The assessment of fowl adenovirus serotype 4 (FAdV-4) challenge in the broiler farms using an in-house ELISA

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Abstract

Since 2021, fowl adenovirus serotype 4 (FAdV-4) has emerged as an increasingly serious threat to the poultry industry in Iran. This highly pathogenic virus has caused widespread damage, including forced eradication of entire flocks, significant increases in mortality rates, and a substantial reduction in growth performance within broiler production systems. As a result, the industry is facing unprecedented economic and operational challenges. To address this ongoing issue, prevent further losses, and ensure the sustainability of poultry production, a vaccination strategy using inactivated vaccines has been implemented. Specifically, broiler breeder farms have been vaccinated against the disease to help curtail its spread and minimize its impact on flock health and production. In this study, a peptide-based enzyme-linked immunosorbent assay (peptide ELISA) was optimized and utilized as a Differentiating Infected from Vaccinated Animals (DIVA) test to assess the prevalence of FAdV-4 infections across broiler farms of Khorasan Razavi province. The province was divided into three geographical zones based on hepatitis hydropericardium syndrome (HHS) outbreak data of the Iran Veterinary Organization (IVO), including high prevalence, medium prevalence, and no history of the disease. Positive sera rates were 86% and 26% in regions with high and medium HHS prevalence, respectively. Notably, no positive sera were detected in areas with no disease history. The study demonstrated a strong correlation between peptide ELISA results and regional FAdV-4 prevalence, confirming the assay's reliability in detecting FAdV-4 exposure in broiler flocks. These findings support its utility as an effective diagnostic and epidemiological tool for current and future disease surveillance.

Keywords

FAdV-4, Hepatitis hydropericardium syndrome, Peptide-based ELISA, DIVA test, Broiler

1. Introduction

Fowl adenoviruses (FAdVs) are non-enveloped viruses containing double-stranded linear DNA genomes and belong to the genus Aviadenovirus. (1). FAdVs are divided into five species (FAdV-A to FAdV-E) (2) and 12 serotypes (FAdV-1 to −8a and −8b to −11) based on the patterns of restriction enzyme digestion and serum cross-neutralization tests, respectively (3)Among the 12 serotypes of adenoviruses, only FAdV-4, classified as FAdV-C, is known to cause hepatitis hydropericardium syndrome (HHS). This emerging immunosuppressive disease mainly affects broilers between 3 and 5 weeks old. (4). It is characterized by a sudden onset and a mortality rate ranging from 30% to 70%. The disease is also marked by the accumulation of clear or amber-colored liquid with aqueous or gelatinous consistency in the pericardial sac and an enlarged and friable liver with infiltration of mononuclear cells. Additionally, basophilic intranuclear inclusions can be found in hepatocytes. (5-7). The first report of the disease was in Angara Goth, Pakistan, in 1987. Subsequent outbreaks have been recorded in many other countries. These outbreaks have resulted in significant economic losses to poultry raisers in countries including the United States. (8), Canada (9), Korea (10), Chile (11), and Slovakia (12). Although the first outbreak of HHS was successfully controlled in Khorasan Razavi province through the implementation of a stamping-out program (13)During the second wave, the disease re-emerged simultaneously in multiple regions through the progeny of a broiler breeder flock. Despite employing effective control measures, the disease remained uncontrolled and spread to more broiler flocks via flock-to-flock transmission within a few months.

These outbreaks caused high mortality and significant economic losses among the broiler flocks. To prevent the further spread of the disease, all broiler breeder flocks in the province were vaccinated with FAdVs, including FAdV-4. Xie *et al.* introduced a peptide-based ELISA for differentiating fowl adenovirus 4 -infected chickens from vaccinated chickens at laboratory scale. To assess the prevalence of HHS in provincial broiler farms, we developed and optimized a peptide-based ELISA. This assay is designed to detect antibodies specific to the non-structural 22K protein of FAdV-4, Which is a key protein involved in the viral replication cycle. During infection, immunogenic epitopes of this protein are expressed within the host, eliciting the production of specific antibodies in infected chickens. Uniquely, this ELISA detects an immune response only in cases of active infection and ongoing viral replication; Accordingly, the peptide-based ELISA serves as a robust and reliable tool for distinguishing between chickens vaccinated with inactivated FAdV-4 vaccines and those naturally infected. It also holds significant value as an epidemiological instrument for assessing the extent of FAdV-4 challenge across broiler farms with varying HHS prevalence rates.

To demonstrate the extent of HHS involvement in the provincial broiler farms, we optimized a peptide-based ELISA as an epidemiological tool to assess the FAdV-4 challenge among broiler farms with varying prevalence rates of HHS.

2. Materials and methods

2.1. Flock sampling

Broiler farms in Khorasan Razavi province were divided into three zones based on disease prevalence data from the provincial veterinary department, including high prevalence, moderate prevalence, and regions without disease reports. The required sample size for each zone was

calculated using the formula N = (Z^2 * p * (1-p))/e^2, resulting in 80, 124, and 384 samples, respectively. Blood sampling was carried out before slaughtering.

2.2. Chicken groups

Forty 14-day-old SPF chicks (Venkateshwara Hatcheries, India) were divided into four groups of 10 each. All chicks were tested using a commercial avian adenovirus ELISA kit from Biochek (Cat No: CK132).

2.2.1. **Group 1 (positive control):** Each bird received 0.5 mL of a 10% tissue suspension of an isolated FAdV-4 isolate (RT60), which was filtered and injected intramuscularly

2.2.2. **Group 2:** Birds were vaccinated with an Indian triple inactivated injectable vaccine (GlobiVac) containing inactivated adenovirus serotypes 4, 8, and 11 intramuscularly. Blood samples were collected at 2, 3, 5, and 8 weeks post-vaccination.

2.2.3. **Group 3:** Birds were vaccinated with a New Zealand bivalent inactivated injectable vaccine (Avi-Mix) containing inactivated adenovirus serotypes 4 and 8 intramuscularly. Blood samples were collected at 2, 3, 5, and 8 weeks post-vaccination.

2.2.4. **Group 4 (negative control):** Each bird received 0.5 mL of phosphate-buffered saline (PBS) intramuscularly.

2.3. Peptide-Based ELISA Optimisation

The peptide-based ELISA was optimized following the protocol by Xie *et al*., (14)with some modifications. A checkerboard assay determined the optimal dilutions for ELISA reagents. The optimal concentrations were peptide (22K-4P_66-88aa): 2 μg/ml, primary antibody: 1:100 dilution, and secondary antibody: 1:4000 dilution. Briefly, 100 μl of the diluted peptide (Thermo Fisher Scientific, United States, Cat. No: AKB10099) in PBS (Sigma, Germany, Cat. No: P4417)

(pH 7.4) was coated onto 96-well ELISA plates and incubated at 37°C for 1 hour. Plates were washed three times with PBST (PBS containing Tween 20) to remove unbound peptides. To minimize nonspecific binding, wells were blocked with 300 µl of blocking buffer (skimmed milk) and incubated at 37°C for 1 hour. Following the blocking step, the plates were washed three times with PBST. Next, 100 µl of positive and negative sera, diluted 1:100 in PBS with a 70:30 skim milk solution, were added to each well. Plates were incubated at 25°C for 40 minutes to allow antigen-antibody interactions, followed by three washes. Then, 100 µl of rabbit anti-chicken antibody, conjugated with horseradish peroxidase (Merck, Germany, Cat. No: A9046) and diluted 1:4000 in PBS with a 70:30 skim milk solution, was added to each well. Plates were incubated at 25°C for 40 minutes, followed by three washes. Colorimetric detection was performed by adding 100 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) solution to each well and incubating it in the dark for 10 minutes. The reaction was stopped by adding 100 µl of 1.6 M sulfuric acid (H2SO4). Absorbance values at 450 nm were measured using an ELISA reader (BioTek ELx800). The ELISA cutoff value was established at an absorbance of 0.250, calculated as the arithmetic mean of 40 SPF sera plus three standard deviations. Sera with absorbance values ≥0.250 were considered positive (Fig. 1).

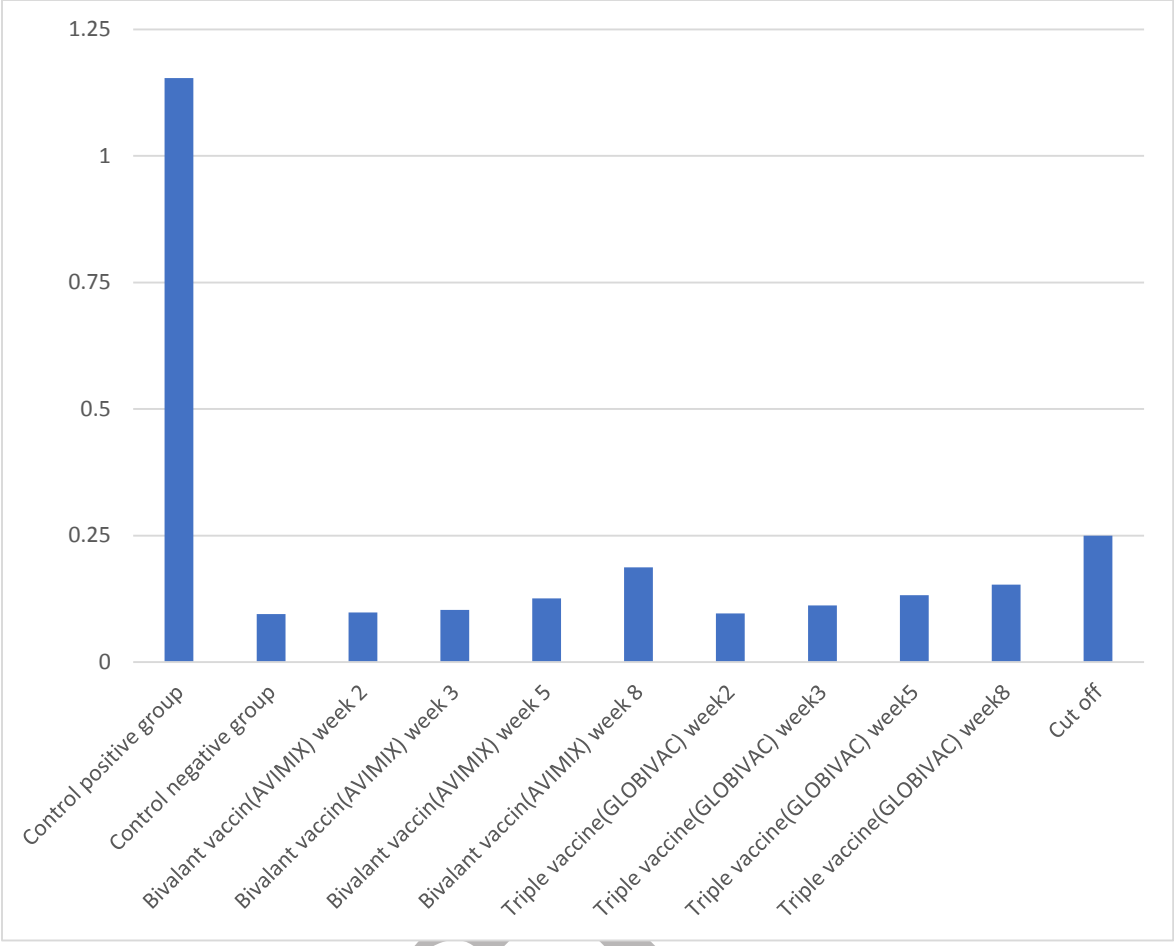

Figure 1: Average serum optical density of test groups with peptide-based ELISA

3. Results

The analysis of sera collected from four chicken groups demonstrated that the peptide-based ELISA was well-optimized. This assay effectively differentiated infected birds from vaccinated ones using a cutoff value of ≥ 0.250. Vaccinated birds and the control negative group were monitored for eight weeks post-injection and remained negative throughout the study. In contrast, the control positive group exhibited a positive response (Fig. 1). Notably, all chicken sera tested negative at 14 days of age using the Biocheck commercial avian adenovirus ELISA kit.

Serum samples from broiler chicken farms revealed a significant correlation between antibody response and the disease risk associated with each region. Specifically,86% of serum samples collected from regions with high disease prevalence tested positive for antibodies, indicating significant exposure to FAdV-4. Only 26% of the samples showed positive antibody responses in regions with moderate disease prevalence. Notably, all sera collected from regions without disease reports were negative, confirming the absence of virus exposure (Fig. 2).

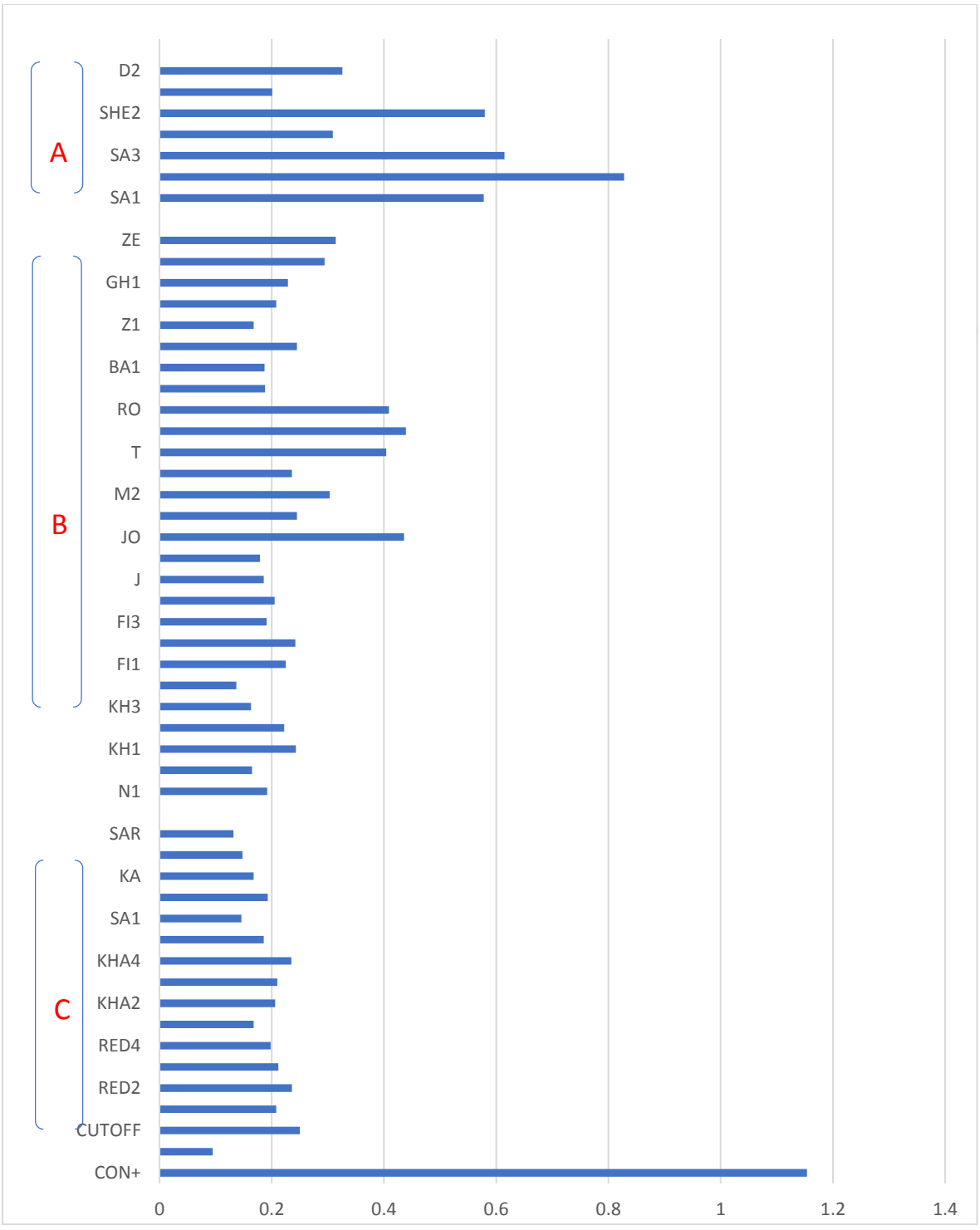

Figure 2: The average optical density of peptide-based ELISA in broiler chicken flocks of the Khorasan Razavi province sampled from areas with high prevalence of disease (A), with moderate prevalence (B), and without history of disease report (C).

4. Discussion

FAdV-4 is a highly contagious virus primarily affecting poultry, particularly broiler chickens aged 3-5 weeks. Over the past three decades, FAdV-4 has caused significant economic losses globally, with documented cases in countries such as Pakistan (15), Iraq (16), India (17), China (18), and Russia (19). The presence of FAdV-4 in neighboring countries has posed a severe risk to Iranian poultry farms. Consequently, veterinary clinicians have included FAdV-4 in differential diagnosis protocols. In March 2021, the first outbreak of HHS was reported in northeastern Iran on a broiler farm, leading to the culling of the affected flock (13). Unfortunately, the disease spread during a second wave, resulting in significant mortality, stunted growth, and uniformity issues in broiler farms. FAdV-4 vaccination was carried out in broiler breeder flocks to mitigate the disease's impact to establish passive immunity. ELISA, with its ability to efficiently process large volumes of samples and its straightforward execution, serves as an ideal test for epidemiological research. Still, the commercial ELISA used for adenovirus detection is group-specific and cannot distinguish between antibodies produced by vaccination and those resulting from natural infection. A peptide-based ELISA was developed to differentiate infected birds from vaccinated ones for FAdV-4 (19) to address this limitation. We also optimized this type of ELISA and applied it to assess viral contamination among broiler flocks of the province. The peptide-based ELISA detects the antibody response following exposure to the live virus, offering a more accurate measure of infection levels in poultry populations.

The findings from the peptide ELISA test revealed that the highest frequency of positive flocks was observed in areas with high disease prevalence. Notably, the test results were negative in

areas with no reported disease. This significant correlation between peptide ELISA results and disease prevalence underlines the test's efficacy in identifying FAdV-4-exposed broiler flocks.

The latent period and intermittent shedding of FAdV-4 pose limitations for cross-sectional studies using the PCR assay (20-22). Therefore, the study's results open up a promising new approach in virus tracking and epidemiological studies. The introduction of infected chicks into healthy flocks can initiate and amplify the circulation of viral pathogens within the primary host population, and, if unchecked, facilitate the rapid dissemination of disease to adjacent farms. In this regard, the peptide-based ELISA represents a valuable diagnostic tool for the early identification of infected chicks, with the added capacity to differentiate progeny from virus-exposed broiler breeder flocks versus those originating from vaccinated and unexposed populations. This diagnostic precision enables the strategic segregation and controlled relocation of healthy chicks to pathogen-free zones while ensuring the appropriate management of infected chicks within contaminated environments. Implementing such measures is critical for preventing the establishment of new infection hotspots in previously unaffected regions and significantly contributes to the overall efficacy of surveillance and biosecurity programs. Additionally, this test can be used as a practical tool for quality control of parent farms before FAdV-4 vaccination. Future research should also consider the broader application of peptide-based ELISA in conjunction with other diagnostic tools to assess FAdV-4 challenge levels in broiler breeders, layer pullet farms, and during the egg-laying period. This combined approach would provide a more comprehensive understanding of the virus's spread within poultry populations and aid in developing more effective control strategies

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Authors' Contribution

Authors' Contribution Study concept and design: RT,AS,VK

analysis: AS, RT,BM,AM

Acquisition of data: AS, RT

Data analysis and interpretation: RT,AS

Manuscript preparation: AS, RT

Critical revision of the manuscript: RT

Study supervision: RT,VK

Sampling:ASS.AS.ARH

Disclosure statement

No potential conflict of interest was reported by the authors.

Ethical statement

The project was found to be in accordance to the ethical principles and the national norms and standards for conducting Medical Research in Iran. The experiments did not include any invasive procedure involving the animals. The study design was reviewed and received approval from the Research Ethics Committees of Faculty of Veterinary Medicine University of Tehran (IR.UT.VETMED.REC.1403.047**).**

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