experiment had a maternal antibody geometric mean titre (GMT) of 4.2 before the oral administration of LaSota on day 7. When examined on day 27 before challenge, vaccinated pigeon and peacock groups (VCh) showed a mean titre of 7.15 and 7.40, respectively, indicating potency as well as appropriate application of vaccines to the birds (Fig. 3a).

We observed variations in severity and duration of clinical signs between challenge isolates and over time post-infection (p.i.). A detailed description of birds in each group together with their clinical signs or sacrificial record is given in Table S2. Briefly, commonly observed signs were general sickness (depression, anorexia), mild respiratory sounds, ocular discharge and reluctance to move. Although severity of clinical signs was more often observed in birds challenged with the virulent peacock-derived viruses, the disease outcome coupled with the gross pathognomonic lesions were comparable for both isolates. The group without pre-vaccination (Ch) showed severe clinical picture with all birds dead or killed by 7 days p.i. (dpi) for both isolates (Fig. 3b, c). Sudden death showed no-to-mild clinical signs was observed particularly in Ch groups and one in VCh group challenged with the peacock isolate. Both the isolates induced nervous signs that included neurological signs such as twisting of the head and neck together on one side, circling movement and opisthotonus. The clinical signs started to appear on 6 days p.i. in group that was challenged (Ch) with the peacock isolate and by 7 days p.i. in birds challenged with the pigeon isolate. Interestingly, the situation was reversed for VCh group, nervous signs were observed on day 9 p.i. in birds challenged with peacock isolate compared to the group challenged with peacock isolate on day 10 p.i. The Ch group showed mild clinical signs till the end of the experiment trial; however the characteristic necropsy lesions were observed on day 8 and day 10 p.i. in birds kept with Ch group of peacock and pigeon isolates, respectively.

Pathogenicity of the isolates was assessed through tissue tropism in birds showing typical gross necropsy lesions of NDV. Various tissues (n=19) from birds revealing typical necropsy lesions were processed. The viral RNA was detected in all tissue samples of birds challenged with peacock isolates. Likewise, tissues of birds challenged with the
pigeon isolate showed presence of RNA in all tissues except liver and kidney probably due to reduced predisposition of isolates for these organs. Together it showed that the peacock isolate had much broader tissue tropism than the pigeon isolate. Shedding of virus was determined for contact bird groups challenged with pigeon and peacock isolates. Cloacal and oropharyngeal swabs were collected on every alternate day till the end of trial period on day 2, 4, 6, 8 and 10. Mock-inoculated chickens remained virus-negative throughout the experiment. Virus shedding was detectable in contact bird groups from day 6 to 10. However, a negligible difference was noted for shedding of the pigeon isolate through the cloaca. Viral shedding was identified for both isolates on day 6, 8 and 10; nevertheless, we found lack of shedding through the cloaca in contact birds challenged with pigeon isolate on day 6.

Sera samples were collected from the survived birds in immunized groups on day 40 (day 13 p.i.) and processed for haemagglutination inhibition (HI) assay. The antibody titres to immunizing LaSota strain, killed vaccine and the respective challenge virus were compared as pre-challenge versus post-challenge immune response (Fig. 3a). We found an increased but varying immune titre for birds in each group; birds challenged with pigeon isolate showed GMT 10.7, while it was 9.3 in birds challenged with the peacock isolate. This varying but overall increase in post-challenge antibody titre may be taken as replication of challenged viruses in both groups. Moreover, pigeon isolate cross-reacted with the sera collected from birds challenged with the peacock isolate and vice versa. However, as was expected, a lower cross-reactivity was observed between the sera collected from LaSota immunized birds and both isolates (pigeon and peacock) (data not shown).

Both strains caused detectable histopathological lesions in affected birds. The microscopic changes were relatively less pronounced in birds challenged with pigeon isolate than
peacock isolate. We found haemorrhages in some areas of tracheal tissue coupled with degenerative changes in the lamina propria and absence of pseudostratified columnar epithelium. Severe congestion particularly in parabronchial blood capillaries was observed in lung tissues. Liver showed fatty changes evidenced by vacuoles of varying sizes in the cytoplasm of many hepatocytes. The portal vein including the sinusoidal capillaries was engorged with red blood cells and, in some areas, Kupffer cells were also observed. Congestion, haemorrhages, degeneration and loss in lymphoid follicles coupled with sloughing of columnar epithelium were evident in caecal tonsils. The microscopic examinations of kidney revealed severe congestion in peritubular capillaries, cellular swelling in renal epithelial cells and coagulative necrosis in renal tubules. Some renal tubules had epithelial cells separated from the basement membrane. Intestine revealed sloughing of epithelial cells in the lumen; a vast majority of the intestinal villi were necrotic and degenerated (Fig. 4).

**DISCUSSION**

The poultry industry in Pakistan has experienced huge economic losses due to continuous outbreaks of ND. It has been proposed that increased genetic divergence between vaccine and field strains may be responsible for vaccine-induced protective immune responses (Shabbir et al., 2012, 2013a; Munir et al., 2012b; Rehmani et al., 2015; Siddique et al., 2013). Since wild birds are known to be reservoir of APMVs, viruses of low virulence could emerge as velogenic NDV that carry mutation in the Fₐ cleavage site (Alexander, 2013). Moreover, phylogenetically related NDVs of class II, possibility causing disease outbreaks, have been isolated from wild birds (Miller et al., 2010) and pigeons (Dortmans et al., 2011). Due to these complications, it is imperative to investigate the evolutionary trends in field strains together with their potential pathobiology in vaccinated and non-vaccinated poultry birds.

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**Fig. 3.** Serological monitoring and survival curves for studied isolates. (a) Comparison of pre- (pre-ch) and post-challenge (post-ch) seroconversion of sera (GMT) samples in chickens that were immunized with LaSota and killed vaccine. Sera were taken prior to challenge on day 27 and post-challenge on day 40. Increase in titre after the virus challenge indicates successful infection and replication of challenged virus in vaccinates. An elevated titre in birds challenged with pigeon isolate (lineage 4/ genotype VI) was observed compared with peacock isolate (lineage 5/genotype VII), indicating partial cross-reactivity to vaccine strains (genotypes II). (b) Percentage survival rates of vaccinates (VCh) and non-vaccinates challenged (Ch) with peacock isolate, or left unchallenged serving as control (non-vaccinated and immunologically naive). (c) Percentage survival rates of VCh and Ch with pigeon isolate or left unchallenged serving as control. Vaccinates and challenged groups were infected with peacock and pigeon isolates on day 28. All birds in challenged group were died on 7 days p.i. Birds in vaccinates group showed partial protection to challenged isolates.
Fig. 4. Microscopic examination of different tissues collected from chicken infected with pigeon (upper panel) and peacock (lower panel) isolates. Arrows indicate lesions in the affected tissues. (a–f) Histopathological lesions in different tissues collected from chickens which were infected with Pigeon/MZS-UVAS-Pak/2014 isolate. (a) Engorgement of red blood cells in the portal vein. (b) Swollen and coagulated renal tubules revealing cellular swelling in epithelial cells and severe congestion in peritubular capillaries. Epithelial cells in some renal tubules are separated from the basement membrane and accumulated in the lumen. (c) Fewer lymphoid follicles, breakdown and sloughing of columnar epithelium are obvious. (d) Prominent haemorrhages and degenerative changes in the lining of the trachea. (e) Congestion in the parabronchial blood capillaries. (f) Primary
pathological changes include excessive epithelial damage and villi disruptions. (g–i) Histopathological lesions in chicken tissues infected with Peacock/QOL-UVAS/Pakistan/2014 and Pigeon/QOL-UVAS/Pakistan/2014. (g) Clear vacuoles with variable sizes and fatty changes in the cytoplasm of many hepatocytes, and nuclei were displaced towards the periphery. Some sinusoidal capillaries were also congested (engorged with nucleated red blood cells). (h) Congestion peribulbar areas, swollen renal epithelial cells, intact basement membrane and sloughing tubular epithelial cells. (i) Congestion and haemorrhages are observed. There is degeneration in the lymphoid follicles. (j) Absence of pseudostratified columnar epithelium, presence of haemorrhages and degenerative changes in the lamina propria. (k) Lung tissues and lining are severely congested. (l) A large number of sloughed off epithelial cells were observed in the lumen of the intestine. Necrotic and degenerated intestinal villi were in abundance. (m–n) Histology of normal tissues: liver, kidney, caecal tonsil, trachea, lung and small intestine collected from the mock-infected group.

The genotypes VI and VII represent the most prevalent group of APMVs in Southeast Asia and all APMVs isolated from wild captive birds either belong to genotype VI or VII (Kim et al., 2007). Two APMV isolates recovered from pigeon and peacock flock were classified as genotype VI/lineage 4 and VII/lineage 5 compared with the vaccine strains (genotype II/lineage 1 and 2). Analysis of the deduced residues at the cleavage site for the isolates in the current study and previously reported isolates from Pakistan indicated co-circulation of different genotypes of APMVs in the country with similar pathogenicity (based on the motif in the cleavage site). This is of importance since invariability in residue pattern has been observed for velogenic NDVs in a given geographical location (Samuel et al., 2013). The presence of polybasic F-protein cleavage site, mean death time (48–64 h), severe clinical and macroscopic and microscopic lesions suggest the virulent nature of these isolates. The sites for glycosylation and cysteine residues are believed to be conserved for F and HN proteins. However, in comparison to each other and to the representative genotype particularly of the vaccine strain, we found differences in the composition of residues for a given glycosylation site and variations in both the number and position of cysteine residues. Furthermore, comparison of functional domains of F and HN protein to other genotypes and vaccine strain revealed several substitutions that were more often in the F protein than in the HN protein. The substitutions particularly in the fusion peptide, hydrophobic regions and transmembrane region of the F protein and neutralizing epitopes of the HN protein could result in altered fusion activity and neutralizing escape variants (Hu et al., 2009, 2010; Hu, 2015; Wang et al., 2015). Taken together, variation in nucleotide and subsequent substitutions/alternations in amino acid profile as observed in this study is consistent with previously described theories of evolution of RNA viruses, particularly the APMVs (Umali et al., 2013; Yu et al., 2001).

Phylogenetic analysis of the complete genome and hypervariable region (F gene, 374 bp) of pigeon-originated APMVs revealed evolutionary relationships to lineage 4/genotype VI (sub-genotype VIIbii), which is predominantly presented by the pigeon isolates (PPMV-1). The APMVs belonging to the same genotype have been reported previously in Sindh (genotype VI) (Khan et al., 2010) and Khyber Pakhtunkhwa (genotype Vic) (Shabbir et al., 2013b) provinces indicating both circulation in the environment and their ability to cause disease outbreaks in chicken flocks. However, the presented complete genetic and clinicopathological characterization of pigeon-originated NDVs (PPMV-1, VIIbii) is the first of its kind in Pakistan. The presented isolate showed a different cleavage motif (112RRQKR117) compared with the viruses isolated before the 1980s (112GRQKR117). Given the significant genetic variability in PPMV-1 viruses belonging to VIIb, the viruses were divided into two major sub-groups (VbI and VIIbii). The isolates belonging to VIIbii have been observed as predominant strains in the latter period of the pigeon-originated pandemic, while cases caused by strains of genotype VIIb have known to have diminished in the late 1980s (Aldous et al., 2003; Awu et al., 2015) imitating selective pressure from vaccine usage (Aldous et al., 2004). Although it is difficult to speculate on the origin of the virus of genotype VIIbii in the current study, its genetic relatedness to an isolate from Russia, closer than relationships to recently reported PPMV-1 viruses from China [(Vla and Vlb) (Awu et al., 2015; Wang et al., 2015)] indicate possible introduction via migration of wild birds.

The peacock isolate clustered within lineage 5/genotype VII (sub-genotype VIIii) was found to be closely related to an isolate previously reported from chickens in Pakistan. Lineage 5/genotype VII is thought to have originated from the Far East with the first isolation from Taiwan in the 1980s (Yang et al., 1999). Since then, there have been detections of this genotype in various parts of the globe (Aldous et al., 2003). A number of velogenic NDV strains, with variable genetic diversity at the sub-lineage level, have been reported from several Asian countries including those that share borders with Pakistan (Aldous et al., 2003; Tirimurugan et al., 2011; Zhang et al., 2011). NDV strains genetically related to sub-genotype VIIia have been previously reported from wild birds, while sub-genotype Vlc, Vlb and novel VIIa were reported from backyard and commercial poultry (Munir et al., 2012b; Rehmani et al., 2015; Siddique et al., 2013). Results presented here demonstrated the identification of lineage 5/genotype VII for the first time in peacock and highlight the transmission potential of such APMV strains among different avian hosts.

Since genotype VI and VII are the most successful APMV groups and the majority of the recent reports on disease outbreaks are associated with either genotype VI or genotype VII, we envisaged to assess the pathobiology of the
representative isolates from each genotype. Moreover, it is important to investigate the infectious nature of isolates that are originated from non-chicken hosts but cluster within the group of APMVs that are reported from domesticated chickens. In the assessment of the clinicopathological impact of these isolates, we observed sudden deaths in challenged birds and in one of the vaccinated group. This was expected, as death with no apparent clinical indications is considered the most noteworthy evidence of velogenic NDVs. Similar observations have been reported by Samuel et al. (2013) in immunologically naïve birds challenged with virulent isolates of African origin. Although severity of observed clinical signs was relatively less for the pigeon isolate than the peacock isolate, the morbidity, mortality and virus shedding was comparable between these groups. Interestingly, all birds in the non-vaccinated group challenged with peacock isolate died on day 7 p.i. due to severe neurological disease. The nervous signs were also prominent in the vaccinated group, potentially indicating lack of complete protection induced by the vaccine.

It has been observed that pigeon-originated APMVs with typical F protein cleavage site for velogenic strains (GRQKRF$^{112}$ or RRKKRF$^{112}$ or RRQKRF$^{112}$) do not result in significant disease in poultry and differences in pathogenicity index vary from moderate to no virulence for chicken (Collins et al., 1994; Dortmans et al., 2011). It has been suggested that a low viral replication rate and an increased antibody level are responsible for the low pathogenicity of pigeon viruses in chickens (Awu et al., 2015). In contrast, some PPMV-1 viruses can gain high pathogenicity in chickens if passed either in chickens or chicken embryos, indicating their potential to cause ND outbreaks (Dortmans et al., 2011), as observed in the current study. Furthermore, variants of genotype VIb originating from pigeon have been shown to produce neurological signs (Ujvari et al., 2003).

While comparing pre- and post-challenge antibody titres, we found varying but an increasing immune response indicating that birds were sufficiently exposed to the virus antigen. The immune response generated by challenge with the pigeon isolate was greater than that generated by the peacock isolate indicating its efficient replication (Fig. 3a). Further nervous signs were evident 1 day earlier in birds challenged with the pigeon isolate compared to the peacock isolate. A potential reason could be the fact that both groups of vaccinates were administered killed vaccine containing genotype VII that may have hindered replication of challenged virus of genotype VII more than genotype VI. Viral shedding together with increase in antibody titre suggests that the commonly practised vaccine schedule and vaccine types (LaSota and killed vaccine of genotype VII) give partial protection from disease, and are unable to protect from infection and virus replication, at least in experimental conditions. Moreover, a lower cross-reactivity was observed between sera collected from LaSota-immunized birds and isolates collected from pigeon and peacock. Taken together, genetic differences observed in the functional domains and neutralization epitopes of the F and HN protein of field isolates and vaccine strains could be attributed to increased virulence and escape from vaccines.

Since there is only one serotype for all APMVs, lentogenic strains (e.g. LaSota or B1) are being used as live-attenuated vaccine to protect birds from velogenic NDVs. These classic vaccines are known to prevent disease but not infection; replication and shedding of the virus occur even in vaccinated animals. Due to the increasing wave of ND outbreaks from 2008, killed vaccines from genotype VII have been included into the vaccination schedule in ND-endemic countries. Given the fact that vaccine strains belong to genotype II and have been isolated approximately 60–70 years ago, whereas most of the circulating strains are clustered within genotype VI or VII, it is plausible that the genetic distances reflect antigen differences and may result in lack of protection against field viruses, disease progression and shedding of virulent APMVs in the environment. Owing to extensive vaccination in broilers (three to four shots of vaccinations in a lifespan of 40 days), it is reasonable that vaccination could be problematic and outweigh the benefits.

Two isolates, originated from pigeon and peacock, were genotypically and pathobiologically characterized. Both isolates had the potential to cause disease and subsequent virus shedding even in vaccinated birds. The results presented may be useful in revising the vaccine schedule being practised currently in Pakistan and other ND-endemic countries. Furthermore, it ascertains the need to establish and maintain active surveillance for NDVs in wild birds.

METHODS

Sampling history and virus stocks. Clinical samples from captive wild birds were collected from two independent outbreaks in private farms around the Lahore district, Punjab, Pakistan. A brief history and relevant details of each isolate are given in Table 1. Trachea, lungs, spleen and caecal tonsils from dead birds (n=15) of each flock were collected aseptically, pooled and processed for virus isolation through the chick embryo inoculation system. Due to extensive vaccination in broilers (three to four times for peacock isolates) (Alexander et al., 1998) followed by identification through spot haemagglutination assay and PCR, as we described earlier (Munir et al., 2012b; Shabbir et al., 2013b). Based on the species of isolation, these isolates were named as Peacock/MZS-UVS-Pak/2014 and Pigeon/MZS-UVS-Pak/2014. Infectivity Embryo Infectious Dose 50 (EID$_{50}$) of these isolates was determined separately in 9–11-day-old embryonated chicken eggs and infectious allantoic fluid of each isolate was pooled together followed by centrifugation at 4000 r.p.m. for 10 min (Allegra XR15 centrifuge). Viruses, titrated by haemagglutination assay, were stored at −40 °C until further use.

Next-generation sequencing. From each isolate, viral RNA was extracted separately using a commercially available RNA extraction kit (QIAamp Viral RNA Mini kit; Qiagen) as per the manufacturer’s instructions. Quantity (NanoDrop) and quality (Qubit fluorometer) of extracted RNA were measured and subjected to next-generation sequencing based whole-genome sequencing (Macrogen). Briefly, a library was prepared with 1 µg of total RNA by Illumina TruSeq mRNA Sample Prep kit (Illumina). The RNA was fragmented using divalent cations under elevated temperature. Using SuperScript II reverse transcriptase (Invitrogen) and random primers, the cleaved RNA fragments
were copied into first and second strand cDNA synthesis using DNA polymerase I and RNase H. The cDNA fragments were then subjected to an end-repair process, the addition of a single ‘A’ base and ligation of the indexing adapters. The products were purified and enriched through PCR to create a final cDNA library. The libraries were quantified using qPCR quantification protocol guide (KAPA Library Quantification kits for Illumina sequencing platforms) and qualified using the TapeStation D1000 ScreenTape (Agilent Technologies). Indexed libraries were then sequenced through a HiSeq 2500 platform (Illumina).

Adapters were trimmed from the raw data and reads mapping to contaminants (rRNA, chicken or human sequences) were removed using both SortMeRNA (Kopylova et al., 2012) and Burrows-Wheeler Alignment tool (BWA-MEM) methods (Li & Durbin, 2010). Unmatched sequence reads were assembled using de novo SPAdes assembler software (version 3.5.0) (Bankevich et al., 2012). All assembled contiguous sequences (contigs) were aligned to the reference genome (LaSota strain, accession number AY845400) using LASTZ (Harris, 2007) to identify and extract maximally aligned viral contigs. To further improve contigs, all raw reads of each segment were mapped back to the assembled contigs. Finally, the consensus sequences from the re-mapping reads and LASTZ contig alignment were obtained using SAMtools command lines (Li et al., 2009).

**Sequence and phylogenetic analysis.** Complete genome sequences of reference strains of each recognized lineage (Aldous et al., 2003) and genotype (Diel et al., 2012) of APMVs as well as the vaccine strain were used to determine percentage similarity of coding regions together with detailed analysis of deduced residues for F and HN protein. To elucidate the phylogenetic relationships between APMV-1 and PPMV-1 viruses reported here and characterized previously from Asia and other parts of the world, we first compiled a dataset of available complete genomes of APMV-1 and PPMV-1. For global and high-level clustering patterns, we executed and sampled every 1000 generations using the default parameters of the priors’ panel. The analysis was based on the GTR+I+G model, which allow significantly changed posterior probability estimates. Phylogenetic relationship was also established with the MEGA version 6.0 software programme using the neighbour-joining method with the Kimura two-parameter model. The evolutionary distances were inferred using the pairwise distance method and expressed as the number of nucleotide substitutions per site giving a statistical significance of the tree topology by 1000 bootstrap resampling of the data (Tamura et al., 2013).

For clarity on association of these viruses with previously characterized Pakistani strains, the lineage-based nomenclature was used in this study as described by Aldous et al. (2003). The corresponding genotypes were also displayed where needed for comparison purposes (Aklous et al., 2003).

**Immunization and challenge experiment.** Clinicopathological assessments of the isolates in immunized, challenged, mock-infected and contact birds were performed individually. Eighty 1-day-old chicks (Hubbard) were procured from a commercial hatchery and raised until the end of the experiment (day 40) at the Experimental Unit in the Department of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan. Feed and water were provided ad libitum.

Birds in vaccinated (VCh) and mock-infected (Mock) groups were divided into three groups named challenged (Ch, n=10), contact (Contact, n=5) and mock-infected (Mock, n=5). Birds in vaccinated (VCh, n=20) were kept in groups of 20 for each isolate. Replicating the vaccination schedule commonly practised by the broiler industry in the country, the birds in VCh group were administered LaSota vaccine (Laprovect) twice on day 7 and 25 and one intramuscular injection of killed vaccine (genotype VII) on day 11 as per the manufacturer’s instructions. The lyophilized vaccine (LaSota, 1000 doses), used for primary immunization, was dissolved in vaccine-provided sterile buffer and administered via eye drops individually to each bird. For boosting, a nationally manufactured and widely used in the field oil-based killed vaccine was applied. The vaccine was administered with a dose of 0.3 ml per bird via subcutaneous route around the neck region.

<table>
<thead>
<tr>
<th>Bird</th>
<th>Age (months)</th>
<th>Birds in flock</th>
<th>Mortality (24 h)</th>
<th>Sample collection date</th>
<th>Vaccination with LaSota strain</th>
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<tr>
<td>Peacock</td>
<td>16</td>
<td>128</td>
<td>5−8</td>
<td>31 March 2014</td>
<td>Yes</td>
<td>Respiratory distress, lack of coordination in movement, head tossing. Necropsy</td>
<td>1:256</td>
<td>10^6.41</td>
<td>112-RQKRF^1,17</td>
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<td>Pigeon</td>
<td>5</td>
<td>56</td>
<td>3−7</td>
<td>13 December 2014</td>
<td>Yes</td>
<td>Dyspnoea, circling of head, greenish diarrhoea. Pinpoint haemorrhages were seen on proventriculus upon necropsy</td>
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Table 1. Brief description of isolates reported in this study

HA, Hemagglutination assay.

were collected 3 months after the initial collection. For global and high-level clustering patterns, we first compiled a dataset of available complete genomes of APMV-1 and PPMV-1. For global and high-level clustering patterns, we first compiled a dataset of available complete genomes of APMV-1 and PPMV-1. Next collected the 3 hypervariable region (374 bp) of F gene from all available sequences. Both these datasets were aligned in BioEdit version 7.2.5 and executed and sampled every 1000 generations using the default parameters of the priors’ panel. The analysis was based on the GTR+I+G model, which allow significantly changed posterior probability estimates. Phylogenetic relationship was also established with the MEGA version 6.0 software programme using the neighbour-joining method with the Kimura two-parameter model. The evolutionary distances were inferred using the pairwise distance method and expressed as the number of nucleotide substitutions per site giving a statistical significance of the tree topology by 1000 bootstrap resampling of the data (Tamura et al., 2013).

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All birds were divided into two groups as vaccines and non-vaccines (n=40 each) and housed separately. For each isolate, birds in non-vaccines were divided into three groups named challenged (Ch, n=10), contact (Contact, n=5) and mock-infected (Mock, n=5). Birds in vaccines (VCh, n=20) were kept in groups of 20 for each isolate. Replicating the vaccination schedule commonly practised by the broiler industry in the country, the birds in VCh group were administered LaSota vaccine (Laprovect) twice on day 7 and 25 and one intramuscular injection of killed vaccine (genotype VII) on day 11 as per the manufacturer’s instructions. The lyophilized vaccine (LaSota, 1000 doses), used for primary immunization, was dissolved in vaccine-provided sterile buffer and administered via eye drops individually to each bird. For boosting, a nationally manufactured and widely used in the field oil-based killed vaccine was applied. The vaccine was administered with a dose of 0.3 ml per bird via subcutaneous route around the neck region.

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Serum antibodies were assessed on day 27 by HI assay before challenge with study isolates. Each bird in the Ch and VCh group was inoculated with 1 ml of 1000 EID₅₀ ml⁻¹ dose of the isolate through the ocularnasal route on day 28. After 8 h challenge, the contact birds were kept together with challenged (Ch) birds of each isolate to understand the horizontal transmission of both viruses. The mock-infected group served as negative control and was administered 1 ml of sterile normal saline. For the next 12 days, birds were monitored twice daily for clinical signs that included depression, anorexia, sneezing/coughing, facial swelling, respiratory sounds, ocular/nasal discharge, conjunctivitis, ruffled feathers, reluctance to move, dyspnoea, greenish diarrhoea, paralysis, tremors, opisthotonus, twisting of head and neck, circling and paresis among others. Until the appearance of clinical signs and necropsy lesions, which are definitive of NDV (haemorrhages in proventriculus and caecal tonsils), a bird from VCh and Ch group was sacrificed daily for detailed necropsy analysis. Based upon typical necropsy lesions of NDV, tissue tropism and histopathology were performed in selected birds. All the remaining birds were maintained to observe clinical signs until the end of study period. Contact birds were sacrificed on every alternative day until 10 days p.i. for necropsy lesions together with collection of oropharyngeal and cloacal swab to monitor shedding of the challenged virus. Birds with severe clinical signs (particularly nervous signs) were euthanized immediately according to the procedures set by the ethical committee.

**Determination of antibody titres.** Sera samples obtained from birds before and after the challenge were tested for antibodies against NDV using haemagglutination inhibition assay described by the OIE Manual of Standard Diagnostic Tests (Afonso et al., 2012). Titres were calculated as the highest reciprocal serum dilution giving complete inhibition in 96-well microtitre plate and antibody titres (1:8 or 2³) or lower were considered negative for NDV/APMVs (Miller et al., 2013). At the end of the experiment, on day 40, all birds in VCh group were sacrificed and blood was processed for HI assay.

**Evaluation of virus shedding and viscerotropism.** The virus shedding from contact birds and tissue tropism in VCh and Ch groups was determined through previously described assays for identification of velogenic and mesogenic strains of NDVs (Wise et al., 2004). Virus shedding was evaluated through oropharyngeal and cloacal swabs, whereas various organs/tissues such as whole blood, brain, harderian gland, tongue, trachea, lung, heart muscle, breast muscle, hair follicles, gizzard, proventriculus, liver, kidney, spleen, bone marrow, intestine, caecal tonsils, bursa and cloacal tissues were processed to track the distribution of challenged viruses.

**Histopathology.** Selected tissues were collected and fixed by immersion in 10% neutral buffered formalin at room temperature for 48 h followed by processing and embedding in paraffin wax. Tissue sections of 5 µm were stained with haematoxylin and eosin and examined for characteristic lesions in 10% neutral buffered formalin at room temperature for 48 h following fixation. The sections were then stained with haematoxylin and eosin and examined for microscopic lesions under light microscope.

**Ethical statement on animal use.** All animal studies and procedures were carried out in strict accordance with the guidance and regulations of Animal Welfare and Health. As part of this process, the work has undergone scrutiny and approval by the ethics committee at the University of Animal and Veterinary Sciences, Lahore, Pakistan.

**ACKNOWLEDGEMENTS**

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