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Original Article

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

Hossein Hosseini[1], Edris Kalirad[2], Pari Karami[2], Zahra Ziafati Kafi[3], Naser Sadri[3], Soroush Sarmadi[3], Alireza Bakhshi[3], Fahimeh Jamiri[3], Omid Eghbali[3], Amir Javadi[4], Rima Morshed[5], Arash Ghalyanchilangeroudi[3*]

1.Department of Clinical Sciences, Faculty of Veterinary Medicine, Karaj Islamic Azad University, Karaj, Iran.
2.Department of Poultry Diseases, Iran Veterinary Organization, Gorgan, Iran.
3.Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
4.Department of poultry Disease, Iranian Veterinary Organization, Qazvin, Iran.
5.*Agriculture and Veterinary Group, Iran Encyclopedia Compiling Foundation, Ministry of Science, Research and Technology, Tehran, Iran.*

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ABSTRACT

Infection with fowl adenovirus is associated with different diseases, including hepatitis hydro pericardium syndrome (HHS), inclusion body hepatitis (IBH), and gizzard erosion. Infection with serotype 4 of fowl adenovirus can lead to HHS, which affects 3-to5-week chickens and can result in high mortality and significant financial losses. The first detection of HHS in Iran was announced in March 2021 in a broiler flock. Fowl adenovirus can be detected using various serological and molecular methods, such as 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 from unvaccinated backyard chicken flocks in Golestan Province, northern Iran, was evaluated using ELISA assay. According to the ELISA results, the overall prevalence was 22.72%, with the highest prevalence found in Saad Abad village at 66.66%. The results also show that the highest antibody titer was found in the Haji Balkhan group (1679.91), and the lowest was found in the Amir Abad group (3.22). Most other titers were between 100 and 300. This study is the first serological investigation of FAdV-4 in Iranian backyard chickens. While the virus can only be detected using molecular techniques, such as PCR, these findings may provide insight into the virus's spread in the northern region of Iran and help develop innovative vaccination strategies.

Corresponding Author:
ghalyana@ut.ac.ir

https://orcid.org/0000-0001-5245-2541

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1. Introduction

Fowl adenovirus (FAdV) is a non-enveloped, double-stranded DNA virus 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 causes different diseases, including 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 aged 3 to 5 weeks. Diseased birds typically exhibit lethargy , anorexia, ruffled feathers ,and yellow mucoid feces (1). The disease causes significant economic losses and has a mortality rate of 20-80%. At necropsy, chickens infected with FAdV-4 exhibit gross lesions, including a balloon-like pericardial sac filled with straw-colored fluid , an enlargedpale liver with necrotic foci, and lesions in other vital organs such as the 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. This is the most common horizontal transmission through the fecal-oral route (5). Furthermore, mechanical transmission via fomites can be another route for horizontal transmission (1, 6). The first reported FAdV-4 outbreak occurred in Pakistan in 1987 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 in a 15-day broiler flock in March 2021 (7). Several efforts have been made to control the global emergence and spread of the disease, including vaccination, equipment disinfection, appropriate ventilation, and restricted biosecurity (8). However, as FAdV is a non-enveloped virus, disinfection is not fully effective in controlling it (9). Therefore, different vaccines, such as inactivated, live attenuated, and recombinant vaccines have been designed to combat the disease as the fundamental strategy to prevent further outbreaks. Inactivated vaccines have become the most widely used vaccines in recent years (10, 11). Backyard poultry, primarily raised in rural areas, contributes to the supply of meat and eggs. According to the Iranian Veterinary Organization, nearly 50 million backyard birds are kept in Iran and provide a source of income for rural communities (12, 13). In Iran, backyard birds are kept using traditional methods without vaccination and adequate biosecurity. The high diversity and density of birds in rural areas combined with the lack of biosecurity dramatically increase the risk of disease transmission between birds. This situation poses a potential threat to industrial flocks that could be infected by the transmission of viruses from backyard flocks (13, 14).

Consequently, detecting infection or previous exposure to infectious diseases in backyard chickens is crucial. Detection can be achieved via serological assays such as enzyme-linked immunosorbent assay (ELISA), virus neutralization (VN), agar gel immunodiffusion (AGI), counterimmunoelectrophoresis, fluorescent antibody techniques, and immunoperoxidase assays, as well as molecular assays, including PCR, real-time PCR and sequencing (15). FAdV-4 is one of the most dangerous diseases that can potentially be transmitted among backyard birds and industrial poultry flocks. A suitable method to detect FAdV-4 is the measurement of acquired specific antibodies through serological methods such as ELISA (16). The northern provinces of Iran, including Mazandaran, Golestan, and Gilan, have the largest populations of backyard birds and industrial poultry farms in Iran. This makes them one of the most critical regions in the country for infectious disease surveillance studies (17). The current study was designed to evaluate the seroprevalence of FAdV-4 in unvaccinated backyard poultry for the first time in Golestan province, Iran, using the ELISA test. It will help estimate the extent to which virus has spread in this province, which is one of most critical zones for industrial poultry production in Iran.

2. Materials and Methods

2.1. Sample Collection

This study examined the prevalence of anti-FAdV-4 antibodies in serum by collectingblood samples from 44 groups of unvaccinated backyard chickens in Golestan province in 2022. The villages where the sampling was performed are indicated in Figure 1. FAdV-4-specific positive and negative serum samples were obtained from the ELISA kit components.

2.2. ELISA Test

Anti-FAdV-4 antibodies were detected in serum samples using a commercially available ELISA kit (Biostone Animal Health Company, Dallas, Texas) (Cat. No.: 10076-02), and the titer of the antibodies was determined. The percentage positivity (PP) of all samples was calculated using the following formula:

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

The results were interpreted as follows:
A. (Mean OD 630 PC) - (Mean OD 630 NC) > 0.1
B. The mean OD of the Positive Control must be ≥ 0.3

2.3. Statistical analyses

The ELISA test results were analyzed using GraphPad Prism software (v9.1.0.221), and the descriptive statistics

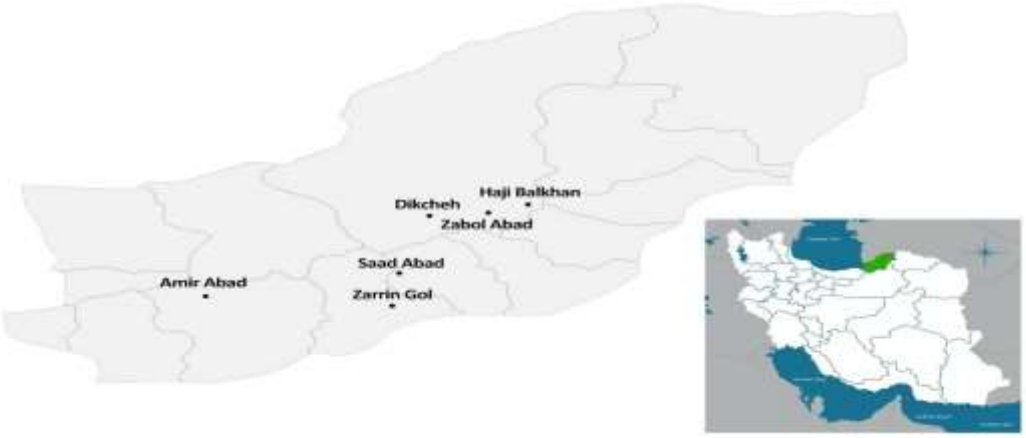

Figure 1. The geographical location of sample collection from backyard chickens in the Golestan province, Iran.

for antibody titers were performed using the same software.

3. Results

ELISA results on the serum of chickens examined in six villages in Golestan Province showed an overall FAdV-4 prevalence of 22.72% (10/44). The percentage prevalence for each village was as follows: Haji Balkhan (14.2%), Zarrin Gol (0%), Amir Abad (28.57%), Zabol Abad (28.57%), Dikcheh (0%), Saad Abad (66.66%) (Figure 2).The highest prevalence was observed in Saad Abad village, while none was observed in Zarrin Gol and Dikcheh villages. Figure 3 shows the mean antibody titers of the investigated groups. Positive titers are recognized by ODs higher than 0.3. The highest antibody titer was 1679.91 in the Haji Balkhan group, and the lowest titer was 3.22 in the Amir Abad group. Most of the remaining titers were in the range of 100 to 300. The Saad Abad group had the highest antibody titer and prevalence percentage , while the Zarrin Gol and Dikcheh groups had the lowest titer and percentage, respectively. There was no significant discrepancy among all groupsexcept for the differences between the Saad Abad and Zarrin Gol groups and between Saad Abad and Dikcheh. Figure 4 shows t he percentage positivity of the six groups.

4. Discussion

Fowl adenovirus, a member of the Adenoviridae family, causes various poultry diseases. HHS is primarily associated with genotype C, while IBH is primarily caused by genotypes D and E (1, 6). These diseases lead to reduced performance and reproduction, as well as increased mortality rates, in both industrial and backyard chickens, particularly in young broilers (18). As backyard and wild birds interact, backyard birds serve as a desirable source for spreading various infectious diseases (19). Also, the lack of biosecurity measures and vaccination in backyard chickens is considered a risk factor for industrial poultry (20, 21). Therefore, veterinary authorities should consider developing surveillance measures for backyard birds. In the current study, ELISA detected antibodies against FAdV-4 in unvaccinated backyard chickens. According to the results, four out of six villages were seropositive for FAdV-4, although the percentage of positive cases varied by village. The highest percentage of positive cases was 66.66% in one village, followed by 28.57% in two other villages and 14.2% in another village. These findings suggest a high incidence rate of FAdV-4 in Golestan province, one of Iran's primary poultry-producing provinces (17). In a study conducted by Jordan et al., blood samples from 43 unvaccinated layer farms on two Caribbean islands were tested using an ELISA test for antibodies against several major infectious diseases, including fowl adenovirus group I (FAdV-1). The study reported 100% positivity for FAdV on one island and 99.35% positivity on the other (22). Specific antibodies against FAdV-4 can be detected using various serologic methods, including ELISA, VN test, immunofluorescence assay (IFA), agar gel diffusion precipitation test (AGPT), and agar gel immunodiffusion (AGID) (15). ELISA is often preferred over other serologic methods for monitoring the presence of antibodies acquired against adenoviruses because it is more sensitive than AGPT and AGID, making it more accurate in detecting FAdV-4-specific antibodies (23). Moreover, the ELISA method has been used to detect group-specific and type -specific antibodies (24).The ' high sensitivity, affordability, ease of use, and reproducibility of ELISA make it an appropriate assay for large-scale epidemiologic assessment of a disease (25). For these reasons, ELISA is an efficient assay for evaluating the prevalence and presence of fowl adenovirus in a given region. However, the primary challenge with these serological tests is interpreting

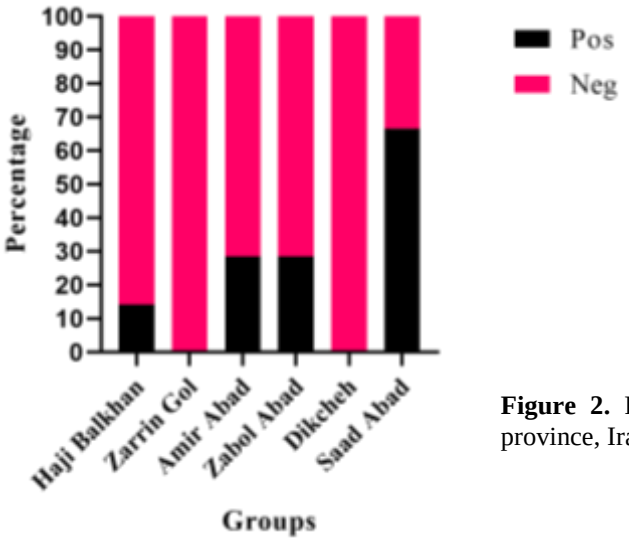

Figure 2. Percent prevalence of FAdV-4 among backyard chickens of the Golestan province, Iran.

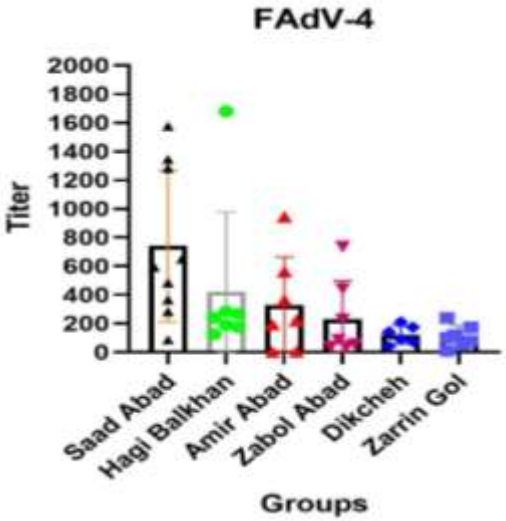

Figure 3. The ELISA mean antibody titer of FAdV-4 among the Golestan province, Iran's backyard chickens.

	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

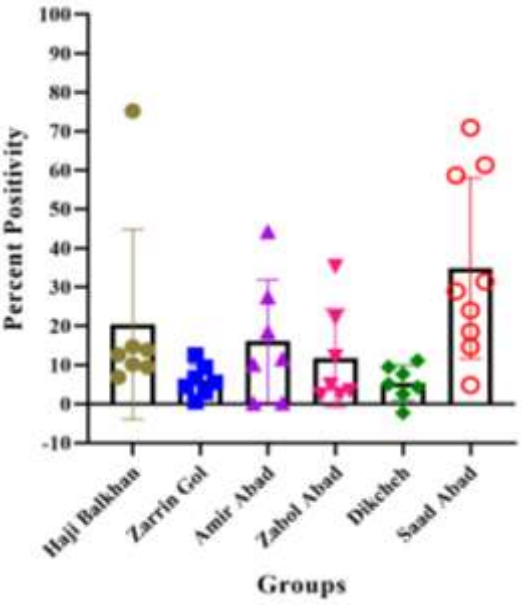

Figure 4. The percent positivity of FAdV-4 among the Golestan province, Iran's backyard chickens.

resultsbecause antibodies against the disease can be found in both healthy and infected birds (1). Therefore, it is difficult to distinguish between an active and a previous infection. In contrast, molecular tests ,such as PCR, can effectively detect active infections, however, they cannot identify animals that have recovered from their last exposure to pathogens (26).

Therefore, combining molecular and serologic tests is recommended in further studies to gain a comprehensive insight into the current spread of infectious diseases in a region. There are no clear reports on the exact serologic or molecular prevalence of HHS in Iran, which borders by Pakistan. As far as we know, this is the first serologic investigation of FAdV-4 disease conducted on backyard unvaccinated chickens in Golestan Province, which is an important province of Iran in the poultry industry. Future studies should evaluate the prevalence of HHS in backyard and industrial chicken flocks in other provinces of Iran. Since Iranian veterinary organization GIS has approved and recommended a certain distance between traditional farms and industrial farms, this distance has been observed between these sites. However, these farms may employ regional workers who keep backyard poultry and are in contact with others who keep backyard chickens. This can help spread the disease, in addition to other mechanical factors, such as vehicles.

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Authors' Contribution

Study concept and design: A. G, H. H.
Analysis and interpretation of data: A. G, Z. ZK.
Drafting of the manuscript: O. E, S. S, A. B, F. J.
Acquisition of Data: E. K, P. K, A. J.
Critical revision of the manuscript for important intellectual content: R. M, H. H.
Study Supervision: A. G.

Ethics

We declare that all ethical standards related to animal health and welfare were respected in the present study.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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Data Availability

The data supporting the findings of this study are available upon request from the corresponding author.

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