

Seroprevalence of Fowl Adenovirus-4 using specific ELISA in backyard chickens, Golestan province, Iran: the first study

Abstract

Infection with fowl adenovirus is associated with different diseases, including hepatitis hydropericardium syndrome (HHS), inclusion body hepatitis (IBH), and gizzard erosion. Infection with serotype 4 of fowl adenovirus can lead to HHS which affects chickens in 3 to 5-week of old and subsequently lead to high mortality and great financial losses. First detection of HHS in Iran was announced in march 2021 in a broiler flock. Detection of fowl adenovirus can be performed by various serological methods, as well as molecular methods like polymerase chain reaction and real-time polymerase chain reaction. In the current study, the level of specific antibodies against the FAdV-4 serotype in 44 blood samples of unvaccinated backyard chickens groups, from Golestan province in northern Iran using was evaluated, using ELISA assay. According to the ELISA results, the overall prevalence was 22.72%, and the highest was found in Saad Abad village, which had 66.66%. The results also show that the highest antibody titer was found in the Haji Balkhan group, with 1679.91, and the lowest in the Amir Abad group, with 3.22. The other titers were mostly between 100 and 300. This study is the first serological investigation of FAdV-4 in backyard chickens of Iran. While the virus can only be detected by molecular techniques such as PCR, these discoveries may offer new perspectives on the spread of the virus in the northern region of Iran and help develop innovative vaccination strategies.

Keywords: Fowl Adenovirus-4, Backyard Chicken, Seroprevalence, ELISA

1. Introduction

Fowl adenovirus (FAdV) is a non-enveloped double-stranded DNA virus which is classified in Adenoviridae family, and aviadenovirus genus. FAdVs can be further divided into five genotypes (A to E) and 12 serotypes (1-3). FAdV infection is responsible for different diseases such as inclusion body hepatitis (IBH), hepatitis hydropericardium syndrome (HHS), and gizzard erosion (1). HHS is an emerging disease caused by serotype 4 of FAdV and severely affects the poultry industry, particularly broiler chickens at the age of 3 to 5 weeks. Diseased birds typically exhibit lethargy and anorexia, as well as ruffled feathers and yellow mucoid feces (1). The disease causes significant losses and has a mortality rate of 20-80%. At necropsy, chickens infected with FAdV-4 show gross lesions, including a balloon-like pericardial sac filled with straw-colored fluid and an enlarged, pale liver with necrotic foci, along with lesions in other vital organs (i.e., spleen, thymus, kidney, and lung) (1, 4). FAdV-4 can be transmitted both vertically and horizontally. Vertical transmission occurs from parent chickens to offspring through the embryonated eggs (1). On the other hand, the virus can be found in all excretions, with the highest titer in feces, which contributes to the most common horizontal transmission through the fecal-oral route (5). Furthermore, mechanical transmission via fomites can also be mentioned as another route for horizontal transmission (1, 6).

In 1987 in Pakistan, FAdV-4 outbreak was reported for the first time, and then spread to several Asian countries, including Iraq, Kuwait, India, Japan and China, as well as some European and South American countries (1). The first reported case of HHS in Iran occurred

٤٣ in a 15-day broiler flock in march 2021 (7). Several efforts have been made to control the
٤٤ global emergence and spread of the disease, including vaccination, equipment disinfection,
٤٥ appropriate ventilation, and restricted biosecurity (8). However, as FAdV is a non-enveloped
٤٦ virus, disinfection is not fully effective in controlling the virus (9). Therefore, different
٤٧ vaccines such as inactivated, live attenuated, and recombinant vaccines designed to combat
٤٨ the disease as the fundamental strategy to prevent further outbreaks. Among these,
٤٩ inactivated vaccines have become the most widely used vaccines in recent years (10, 11).

٥٠ Backyard poultry, primarily raised in rural areas, contributes to the supply of meat and eggs.
٥١ According to the Iranian Veterinary Organization, nearly 50 million backyard birds are kept
٥٢ in Iran, providing a source of income for rural communities (12, 13). In Iran, backyard birds
٥٣ are kept using traditional methods without vaccination and adequate biosecurity. The high
٥٤ diversity and density of birds in rural areas combined with the lack of biosecurity
٥٥ dramatically increase the risk of disease transmission between birds. This situation poses a
٥٦ potential threat to industrial flocks that could be infected by the transmission of viruses from
٥٧ backyard flocks (13, 14). Consequently, detecting infection or previous exposure to infectious
٥٨ diseases in backyard chickens is crucial. Detection can be achieved via serological assays
٥٩ such as enzyme-linked immunosorbent assay (ELISA), virus neutralization (VN), agar gel
٦٠ immunodiffusion (AGI), counterimmunoelectrophoresis, fluorescent antibody techniques,
٦١ and immunoperoxidase assays, as well as molecular assays, including PCR, real-time PCR
٦٢ and sequencing (15). FAdV-4 is one of the most dangerous diseases that can potentially be
٦٣ transmitted between backyard birds and industrial poultry flocks. A suitable method to detect
٦٤ FAdV-4 is the measurement of acquired specific antibodies through serological methods such
٦٥ as ELISA (16).

٦٦ The northern provinces of Iran, including Mazandaran, Golestan, and Gilan, house the
٦٧ majority of the population of backyard birds and industrial poultry farms in Iran. This issue
٦٨ makes them one of the most critical regions in the country for the study of infectious disease
٦٩ surveillance (17). Current study was designed to evaluate the seroprevalence of FAdV-4 in
٧٠ unvaccinated backyard poultries for the first time in Golestan province, Iran, using the
٧١ ELISA test. It will help estimate the extent of virus spread in this province, one of Iran's most
٧٢ critical zones for industrial poultry production.

٧٣ **2. Material and Methods**

٧٤ **2.1. Sample collection**

٧٥ In this study, blood samples were randomly collected from 44 backyard chickens groups, in
٧٦ Golestan province who had not been vaccinated against FAdV-4 to determine the prevalence
٧٧ of anti-FAdV-4 antibodies in serum in 2022. The villages where the sampling was performed
٧٨ are indicated in Figure 1. FAdV-4-specific positive and negative serum samples were
٧٩ obtained from the components of the ELISA kit.

٨٠ **2.2. ELISA assay**

٨١ The detection of anti-FAdV-4 antibodies in the serum samples was carried out using a
٨٢ commercially available ELISA kit (Biostone Animal Health Company, Dallas, Texas) (Cat.

٨٣ No.: 10076-02), and the titer of the antibodies was determined. The percentage positivity (PP)
٨٤ of all samples was calculated using the following formula:
٨٥

$$\text{PP} = \frac{\text{OD630 test sample} - \text{OD630 NC}}{\text{OD630 PC} - \text{OD630 NC}} \times 100\%$$

٨٧

٨٨ The results were interpreted as follows:

٨٩ A. (Mean OD 630 PC) - (Mean OD 630 NC) > 0.1

٩٠ B. The mean OD of the Positive Control must be ≥ 0.3

٩١

٩٢ 2.3. Statistical analyses

٩٣ ELISA test results were managed using GraphPad Prism software (v9.1.0.221), and
٩٤ descriptive statistics for antibody titers were performed using the same software.

٩٥ 3. Results

٩٦ The result of ELISA on the serum of the examined chickens in six villages of Golestan
٩٧ province showed an overall prevalence of 22.72% (10/44) for FAdV-4. The percentage
٩٨ prevalence for each village was as follows: Haji Balkhan (14.2%), Zarrin Gol (0%), Amir
٩٩ Abad (28.57%), Zabol Abad (28.57%), Dikcheh (0%), Saad Abad (66.66%) (Figure 2).
١٠٠ While the highest prevalence was observed in Saad Abad village, none was observed in
١٠١ Zarrin Gol and Dikcheh villages.

١٠٢ The mean antibody titers of the investigated groups are shown in Figure 3. Positive titers
١٠٣ are recognized by ODs higher than 0.3. The highest antibody titer was 1679.91 in the Haji
١٠٤ Balkhan group, and the lowest titer was 3.22 in the Amir Abad group; the remaining titers
١٠٥ were mostly in the range of 100 to 300. The Saad Abad group had the highest antibody titer
١٠٦ and the highest percentage of prevalence, while the Zarrin Gol and Dikcheh groups had the
١٠٧ lowest titer and percentage, respectively. There was no significant discrepancy between all
١٠٨ groups, except for the differences between the Saad Abad and Zarrin Gol groups and between
١٠٩ Saad Abad and Dikcheh. The percentage positivity of the six groups is also shown in Figure
١١٠ 4.

١١١ 4. Discussion

١١٢ Fowl adenovirus, a member of the Adenoviridae family, causes various poultry diseases.
١١٣ HHS is mainly associated with genotype C, while IBH is primarily caused by genotypes D
١١٤ and E (1, 6). These diseases lead to reduced performance and reproduction as well as
١١٥ increased mortality rates in both industrial and backyard chickens, especially in young
١١٦ broilers (18). As backyard and wild birds interact, backyard birds serve as a desirable source
١١٧ for spreading various infectious diseases (19). Also, backyard chickens are considered risk
١١٨ factors for industrial poultry flocks due to the lack of biosecurity measures and vaccination
١١٩ (20, 21). Therefore, veterinary authorities should consider developing surveillance measures
١٢٠ for backyard birds.

١٢١ In the current study, ELISA determined antibodies against FAdV-4 in unvaccinated backyard
١٢٢ chickens. According to the results, four out of six villages were seropositive for FAdV-4,

123 although the percentage of positive cases varied between villages. The highest percentage of
124 positive cases was 66.66% in one village, followed by similar rates of 28.57% in two other
125 villages and 14.2% in another. These findings indicate a high incidence rate of FAdV-4 in
126 Golestan province, as one of Iran's primary poultry-producing provinces (17). In a study
127 conducted by Jordan et al., blood samples from 43 unvaccinated layer farms on two islands in
128 the Caribbean were tested for antibodies against several major infectious diseases, including
129 fowl adenovirus group I, using an ELISA assay. The study reported 100% positivity for
130 FAdV on one island and 99.35% positivity on the other island (22).

131 Detection of specific antibodies against FAdV-4 can be performed by various serologic
132 methods, including ELISA, VN test, immunofluorescence assay (IFA), agar gel diffusion
133 precipitation test (AGPT), and agar gel immunodiffusion (AGID) (15). Among these
134 serologic methods, ELISA is often preferred for monitoring the presence of antibodies
135 acquired against adenoviruses because it demonstrates higher sensitivity compared to several
136 other serologic methods, including AGPT and AGID, making it more accurate in detecting
137 specific antibodies to FAdV-4 (23). Moreover, ELISA method was employed for detection of
138 group specific antibodies and type specific antibodies (24). ELISA's high sensitivity,
139 affordability, ease of use, and reproducibility make it an appropriate assay for large-scale
140 epidemiologic assessment of a disease (25). For above reasons, ELISA is an efficient assay
141 for evaluation the prevalence and presence of fowl adenovirus in a given region. However,
142 the primary challenge with these serological tests lies in interpreting their results, as
143 antibodies against disease can be found in healthy and infected birds (1). Therefore, it is
144 difficult to distinguish between an active infection and a previous infection. In contrast,
145 molecular tests such as PCR can effectively detect active infections, although they cannot
146 identify animals recovered from last exposure to pathogens (26). Therefore, it is recommend
147 combining molecular and serologic tests in further studies to gain a comprehensive insight
148 into the current spread of infectious diseases in a region. There are no clear reports on the
149 exact serologic or molecular prevalence of HHS in Iran which is bordered by Pakistan. As fat
150 as we know, this is the first serologic investigation of FAdV-4 disease conducted on backyard
151 unvaccinated chickens in Golestan province, which is an important province of Iran in the
152 poultry industry. Evaluation of the prevalence of HHS in backyard and industrial chicken
153 flocks in other provinces of Iran would be necessary and recommended for future studies.

154 Since Iranian veterinary organization GIS has approved and recommended a certain distance
155 between traditional farms and industrial farms, this distance has been observed between these
156 sites. However, these farms may employ regional workers who keep backyards poultries and
157 are in connection with other persons who keep backyards chickens, this can help to spread the
158 disease in addition to other mechanical factors like vehicles.

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۱۶۴ **Author s Contributions**

۱۶۵ Study concept and design: Arash Ghalyanchilangeroudi, Hossein Hossieni

۱۶۶ Analysis and interpretation of data: Arash Ghalyanchilangeroudi, Zahra Ziafati Kafi

۱۶۷ Drafting of the manuscript: Omid Eghbali, Soroush Sarmadi, Alireza Bakhshi, Fahimeh
۱۶۸ Jamiri

۱۶۹ Acquisition of Data: Edris Kalirad, Pari Karami, Amir Javadi

۱۷۰ Critical revision of the manuscript for important intellectual content: Rima Morshed, Hossein
۱۷۱ Hosseini

۱۷۲ Study Supervision: Arash Ghalyanchilangeroudi

۱۷۳ **Ethic**

۱۷۴ We declare that all ethical standards related to animal health and welfare have been respected
۱۷۵ in present study.

۱۷۶ **Conflict of interest**

۱۷۷ The authors declare no conflict of interest.

۱۷۸ **Funding**

۱۷۹ No grant from funding agencies was provided for current study.

۱۸۰ **Data Availability**

۱۸۱ The data that support the findings of this study are available on request from the
۱۸۲ corresponding author.

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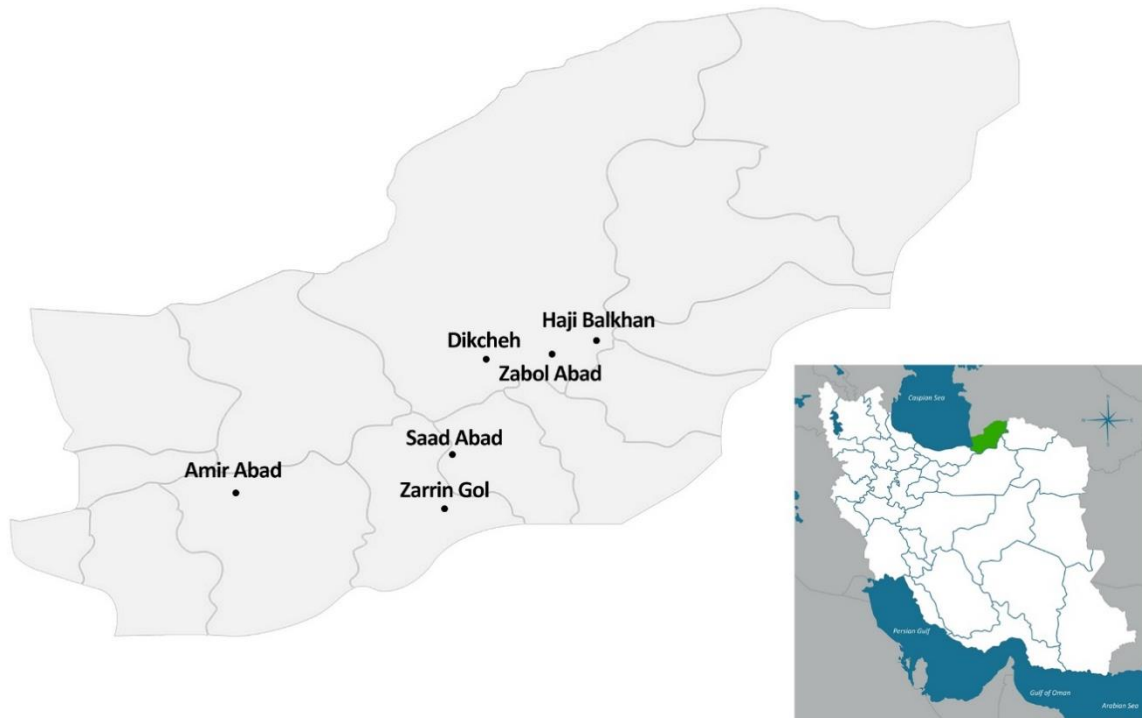
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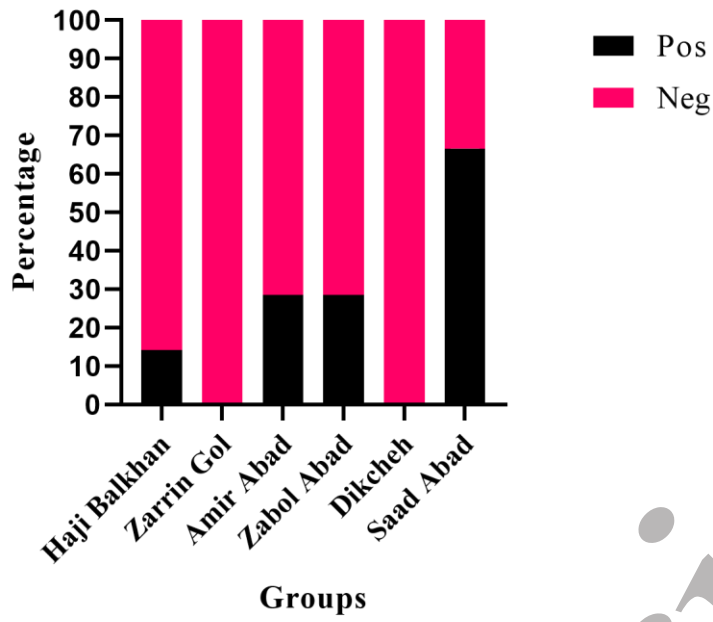
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۲۶۴ Figure 1. The geographical location of sample collection from backyard chickens in the Golestan
۲۶۵ province, Iran.

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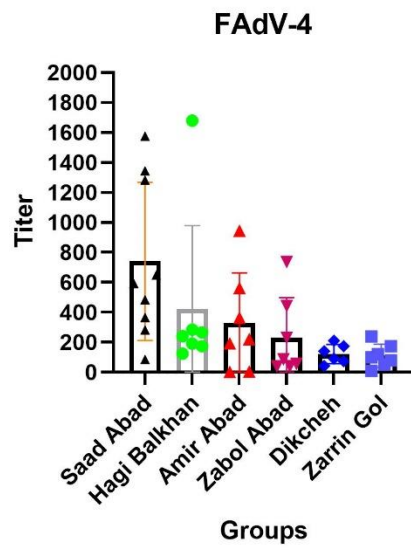
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Figure 2. Percent prevalence of FAdV-4 among backyard chickens of the Golestan province, Iran.

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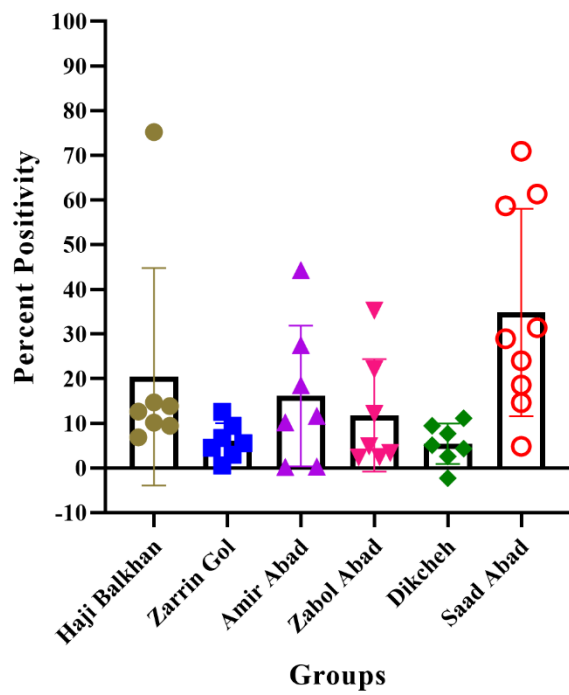
	Hagi Balkhan	Zarrin Gol	Amir Abad	Zabol Abad	Dikcheh	Saad Abad
Mean	422.7	110.1	327.0	232.6	122.2	740.2
Std. Deviation	557.2	77.34	335.5	265.7	63.90	528.1

Figure 3. The ELISA mean antibody titer of FAdV-4 among the Golestan province, Iran backyard chickens.

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Figure 4. The percent positivity of FAdV-4 among the Golestan province, Iran backyard chickens.

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