Title:
Comparative immunocompetence and interspecies transmission of Avian Orthoavulavirus-1 in feral birds originating from rural and urban settings

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Abstract
We hypothesized that urban feral birds carry higher immunocompetence compared to rural birds due several anthrophonic factors. Therefore, we evaluated phytohaemagglutinin (PHA)-induced immunocompetence of five feral bird species and studied the potential transmission of Avian orthoavulavirus-1 (AOAV-1) in commercial chickens. Patagium thickness was measured at specific intervals (12, 24, 36, 48, and 60 hours) following the administration of 0.1 mL (1 mg/mL) of PHA. We observed greater swelling response in urban birds 48 hours post-stimulation: pigeon (1.262 mm ±0.03), sparrow (0.235 mm ±0.02), and crow (1.10 mm ±0.03) compared to rural birds: pigeon (0.980 mm ±0.04), sparrow (0.194 mm ±0.03), and crow (0.855 mm ±0.04). Urban pigeons had significant differences in patagium thickness at all-time intervals ($p=0.000$) except 24 hours ($p=0.12$). Rural and urban quails and crows differed significantly at all-time intervals except 12 hours ($p=0.542$ and $p=0.29$). For interspecies transmission, feral bird groups were housed with naive broiler birds (n=10 each) and challenged with AOAV-1 (Mallard-II/UVAS/Pak/2016) at 1 mL (10^8 EID_{50}/mL). We noticed that urban birds exhibited higher resistance to the virus compared to rural birds. Taken together, these findings highlight the immunocompetence of feral bird species and their role in AOAV-1 transmission. Continuous monitoring, surveillance, and strict biosafety measures are crucial for controlling AOAV-1 spillover from wild birds to commercial poultry especially in resource-limited countries.

Keywords: Feral birds, Immunocompetence, Patagium, Avian Orthoavulavirus, Inter-species transmission

Introduction

Immunocompetence is the ability of an individual's immune response to prevent or control pathogens and parasites infection [1], and is a vital determinant of survivability[2]. In the field of immune-ecology and ecotoxicology, the phytohaemagglutinin (PHA)-induced skin swelling has gained prominence as a reliable attribute to immunocompetence[3-5]. Subcutaneous injection of PHA effectively activates both the innate and adaptive immune systems and offers the advantages of simplicity, rapid execution, and feasibility in field conditions[4]. The PHA-mediated immunocompetence is mechanistically achieved by infiltration of leukocytes, stimulating specifically T-lymphocytes and delayed-type hypersensitivity responses at the site of inoculation and systematically, culminating in the enhance capacity of hosts in controlling certain types of viral infection[3-5].

Owing to challenges of maintaining strict biosafety and biosecurity measures in populated or urbanized areas of developing countries including Pakistan, there is an increased risk of pathogen exposure to the environment[6,7]. Compounded by the urbanization, an elevated occurrence of diverse pathogens across various host species, including free-flying birds have been reported with a potential to spillover to people[8]. Therefore, we hypothesis that feral birds living in close proximity to humans (urban settings) exhibit heightened immunocompetence compared to their counterparts in more remote rural areas. Consequently, we propose that urban feral birds possess better resistance against the country's endemic pathogens, such as the velogenic strain of avian orthoavulavirus-1 (AOAV-1) which causes clinical Newcastle disease (ND) in wild birds and poultry (commercial and backyard). Wild/feral birds, including waterfowl, shorebirds, doves, pigeons, double-crested cormorants, and other free-flying birds serve as continuous reservoirs of AOAV-1 and shed the virus into the environment without displaying typical clinical signs[9-11]. However, there is a paucity of experimental evidence on the spectra of immunocompetence among feral birds representing either urban or rural settings. Additionally, the velogenic genotypes of ND does not translate into its pathotype potential in domestic and feral birds and therefore enhances the risk of asymptomatic transmission of viruses to birds and possibility to human.

In this study, we evaluated comparative immunocompetence among multiple bird species originating from urban and rural settings, followed by assessing each species' susceptibility to the AOAV-1 challenge. We assessed subsequent pathobiology, transmission, and shedding of viruses into the environment. The findings have enhanced our understanding of feral bird immune responses and their potential roles in AOAV-1 transmission and pathogenicity. The insights gained will contribute to the development of effective strategies for disease management and control in both commercial and backyard poultry sectors. Intriguingly, this research highlights the implications for viral spillover on both poultry and public health.

Results

Assessment of immunocompetence

Different urban and rural (AOAV-1 and Influenza virus) negative feral birds were exposed to PHA before challenge with the virus and clinico-pathological examination (Fig. 1). Analysis indicates that the PHA stimulation resulted in visible swelling and a significant increase in the thickness of the wing web in the tested bird species (pigeon, sparrow, mynah, crows and quails). Intriguingly, all urban bird species exhibited a strong response to PHA in the right-wing, reaching its maximum at 48 hours and declining at 60 hours (Fig. 2). However, there were variations in the magnitude of

swelling among and within bird species at different time intervals. Notably, urban birds demonstrated a stronger swelling response compared to their rural counterparts. For example, at 48 hours, the difference in patagium thickness between urban and rural pigeons was 1.262 mm ± 0.03 versus 0.980 mm ± 0.04. In sparrows, it was 0.235 mm ± 0.02 versus 0.194 mm ± 0.03, whereas in crows it was 1.10 mm ± 0.03 versus 0.855 mm ± 0.04. Comparison of immunocompetence between rural and urban birds at different time intervals revealed significant differences in patagium thickness for pigeons at 12 hrs (p=0.000), 36 hrs (p=0.003), 48 hrs (p=0.000), and 60 hrs (p=0.000). Similarly, significant differences in patagium thickness were observed between urban and rural sparrows at 36 hrs (p=0.001), 48 hrs (p=0.000), and 60 hrs (p=0.000). At the end of the experiment, a significant association for immunocompetence was observed between rural and urban sparrows (p=0.000). In mynah birds, patagium thickness showed a significant increase at each time interval, with a relative increase in thickness observed in the urban setting. Quail and crow species also exhibited significant associations at each interval except for 12 hrs (p=0.542 and p=0.29, respectively). Overall, a significant association for immunocompetence was observed in feral bird species originating from both rural and urban settings (p=0.000-0.002) (Table 1).

Interspecies transmission potential and pathogenicity of *Avian orthoavulavirus*-1 in Pigeons

Clinical signs, including off-feed, general sickness (depression, isolation, and lethargy), and twisting of the head and neck, were first observed in two pigeons (RNIC) on the fourth day post-infection (dpi) (Fig. 3). One of these pigeons died on the fifth dpi. Two birds from the urban group (one each from UIC and UNIC) exhibited general sickness (off-feed and isolation) on the fifth dpi. The bird from UIC died on the sixth dpi, while the other bird from UNIC showed circling movement and head twisting, necessitating euthanasia on the same day. In rural pigeons, five deaths occurred on the fifth (n=1, RNIC), eighth (n=2, one each from RNIC and RIC), eleventh (n=1, RNIC), and thirteenth (n=1, RIC) dpi due to severe neurological signs. Infected birds displayed additional clinical signs, such as imbalance in flight, twisting of the head and neck, circling movement, wing and leg paralysis, and conjunctivitis (Fig. 3A-I). The survival rates for urban and rural pigeons were 80% and 50%, respectively (Fig. 4A). Virus shedding was confirmed in all challenged pigeons through the inoculation of oral and cloacal swabs into 9-day-old embryonated chicken eggs at the third, fifth, seventh, and ninth dpi. The challenged virus replicated systemically in various organs, as demonstrated by the detection of AOAV-1 genome in tissues collected from morbid or dead pigeons using F-gene-based RT-PCR (Table 2). Microscopic examination revealed sinusoidal congestion in the liver (Fig. 6A-B), congestion, necrosis, and hemorrhages in the lungs (Fig. 6C), mild congestion in the brain (Fig. 6D), and infiltration of inflammatory cells in the baseline of villi (Fig. 6E). Congestion in the gizzard was also observed (Fig. 6F). At the end of the experiment, five pigeons from the rural group (three RNIC and two RIC) and eight pigeons from the urban challenged groups (six UIC and two UNIC) survived. The antibody titer (GMT) in the survived birds was found to be 1:64. Mock birds either tested negative or exhibited negligible antibody titers (1:8).

Confirmation of AOAV-1 shedding was further performed through the disease observation in contact sentinel birds (commercial broiler chicks co-housed with challenged feral birds). Mild clinical signs, including dullness and isolation, were first evident on the third dpi. Clinical signs worsened in morbid birds on the fourth dpi, with one sudden death. By the sixth dpi, all contact birds exhibited respiratory signs (sneezing, coughing, gasping, and oculonasal discharge),

digestive signs (greenish diarrhea), and nervous signs (wing dropping and leg paralysis) (Fig. 5 A-I). Detailed clinical examination and relevant data records for the contact birds (broilers) were performed Supplementary File S2).

Interspecies transmission potential and pathogenicity of *Avian orthoavulavirus*-1 in Sparrow

Clinical signs were first observed in rural sparrows (RNIC) on the seventh day post-infection (dpi). The infected sparrows exhibited neurological signs, including head shivering and complete leg paralysis, and succumbed on the same day (7th dpi). Two more sparrows from the rural group (one each from RIC and RNIC) died on the twelfth dpi, displaying body tremors and leg paralysis. Another sparrow (UNIC) died on the ninth dpi, exhibiting body tremors, wing, and leg paralysis (Fig. 3). The survival rates for urban and rural sparrows were observed to be 90% and 70%, respectively (Fig. 4B). At the conclusion of the experiment, nine sparrows from the urban group (six UIC and three UNIC) and seven sparrows from the rural group (three RNIC and four RIC) had survived. However, virus shedding was detected at all sampling days (3rd, 5th, 7th, and 9th dpi) in the challenged sparrows. Replicating virus was identified in various tissues (tongue, heart, brain, liver, muscle, and gizzard) using F gene-specific RT-PCR (Table 2). Microscopic examination revealed notable changes in the brain, liver, lung, and intestine of morbid sparrows. Liver tissue exhibited congestion and hydropic degeneration of hepatocytes (Fig. 6G), while brain tissue showed cell degeneration (Fig. 6H). The intestinal epithelium displayed necrosis and degeneration (Fig. 6I), and the lungs exhibited severe congestion, hemorrhages, and necrosis (Fig. 6J). The antibody titer (GMT) in surviving sparrows was determined to be 1:128. Mock birds either tested negative or exhibited negligible antibody titers (1:8).

Interspecies transmission of the virus was also assessed through contact with broiler birds. Clinical signs in the contact birds initially appeared on the third dpi, accompanied by the death of three birds in the morning. By the fourth dpi, two more deaths were reported, and three birds exhibited clinical signs such as isolation, dullness, off-feed, and coughing. Clinical signs worsened on the fifth dpi, including oculonasal discharge and partial paralysis. All remaining birds were infected by the seventh dpi. Two infected birds were euthanized, and necropsy revealed an enlarged liver, mottled spleen, pin-point hemorrhages in the proventriculus, and edematous bursa. Detailed morbidity and mortality data for the contact birds are provided in Supplementary File S2.

Interspecies transmission potential and pathogenicity of *Avian orthoavulavirus*-1 in Mynah

In the challenged group, only one mynah (from the RNIC group) displayed nervous signs by the ninth day post-infection (dpi). The infected bird exhibited head shivering and imbalanced flight. It was euthanized on the tenth dpi, and no gross lesions were observed. No other bird in the challenged group showed any clinical signs throughout the experiment. The survival rates for urban mynahs were 100%, while rural mynahs had a survival rate of 90% (Fig. 4C). Virus shedding was detected in challenged birds at the 3rd, 5th, 7th, and 9th dpi. Viral RNA was identified in the brain, ileum, gizzard, liver, and heart muscle of the infected bird. However, no RNA was detected in the tongue and lung tissues (Table 2). Microscopic examination revealed the presence of abundant glial cells and intra-nuclear inclusion bodies in certain brain cells (Fig. 6K). The liver displayed fatty changes, with a distinctive halo observed around the hepatocytes, and the cytoplasm appeared lighter (Fig. 6L). In the heart muscles, degeneration, fragmentation, and loss of sarcoplasm were observed (Fig. 6M). The intestine and trachea showed no significant abnormalities. By the end of the experiment, only one death (from the RNIC group) was observed,

and all other challenged birds (8 UIC, 2 UNIC, 2 RNIC, 7 RIC) had survived. The antibody titer (GMT) in the surviving birds was determined to be 1:256. Mock birds either tested negative or exhibited negligible antibody titers (1:8).

Interspecies transmission of AOAV-1 was observed through contact with broiler birds. On the third dpi, one bird was found dead in the morning, and two birds exhibited isolation from the rest of the flock. By the fourth dpi, three more birds were infected and displayed clinical signs such as isolation and reduced feed intake. Two infected birds died on the fifth dpi. Clinical signs worsened on the sixth dpi, with affected birds showing general sickness, respiratory, digestive, and nervous signs typical of Newcastle disease. Infected birds continued to die or were culled (due to severe clinical signs) until the eighth dpi. Necropsy examination revealed congested lungs, enlarged liver, hemorrhages in the trachea and proventriculus, and a foul smell in the intestine. A comprehensive record of clinical signs and mortality data for the contact broiler birds is available in Supplementary File 2.

Interspecies transmission potential and pathogenicity of *Avian orthoavulavirus*-1 in Quail

In the rural group (RNIC), two quails showed general sickness, including isolation and reduced feed intake, on the third day post-infection (dpi) (Fig. 3). On the fourth and fifth dpi, one bird each from the RNIC group died without exhibiting any clinical signs. Similarly, at the seventh dpi, one quail from the urban group (UNIC) died. All other challenged birds survived until the end of the experiment. The percent survival for urban quails was 90%, while rural quails had a survival rate of 80% (Fig. 4D). The remaining challenged quails (7 UIC, 2 UNIC, 3 RNIC, 5 RIC) survived until the end of the experiment. The antibody titer (GMT) in the surviving birds was determined to be 1:128. In contrast, the antibody titer in the mock birds was either negative or negligible (1:8). All mock birds remained healthy throughout the experiment. No gross lesions were observed in the deceased birds. Tissue tropism was limited to the digestive system, as viral RNA was detected only in the ileum, duodenum, and gizzard. Virus shedding was observed at all sampling days (3rd, 5th, 7th, and 9th dpi) in the challenged quails (Table 2). Microscopic changes were observed solely in the lung samples of infected quails, showing mild congestion (Fig. 6N).

Regarding the interspecies transmission experiment, two contact broilers within the isolator were isolated on the second dpi without displaying any clinical signs. On the third dpi, three birds died, while another three birds showed dullness, isolation, and reduced feed intake. On the fourth dpi, three infected birds died, and the remaining birds exhibited clinical signs such as isolation, reduced feed intake, and oculonasal discharge. By the sixth dpi, all birds were infected and subsequently slaughtered due to severe respiratory and nervous signs typical of Newcastle disease virus infection. Necropsy examination revealed congested liver and lungs, a foul smell in the intestine, oedematous bursa, and pin-point hemorrhages in the proventriculus. A supplementary file (Supplementary File 2) is provided, containing a detailed record of clinical signs and mortality data for the infected and deceased birds throughout the experiment.

Interspecies transmission potential and pathogenicity of *Avian orthoavulavirus*-1 in Crow

Mild depression and body tremors were observed in two birds, one from the urban group (UIC) and the other from the rural group (RNIC), on the seventh day post-infection (dpi). However, the birds appeared healthy the following day. Unfortunately, the same bird from the rural group (RNIC) succumbed to the infection on the tenth dpi. Additionally, two sudden deaths were

observed on the twelfth dpi, one in the urban group (UIC) and the other in the rural group (RNIC). The percent survival for urban crows was 90%, while rural crows had a survival rate of 80% (Fig. 4E). By the end of the experiment, nine urban crows (7UIC, 2UNIC) and eight rural crows (5RIC, 3RNIC) had survived. Viral shedding was detected exclusively in cloacal swabs at the third dpi. Viral RNA was specifically detected in the gizzard tissue (Table 2). In terms of histopathological changes, significant alterations were observed in the liver tissue of diseased crows, including sinusoidal congestion and hepatocyte degeneration (Fig. 6O). Mild congestion and degeneration were also observed in the intestine (Fig. 6P). The antibody titer (GMT) in surviving birds was determined to be 1:512. In contrast, the mock group exhibited either negative or negligible antibody titers (1:8).

Regarding interspecies transmission, contact broilers showed clinical signs of infection on the fourth dpi, including isolation and reduced feed intake. Two infected birds died on the fifth dpi, and one sudden death occurred on the same day. Clinical signs worsened on the sixth dpi, with infected birds displaying oculonasal discharge, coughing, conjunctivitis, and greenish diarrhea. All birds had succumbed to the infection by the eighth dpi. Necropsy examination revealed hemorrhages in the trachea and proventriculus. A supplementary file (Supplementary File 2) is provided, containing a detailed record of clinical signs and mortality data for infected and deceased birds throughout the experiment.

Discussion

Immunocompetence is a critical factor in an organism's ability to mount an effective immune response against pathogens. In this study, we aimed to investigate and compare the immunocompetence of feral birds from urban and rural environments, focusing on their response to the phytohaemagglutinin (PHA) skin test and their susceptibility to avian orthoavulavirus 1 (AOAV-1) infection. By examining these aspects, we gained insights into the potential differences in immune function between urban and rural bird populations and their implications for disease transmission dynamics. The PHA skin test is a widely used method to assess immunocompetence in various animal species including birds[12-14]. It involves injecting PHA, a lectin derived from red kidney beans, into the skin and measuring the subsequent inflammatory response. The PHA skin test serves as an indirect indicator of immune function, as it stimulates a T-cell-mediated immune response. The stronger the response, the more competent the immune system is considered to be[15-17].

In our study, we found that feral birds from urban environments exhibited a significantly stronger response to the PHA skin test compared to their rural counterparts [15-17]. This finding aligns with previous research conducted on other bird species, such as bullfinches and blackbirds, which also demonstrated enhanced immunocompetence in urban populations [18,19]. These studies suggest that urban environments can positively influence the immune function of feral birds.

Urban environments are characterized by a higher level of human-associated activities, such as increased availability of food resources, exposure to artificial light at night, and altered landscapes. These factors can influence the behavior, physiology, and immune priming of urban bird populations. For example, urban birds often have access to anthropogenic food sources, which can result in a more stable and abundant food supply compared to their rural counterparts. This nutritional advantage may contribute to enhanced immune function in urban birds[20,21]. Additionally, exposure to artificial light at night, a common feature of urban areas, can disrupt

circadian rhythms in birds. Disrupted circadian rhythms have been shown to influence immune function, potentially leading to alterations in immunocompetence[22]. Furthermore, urban landscapes may differ significantly from natural habitats, with urban birds facing unique challenges such as increased pollution levels, noise pollution, and a higher prevalence of parasites and pathogens[23]. These environmental stressors can stimulate immune responses in urban birds, contributing to their enhanced immunocompetence.

Apart from examining immunocompetence, we also investigated the susceptibility of feral birds to AOAV-1 infection. AOAV-1, commonly known as Newcastle disease virus (NDV), is a highly contagious and economically significant pathogen that affects various avian species, including both domestic and wild birds[24-26]. Understanding the susceptibility of feral birds to AOAV-1 is crucial for assessing their role as potential carriers and transmitters of the virus. Several studies have explored the susceptibility of feral birds to AOAV-1 infection. Research conducted on feral pigeons (*Columba livia*) and house sparrows (*Passer domesticus*) has shown that these species can be infected with AOAV-1 and shed the virus[27-32]. Our study expands on these findings by investigating the susceptibility of additional feral bird species including sparrows, crows, and quails, which are commonly found in both urban and rural environments.

Our results revealed that sparrows, crows, and quails can harbor and shed AOAV-1, indicating their potential role in the transmission of NDV to commercial poultry populations. These findings are consistent with studies that have demonstrated the ability of feral bird species to act as reservoirs or carriers of NDV[33,34]. The presence of AOAV-1 in feral bird populations poses a significant challenge for disease control efforts, as it increases the risk of introducing the virus to commercial poultry farms and potentially causing outbreaks.

The ability of feral birds to carry and shed AOAV-1 can be attributed to several factors. Firstly, these bird species often share habitats with domestic poultry, allowing for close contact and potential transmission of the virus through direct or indirect means[35-37]. The close proximity between feral birds and poultry creates opportunities for virus transmission, especially in areas with poor biosecurity measures. Secondly, feral birds, especially sparrows and crows, are known to exhibit gregarious behavior, forming large aggregations in urban and rural areas. These aggregations create opportunities for virus transmission among individuals within the bird population and potentially to other avian species, including commercial poultry[38,39].

It is important to note the limitations of our study. Firstly, our investigation of feral bird species was limited to sparrows, crows, and quails, overlooking other potential reservoirs or transmitters of AOAV-1. Future studies should consider expanding the scope to include a broader range of feral bird species to obtain a more comprehensive understanding of their role in ND dynamics. Additionally, the study focused on a specific geographical region (Pakistan), and the findings may vary to other regions with different bird populations and environmental conditions.

In conclusion, our study provides valuable insights into the immunocompetence of feral birds and their role in disease transmission, specifically focusing on sparrows, crows, and quails as potential carriers and transmitters of AOAV-1. The findings highlight the enhanced immunocompetence of urban bird populations and emphasize the importance of implementing effective disease management strategies to mitigate the risk of ND transmission from feral birds to commercial poultry populations. Strict biosafety and biosecurity measures, such as adequate fencing, bird-proofing facilities, and regular surveillance of feral bird populations, should be implemented to prevent disease transmission. Furthermore, our study underscores the need for continued research and surveillance efforts to enhance our understanding of the complex interactions between feral

bird populations and the transmission of avian viruses such as avian influenza which continue to pose risk for people. Investigations across different regions and a broader range of feral bird species would contribute to a more comprehensive understanding of the dynamics of AOAV-1 transmission. By gaining further knowledge about the virus challenge and exposure in feral birds, we can develop more targeted and effective strategies for disease prevention and control in both domestic poultry and wild bird populations.

Methods

Ethical Statement

The handling, sample collection, and processing of birds were conducted in strict adherence to the Animal Welfare and Health regulations and institutional guidelines. The Ethical Review Committee for the Use of Laboratory Animals (ERCULA) at the University of Veterinary and Animal Sciences, Lahore, Pakistan, granted approval for the procedures (Permit Number: ORIC/DR-70).

Experimental Birds Screening and Inclusion

A diverse range of feral bird species (n=40 each) was included in the study without considering age or gender. The species comprised pigeon (*Columba livia*; 190-240g), sparrow (*Passer domesticus*; 22-28g), mynah (*Acridotheres tristis*; 84-110g), quail (*Coturnix coturnix*; 90-140g), and crow (*Corvus splendens*; 210-250g). Determination of age and gender was not possible due to the birds being either purchased from live bird markets or captured from villages. Additionally, previous studies have shown that the magnitude of PHA-induced swelling is independent of gender and sex (L. Martin et al., 2006). Rural birds (Rb) were captured in mist nets from fields/semi-forest areas situated far away from villages or populated regions within the Punjab province. Urban birds (Ub) were procured from a live bird market in close proximity to Hazrat Data Ganj Bukhsh, district Lahore. All birds were appropriately caged, tagged, and underwent a two-week acclimatization period, during which they had access to ad libitum feed and water. The birds were screened for antibodies against common respiratory pathogens, such as AOAV-1 and avian influenza virus (AIV), using the hemagglutination inhibition (HI) assay. Additionally, oral and cloacal swabs were collected for individual processing through RT-PCR targeting the F gene for AOAV-1 (Muhammad Munir et al., 2010) and the M gene for avian influenza virus (Ali et al., 2017). Each swab was also subjected to virus isolation via egg inoculation (M. Stear, 2005). Only birds that were naïve to antibodies or had antibody concentrations less than 1:8, and were free from AOAV-1 and AIV genomes and viruses, were included in the study (Rb=20 and Ub=20 for each species).

Assessment of Immunocompetence in Feral Birds Originating from Rural and Urban Settings

The immunocompetence of each bird was evaluated using the PHA skin test. The PHA mitogen, prepared in cell culture-grade phosphate-buffered saline (1 mg/mL), was injected into the right patagium using an insulin syringe (1 mL), while the left patagium received an equivalent volume of PBS as a control. The swelling of the injected tissue was measured using a digital caliper, subtracting the initial wing thickness from the measurements taken at 12, 24, 36, 48, and 60 hours after injection. Birds exhibiting visible and sustained swelling for at least 48 hours were considered immunocompetent, whereas those without such swelling were classified as non-immunocompetent.

Pathogenicity of AOAV-1 and Interspecies Transmission Potential

To assess the pathogenicity of the velogenic strain of AOAV-1 (KY967612; Mallard-II/UVAS/Pak/2016) originating from Anseriformes and its potential for interspecies transmission, feral birds from rural and urban settings were challenged with the virus. After a two-week interval to minimize the effect of PHA, birds in group A were intraocularly and intranasally challenged with 1 mL of the AOAV-1 isolate (10^8 EID_{50}/mL). Birds in group B, serving as controls, were mock-inoculated with an equal volume of PBS via the same routes. After 8 hours, broiler birds (n=10 each) were introduced into the same shed to assess the interspecies transmission potential of AOAV-1. Feral birds and broiler chickens shared feed and water within their respective sheds, allowing for potential contact and virus transmission.

Clinical Examination and Sample Collection

Following AOAV-1 challenge, the birds were closely monitored for clinical symptoms over a 15-day period. Clinical examinations were conducted every 12 hours to observe signs such as sudden death, depression, inappetence, ruffled feathers, greenish diarrhea, torticollis, and conjunctivitis. Oropharyngeal and cloacal swabs were collected on the 3rd, 5th, 7th, and 9th day post-infection (dpi) and stored at -80 ºC until further analysis. RT-PCR was performed to detect the viral genome, and virus isolation was carried out using 9-11-day-old chicken embryonated eggs following established protocols (M. Stear, 2005). Morbid birds displaying clinical symptoms resembling Newcastle Disease were euthanized to collect tissue samples, including the brain, heart, liver, lungs, small intestine, gizzard, proventriculus, colon, breast muscle, and tongue. Genomic RNA was extracted from the tissue samples using the QIamp Viral RNA mini kit (Qiagen®, USA), and subsequent RT-PCR targeting the F gene was performed (Muhammad Munir et al., 2010). Tissue sections were prepared and stained with hematoxylin and eosin for microscopic examination of histopathological changes. A complete study design was shown in Fig. 1.

Statistical Analysis

Descriptive statistical analysis was employed to determine the mean and standard deviation of the immunocompetence response. The swelling response between rural and urban bird groups at different time intervals was compared using Repeated Measure Design and independent t-tests. Kaplan-Meier survival curves were generated to illustrate the percent survival of challenged birds against AOAV-1. Statistical analyses were performed using SPSS version 23, and the significance level was set at $p < 0.05$.

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Author's contribution

MH and MZS conceived and designed the work. MH, ZR and AR performed laboratory procedures and relevant methods. MH, MB and MZS are involved in data analysis. MZS, MM, MAS, and ZR provided necessary laboratory resources and consumables. MH, AR and MZS wrote the draft and edited it.

Competing interest

There is no competing interest among authors.

Supplementary data

The supplementary files S1 and S2 are related to this article.

Figures Legends

Figure 1. An elaborative experimental plan for assessing immunocompetence in urban and rural birds.
Figure 2. Comparative assessment of immunocompetence in urban and rural feral birds following phytohemagglutinin (PHA) injection at different time intervals
Figure 3. Clinical manifestations in pigeons, sparrows, and quails experimentally infected with a velogenic strain of AOAV-1 (Mallard II/UVAS/Pak/2016). Infected pigeons displayed clinical signs such as eyelid edema (a, b), leg paralysis I, wing paralysis (d, e), greenish diarrhea, and head twisting (f). Infected sparrows showed wing and leg paralysis (g, h), while infected quails exhibited isolation and depression (i).
Figure 4. Comparative survival rates of urban and rural bird species challenged with AOAV-1 (Mallard/II/UVAS/PAK2016), including control groups (Unchallenged). Bird species represented as follows: a = Pigeon, b = Sparrow, c = Mynah, d = Quail, and e = Crow. U = Urban (Challenged), R = Rural (Challenged), UC = Urban Control, and RC = Rural Control.
Figure 5. Interspecies transmission of Mallard-II/UVAS/Pak/2016 isolate from infected feral birds to non-infected and non-vaccinated broiler chickens
Figure 6: Microscopic examination of histopathological changes at varied resolutions in various tissues of pigeons experimentally infected with Mallard-II/UVAS/Pak/2016 isolate. Arrows indicate pathological lesions in the liver (a, b), luI(c), brain (d), Ili (e), and gizzard (f). Additionally, pathological lesions were observed in sparrow liver (g), brain (h), small intestine (i), and lung (j), mynah brain (k), liver (l), heart (m), quail lungs (n), and crow liver (o) and small intestine (p).

Tables
Table 1. Comparative assessment of immunocompetence in rural and urban feral birds across various time intervals
Table 2. A comprehensive summary of observations of clinical signs, virus shedding, and tissue tropism in feral birds challenged with Avian Orthoavulavirus-1 of Anseriformes origin.

Supplementary Figure legends

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