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A novel indirect ELISA for serological surveillance of fowl adenovirus in chickens: utilizing a regionally relevant field strain

Una nueva prueba ELISA indirecta para La vigilancia serológica del adenovirus aviar en pollos: Uso de una cepa de campo con relevancia regional

Beatriz Padron[1]*, Osman Erganis[1]

[1] Selçuk University, Faculty of Veterinary Medicine, Microbiology Department, Konya, Türkiye.

**Corresponding author: beatriz_padron_perez@hotmail.com*

ABSTRACT

Fowl adenoviruses are significant avian pathogens that cause considerable economic losses globally, associated with diseases such as inclusion of body hepatitis, hydropericardium syndrome, and gizzard erosion. There is a critical need for rapid, sensitive, and high-throughput diagnostic tools for seroepidemiologic surveillance and evaluating vaccine efficacy, as the traditional diagnostic methods are limited due to the high viral diversity, which often hinders the effectiveness of group-specific targeted commercial ELISAs, as the antigens may not align with regionally prevalent serotypes. This study details the development and validation of an indirect ELISA designed for the detection of anti-Fowl adenoviruses antibodies in chickens, utilizing an antigen derived from a field strain of Fowl adenoviruses from Türkiye and propagated in cell culture. The assay was validated using 109 serum samples, benchmarked against the commercial BioCheck Fowl adenoviruses-1 ELISA CK132 kit. The developed ELISA demonstrated an optimal sample dilution of 1:400 and a cutoff value of 0.125. It achieved a sensitivity of 90.20 % and a specificity of 100 %. A Cohen's kappa index of 0.675 indicated good agreement with the reference method. Furthermore, the assay exhibited good precision, with intra-assay coefficients of variation (CV %) ranging from 6.24 % to 11.03 % and an inter-assay CV % of 16.20 %, both within acceptable limits. This novel ELISA, employing a regionally relevant field strain, offers a cost-effective and specific tool for Fowl adenoviruses serological surveillance and vaccine monitoring, particularly beneficial in regions where circulating strains vary from vaccine strains.

Key words: ELISA; fowl adenovirus; validation; serology; poultry

RESUMEN

Los adenovirus aviares son importantes patógenos que causan considerables pérdidas económicas a nivel mundial y que están asociados a enfermedades como la hepatitis por cuerpos de inclusión, el síndrome de hidropericardio y la erosión de molleja. Existe una necesidad crítica de herramientas de diagnóstico rápidas, sensibles y de alto rendimiento para la vigilancia seroepidemiológica y la evaluación de la eficacia de las vacunas, ya que los métodos de diagnóstico tradicionales son limitados debido a la gran diversidad viral, lo que a menudo dificulta la eficacia de los ELISA comerciales específicos para cada grupo, puesto que los antígenos pueden no coincidir con los serotipos prevalentes en la región. Este estudio detalla el desarrollo y la validación de un ELISA indirecto diseñado para la detección de anticuerpos frente a los adenovirus aviares en pollos, utilizando un antígeno derivado de una cepa de campo de adenovirus aviares-E de Turquía y propagada en cultivo celular. El ensayo se validó utilizando 109 muestras de suero, comparadas con el kit comercial BioCheck adenovirus aviares-1 ELISA CK132. La prueba desarrollada demostró una dilución óptima de la muestra de 1:400 y un valor de corte de 0,125. Alcanzó una sensibilidad del 90,20 % y una especificidad del 100 %. Un índice kappa de Cohen de 0,675 indicó una buena concordancia con el método de referencia. Además, el ensayo mostró una buena precisión, con coeficientes de variación intraensayo (CV %) que oscilaron entre el 6,24 % y el 11,03 % y un CV % interensayo del 16,20 %, ambos dentro de los límites aceptables. Este novedoso ELISA, que emplea una cepa de campo relevante a nivel regional, ofrece una herramienta específica y eficiente con relación al coste para la vigilancia serológica del adenovirus aviar y el seguimiento de la vacunación, lo que resulta especialmente beneficioso en regiones donde las cepas circulantes varían con respecto a las cepas de las vacunas.

Palabras clave: ELISA; adenovirus aviar; validación; serología; avicultura

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INTRODUCTION

Fowl adenoviruses, classified within the *Aviadenovirus* genus of the Adenoviridae family, are segmented into three groups: Fowl adenoviruses (FAdV)-I, FAdV-II, and FAdV-III [1]. Group I is further subdivided into five species, encompassing twelve serotypes (1–8a, 8b–11) distinguished by their virus-neutralization profiles and genomic composition [2 , 3]. Genotype A includes FAdV-1; Genotype B includes FAdV-5; Genotype C includes FAdV-4; Genotype D groups FAdV-2, FAdV-3, FAdV-9 and FAdV-11; y Genotype E groups FAdV-6, FAdV-7, FAdV-8a and FAdV-8b [4]. Group II includes viruses responsible for hemorrhagic enteritis in turkeys and marble spleen disease in pheasants, while Group III is predominantly associated with egg drop syndrome virus [5].

Although FAdVs were historically regarded as non-pathogenic, they have since emerged as significant agents of disease in poultry, contributing to considerable global economic losses through outbreaks of inclusion body hepatitis (IBH), hydropericardium syndrome (HPS), and gizzard erosion (GE). Specifically, IBH is frequently linked to FAdV-2, FAdV-11, FAdV-8a, and FAdV-8b serotypes; GE is predominantly caused by FAdV-1; and HPS is attributed to FAdV-4 [6 , 7 , 8].

Adenoviridae family infections are present worldwide and the prevalence of specific serotypes varies across different geographical regions. For example, FAdV-4 is predominant in Asia, while FAdV-1 and FAdV-8b are more common in Europe and America [9]. In the case of Türkiye, the first cases were reported in 2020 by Şahindokuyucu *et al*. [10] and Çizmecigil *et al.* [11] and show that at least serotypes 8b and 11 are circulating; however, further information is still scarce.

Diagnostic methodologies for FAdV detection have undergone considerable advancement. Current antigen detection methods encompass polymerase chain reaction, quantitative PCR, loop-mediated isothermal amplification, high-resolution melting analysis, and sandwich enzyme-linked immunosorbent assays [12 , 13 , 14].

Traditional serological assays like agar gel precipitation and virus neutralization tests suffer from limited sensitivity and scalability. Consequently, the emergence of rapid, sensitive, and high-throughput Enzyme-Linked ImmunoSorbent Assay (ELISA) platforms is essential for accurate sero epidemiological surveillance and the assessment of vaccine efficacy, particularly considering potentially subclinical infections. Nonetheless, the substantial viral diversity presents a hurdle due to limited cross-protection among different strains [9 , 15].

Recent advancements include recombinant fiber- and hexon-based ELISAs tailored to specific serotypes, notably FAdV-4 [9 , 16 , 17].

This research outlines the creation and validation of an indirect ELISA employing a cell-cultured FAdV-E field strain from Türkiye. The primary goal was to develop a more appropriate and regionally relevant method for FAdV serological surveillance, by addressing the limitations of commercial ELISAs, which often utilize group-specific antigens that may not align with regionally prevalent outbreaks.

MATERIALS AND METHODS

Serum samples

The serum samples utilized in this study were gathered from different poultry (*Gallus gallus domesticus*) farms across Türkiye and sent to the Microbiology Department of the Veterinary Faculty of Selçuk University (Konya, Türkiye), where each blood sample was processed upon arrival and the serum was separated and stored at -40 °C (Ugur, UED 480 DGT D/S R64, Türkiye) until analysis. In order to assess the new ELISA and evaluate its reliability, only the samples that, independently of this study, were also sent to an external laboratory for diagnostic or control purposes and were analyzed using the commercial FAdV-1 ELISA CK132 kit (Biochek, Ascot, UK) were included in the study; a total of 109 samples.

ELISA plate coating

The antigen used was a strain of FAdV (SÜVF Stock Number 289), which had previously caused an IBH outbreak in southwestern Türkiye. The amplified hexon L1 gene fragment was sequenced directly using Sanger and a BLAST search (https://blast .ncbi.nlm.nih.gov) was used to compare these sequences with other isolates. SÜVF 289 was found to be related to species E with 100.00 % (e.g. OM858818.1, MK572861.1, MK572859.1) sequence identity. The isolate was then produced in a Vero monolayer cell line. FAdV antigen was measured using Qubit Protein Assay Kit (Invitrogen, Q33212, USA), diluted to a concentration of 5 µg/mL in a 0.05 M carbonate-bicarbonate buffer (pH 9.6) and distributed at a ratio of 100 µL/well into a polystyrene 96-well microplate. The plates were then incubated at +4 ºC (Altus, Al 328B, Türkiye) overnight, and at the conclusion of the incubation phase, they were washed three times with 300 µL of phosphate-buffered saline (PBS) (pH: 7.2) + 0.05 % Tween 20. To block antigen-unbound polystyrene surfaces, 100 µL of PBS (pH: 7.2) containing sterile 5 % skim milk powder (LabM, MC027, England) was distributed into each well and incubated at room temperature for 2 hours (h), followed by three washes with 300 µL of PBS (pH: 7.2) + 0.05 % Tween 20. The plates were then dried and kept at +4 °C (Altus, Al 328B, Türkiye) until needed [18 , 19 , 20 , 21].

ELISA procedure

To achieve the best possible test performance, the optimal sample dilution was determined. To optimize assay performance, a systematic evaluation of various serum dilutions was conducted to identify the minimum concentration yielding optimal signal, thereby ensuring accurate antibody detection while minimizing sample volume. This process utilized seven positive and one negative serum, whose titers and optical density values had been previously determined using the afore mentioned commercial ELISA kit.

Afterwards, the 109 sera were diluted using PBS buffer (pH 7.2) at a 1:400 concentration and distributed in duplicate into the coated wells (100 µL each), along with the positive (n = 1) and negative (n = 2) controls. The plates were kept at +4 °C overnight. After a washing step with PBS (pH: 7.2) + 0.05 % Tween 20, 100 µL of rabbit anti-chicken IgY (IgG) peroxidase conjugate (Sigma A9046, USA) diluted to 1/20,000 using a 0,05 M carbonate-bicarbonate buffer (pH 9.6) was added to the wells and allowed to incubate for 1 h at room temperature.

The plates were washed three times using the washing buffer, while the substrate, δ-phenylenediamine 98 dihydrochloride (δ-phenylenediamine dihydrochloride tablets, Sigma P8287, USA), was prepared by adding it to a phosphate-citrate solution (sodium perborate-capped phosphate-citrate buffer, Sigma P4922, USA). 100 µL of substrate was added to each well and incubated in the dark at room temperature for 30 minutes. Upon conclusion of the incubation period, 50 µL of stop solution (0.5 M H_2SO_4) was added to each well, and the results were evaluated by assessing the optical density (OD) at 450 nm with the help of an ELISA microplate reader (BioTek 800TS, USA) [18 , 19 , 20 , 21].

Cutoff value determination

To determine the optimal cutoff value for the developed ELISA test, a Receiver Operating Characteristic (ROC) curve analysis was performed using SPSS 26 software, based on 22 individual measurements. The cutoff point closest to the upper-left corner of the ROC curve was selected, as it represents the best trade-off between sensitivity and specificity [22].

Definition of the standard

A positive and a negative standard were selected from sera identified as positive and negative by the commercial FAdV antibody kit. Both standards were distributed in duplicate on each of the tested plates. The inclusion of internal standards on each plate allowed for normalization across runs, accounting for inter-assay variability and ensuring the reliability of quantitative measurements [23].

FAdV ELISA test validation

The FAdV Ab ELISA developed in this study was validated by comparing its performance with that of the FAdV-1 ELISA CK132 kit, the prevalent serologic test in Türkiye, using 109 serum samples. The sample size utilized in this study aligns with the quantities employed in previous literature for validating ELISA tests [22 , 24 , 25 , 26].

Diagnostic parameters such as diagnostic accuracy, sensitivity and specificity offer a comprehensive assessment of the assay's performance characteristics, offering insights into its practical utility for disease detection and epidemiological studies [25]. These were calculated using the selected cutoff value, based on the true positives (TP), false positives (FP), false negatives (FN), and true negatives (TN) obtained when compared to the commercial FAdV Ab kit. On the sensitivity and specificity parameters, a 95 % confidence interval was applied [22].

True positives, FP, FN and TN

$$\text{Diagnostic accuracy} = (TP + TN) / (TP + FP + FN + TN)$$

$$\text{Sensitivity} = TP / (TP + FN) * 100$$

$$\text{Specificity} = TN / (FP + TN) * 100$$

The precision of the assay was evaluated through both intra-assay and inter-assay variability analyses. Intra-assay variability was assessed using the 109 clinical samples, each tested in duplicate within the same microplate. For each sample, the coefficient of variation (CV %) was computed. Intra-assay CVs were computed separately for each plate, allowing for plate-specific precision profiling. Inter-assay variability was determined using two reference samples analyzed in duplicate across all four plates (n = 8 measurements per sample) [22].

The CV % for each reference sample was calculated, reflecting reproducibility across independent assay runs. To further minimize variability between assay runs and improve the interpretability of ELISA results, normalization techniques based on the reference samples were implemented across different experimental sessions. This methodological approach is designed to mitigate variations stemming from procedural differences across assays, thereby improving data consistency and overall dependability [27].

Statistical analysis

Receiver Operating Characteristic analysis was performed to determine the cutoff value. SPSS version 26 (SPSS Inc., Chicago, IL, USA) software was used. The remaining calculations were performed using Microsoft Excel for Microsoft 365, version 2401 (Build 16.0.17231.20182), included in Microsoft Office Professional Plus 2024 (Microsoft Corporation, Redmond, WA, USA). In all analyses, $P < 0.05$ was defined as "significant". For comparative assays, Cohen's kappa index was employed to quantify the degree of agreement, where P0 represents the observed probability of agreement and Pe denotes the probability of agreement occurring by chance [22].

$$\text{Cohen's kappa index} = (P0 – Pe) / (1 – Pe)$$

RESULTS AND DISCUSSION

This work contributes to the development of a novel ELISA test for detecting anti-FAdV antibodies in chickens. The developed test was validated against the FAdV-1 ELISA CK132 kit (Biochek, Ascot, UK) as a reference method because it is widely used and accepted in the poultry sector of Türkiye. When benchmarked against the commercial kit, along with a moderate agreement, the assay demonstrated a high specificity.

Sample dilution selection

The preliminary ELISA test was conducted using seven positive and one negative samples, diluted from 1:100 to 1:204,800. A good performance was achieved with sera diluted to 1:400 (FIG. 1). As expected, the OD values increased with lower dilutions. Nevertheless, the capacity of the assay to perform reliably with small sample volumes is a key advantage for commercial implementation and routine high-throughput testing.

Cutoff value determination and validation

The cutoff threshold for the in-house ELISA was established based on the ROC curve analysis at 0.125. A total of 109 serum samples were analyzed and the assay correctly identified 92 of the 102 positive samples (antibody detection rate: 81.65 %, sensitivity: 90.20 %; 95 % CI: 82.7–95.7 %) and all the negative samples (specificity: 100 %; 95 % CI: 59.3–100 %) (FIG. 2).

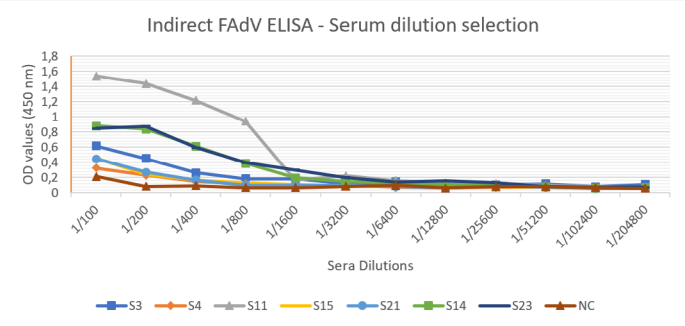

FIGURE 1. Indirect Fowl Adenovirus (FAdV) ELISA showing optical density at 450 nm (OD450) for serum samples diluted 1:100 to 1:204,800 (two-fold serial dilutions). Samples: S3, S4, S11, S15, S21, S14, S23 (positive sera), NC (negative control: negative serum). Colors correspond to sample IDs as labelled.

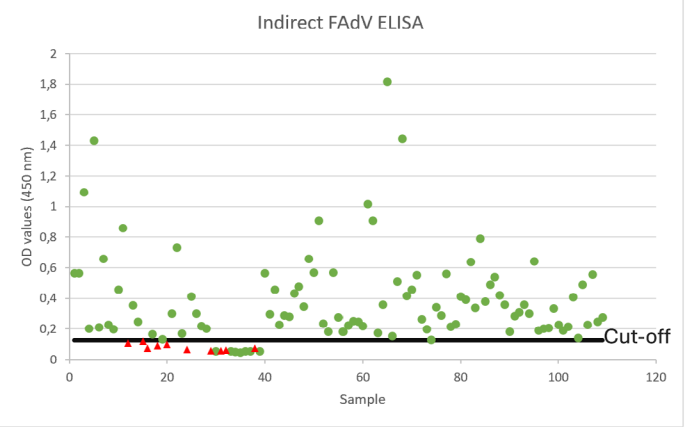

FIGURE 2. Comparison of OD450 values from the developed ELISA versus the reference commercial FAdV ELISA. Each point represents the OD450 value for each individual serum sample numbered 1–109. Red triangles are samples classified as positive by the reference kit but negative by the developed ELISA (discordant false negatives). Horizontal dashed line = positivity cutoff for the developed ELISA, calculated using the Receiver Operating Characteristic (ROC); samples with OD450 below this line were considered negative. OD = optical density; FAdV = fowl adenovirus; NC = negative control.

As the commercial ELISA was used as the standard method, the diagnostic accuracy, as well as the overall agreement between the two methods, was 90.83 % (95% CI: 85.4–96.2 %), with a Cohen's Kappa index of 0.675, indicating good agreement beyond chance. No FP were observed, while 10 TP samples were not detected by the novel assay.

The performance demonstrated, particularly the high specificity, indicates the assay's capacity to minimize FP, which is crucial for effective disease surveillance and management [28]. On the other hand, the occurrence of 10 FN results among the 109 analyzed serum samples suggests that the assay may have reduced sensitivity in samples with low antibody titers, variations in the immune response or infections by non-E serotypes. This limitation should be considered, in contexts requiring maximal case detection, although the assay remains suitable for population-level surveillance and vaccine monitoring.

Importantly, the present study did not evaluate the ability of the developed ELISA to differentiate infection from vaccination. This distinction requires specifically designed experimental infections or paired serological profiling, which were beyond the scope of the current validation. Likewise, the assay was not tested in regions with high serotype diversity. The assay should thus be interpreted as a regionally adapted tool optimized for genotype E exposure, rather than a universal replacement for existing commercial platforms.

These findings are consistent with previous studies that have reported variability in the sensitivity of ELISA assays depending on the antigen used, the stage of infection, and the animal species evaluated. In this regard, optimization of the ELISA protocol, including the selection of recombinant antigens or cross-validation with other molecular techniques, could improve its diagnostic performance, as it was demonstrated by Pan *et al.* [8], Feichtner *et al.* [9] and Shao *et al* [29]. The mean intra-assay CV % ranged from 6.24 % to 11.03 % across plates. Although the proportion of samples exceeding an inter-assay variation threshold of 15 % is higher than ideal (17.43 %), it remains within an acceptable range according to general guidelines [30, 31, 32, 33]. On the other hand, the inter-assay CV % was 16.20 %, which raised some doubts about the use of serum as a control, as it may introduce variability and inconsistencies between assay plates due to the inherent nature of the sample, a phenomenon previously documented in other ELISA methodologies [34], and which could be mitigated by developing a recombinant protein standard.

Interpretation of discordant results and assay scope

The absence of FP underscores the capacity of the assay to minimize erroneous classification at the individual-sample level, which is essential for surveillance and control decisions. The ten FN raise important considerations: they could reflect low antibody titers in certain samples, variability in host responses, or antigenic mismatch between the coating antigen and some infecting serotypes. In this instance, the BioChek ELISA's capacity to detect antibodies against all 12 FAdV serotypes suggests that serum samples negative in this assay may possess antibodies targeting other FAdV serotypes, Importantly, the ELISA in this study was coated with a Turkish field strain of genotype E, so its sensitivity is optimized for detecting antibodies against FAdV-E (including 8a/8b) and for measuring vaccine-related responses. Given that previous surveillance has documented circulation of 8b and 11 [9, 10], sera negative in this genotype-E coated assay may harbour antibodies induced by infections with non-E serotypes (e.g., serotype 11) or display antibody profiles shaped by vaccination; this pattern has been noted in earlier reports [35] and can contribute to discordant results.

Assay format, antigen choice, and practical relevance

The indirect ELISA format was chosen due to its potential for higher sensitivity compared to competitive and sandwich formats, which target a limited number of epitopes [36, 37]. A key advantage of this study is the utilization of an antigen derived from a Turkish field strain of FAdV-E as the coating antigen; this regional adaptation enhances the diagnostic utility, as current commercial ELISAs often target group-specific FAdV antigens, which may not always align with the specific serotypes prevalent in a given geographical area [8]; in this case, serotypes belonging to this genotype (especially 8b) have been previously reported in the country [10, 11]. This correspondence between the antigen genotype and the serotypes detected in local outbreaks reinforces the epidemiological relevance of the ELISA developed and its usefulness for regional surveillance This regional antigen choice improves epidemiological interpretation for Egenotype

exposure and vaccine monitoring, but it also narrows serotype breadth, reducing sensitivity for antibodies primarily directed against Dlineage serotypes such as 11, and thereby explaining some FN.

The ELISA design, although with several reagents currently imported, employs a field-derived antigen (SÜVF-289), which increases the assay's epidemiological relevance for local strains and may reduce reliance on imported commercial kits, offering a significant economic advantage. Even though a detailed cost analysis is not available per sample, the use of antigens produced in-house and generic reagents allows for reducing operating costs, especially in comparison with imported commercial kits that can be disproportionate in relation to local purchasing power. This feature makes it a more accessible tool for regional laboratories and low-cost surveillance programs, favoring their large-scale implementation

Limitations, future optimization and recommended application

The study presents certain limitations, such as a limited sample size and the absence of interlaboratory validation, that warrant further investigation. In addition, no information was provided about the samples, including vaccination status, which could significantly influence the interpretation of serological results by distinguishing between infection-induced and vaccine-induced immunity [35], as although heterologous reactions are present, homologous reactions are known to be stronger [38]. A genotypeE coated ELISA helps identify FAdV-E exposure and vaccine responses, but should be complemented by assays incorporating genotype D antigens or by a multiplex panel that includes both D and E antigens to resolve infections by the other genotypes present in Türkiye. This future optimization should also include the usage of recombinant or thoroughly characterized protein standards for plate normalization to counteract the higher-than-expected inter-assay variability. In addition, future studies incorporating controlled vaccination–challenge designs and broader serotype panels will be essential to further refine the assay's diagnostic scope and comparative performance.

Overall, the assay's high specificity, reasonable precision, and regional antigen suitability make it a suitable option for population-level serological surveillance and vaccine monitoring. Nonetheless, using it alongside reference methods is advisable in epidemiological research or clinical cases demanding the highest sensitivity.

CONCLUSIONS AND IMPLICATIONS

In summary, the successful creation and validation of this indirect ELISA, which uniquely employs a regionally relevant field FAdV-E strain from Southwest Türkiye, establishes it as a robust and highly specific diagnostic instrument tailored to local epidemiological conditions. The assay offers cost-effective and practical value for population-level serological surveillance and vaccine-monitoring programs, particularly in regions where genotype E is known to circulate. However, its ability to distinguish vaccine-induced from infection-induced antibodies or to outperform commercial kits in areas with high serotype diversity has not yet been evaluated and future studies incorporating controlled vaccination–challenge designs and broader serotype panels will be essential to further refine the diagnostic scope and comparative performance. This assay significantly enhances FAdV serological surveillance and vaccine monitoring by reflecting the local epidemiological conditions within poultry populations. Moreover, its practical utility should be considered in the context of regional disease control programs, where a clear distinction between individual-level diagnosis and population-level surveillance is essential for guiding intervention strategies and evaluating vaccine coverage.

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Conflict of interest

The authors have no conflicts of interest that could be perceived as prejudicing the impartiality of the study.

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