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Establishment of an RAA-CRISPR/Cas12a-based diagnostic method for the detection of fowl adenovirus serotype 4 virus in chickens and wild birds

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Abstract

Fowl adenovirus (FAdV) serotype 4, recognized as the causative agent of hydropericardium syndrome (HPS) in chickens, causes substantial economic losses in poultry farming. To develop a simple, rapid, and reliable diagnostic method for the timely detection of FAdV-4 nucleic acid, we integrated the CRISPR/Cas12a system with recombinase-aided amplification (RAA). This approach enables visual detection of FAdV-4 with a sensitivity of one genome copy. The results can be obtained within 40 to 50 min without the need for complex instrumentation, making it ideal for remote field applications. Using this method, we investigated the prevalence of FAdV-4 in both common farm poultry and wild birds. Our results indicated that the FAdV-4-positive rate in wild birds was 51.19%, suggesting that wild birds may serve as specific reservoirs for this virus. In summary, we present a sensitive, swift, accurate, and inexpensive detection method for FAdV-4, along with an investigation of its epidemic situation in birds. Our study advances the detection and epidemiological understanding of FAdV-4 transmission among farm poultry and wild birds.

Keywords Fowl adenovirus, Enzymatic recombinase amplification, CRISPR/Cas12a, Diagnosis

Introduction

The adenovirus family Adenoviridae is classified into mammalian adenoviruses and fowl adenoviruses on the basis of differences in their infection reservoirs (Andavar et al. 2023). There are 12 subtypes of poultry adenoviruses. Fowl adenovirus (FAdV) serotype-4, which is considered the causative pathogen of hydropericardium syndrome (HPS), causes significant economic losses to poultry farms. FAdV-4 infection manifests severe symptoms such as lethargy, disordered feathers, loss of appetite, and green excretion, leading to typical pathological changes such as hepatitis, pericardial effusion, and nephritis (Grgic et al. 2013; Sultan et al. 2021). FAdV-4 was first reported in Pakistan in 1987, where it was named 'Angara Disease' because it was first identified in Angara Goth, near Karachi. Subsequently, outbreaks of FAdV-4 infection have spread to other countries,

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including Iraq, Japan, Russia, Korea, and China (Abdul-Aziz and Hasan 1995; Li et al. 2016; Lobanov et al. 2000).

FAdV-4 infections are typically endemic in poultry farms, with broiler chickens being the most affected targets. These chickens are most likely to develop hydropericardium syndrome (HPS) following FAdV-4 infection. The mortality rate in FAdV-4-infected chickens aged three to five weeks can reach as high as 80% (Xie et al. 2023; Grgic et al. 2013). FAdV-4 was also detected and isolated from waterfowl, including ducks, geese ducks and mandarin ducks (Manzoor et al. 2013; Pan et al. 2017a; Roy et al. 2004). Recently, wild birds, such as pigeons, ostriches, crows, and quails, were shown to be infected with FAdV-4 (Kumar et al. 2010). Wild birds may act as natural reservoirs for FAdV-4, contributing to the pandemic of FAdV-4 infection (Shen et al. 2019). The epidemiological investigation of FAdV-4 in both farm and wild birds can enhance our understanding of the transmission dynamics among various host species, offering valuable insights for the control and prevention of FAdV-4.

FAdV-4 is an unencapsulated double-stranded DNA virus that expresses both major structural and nonstructural proteins. Hexon, penton base, fiber 1, and fiber 2 are the main structural proteins of the FAdV-4 capsid. Hexon, a monomer of FAdV-4, consists of two conserved pedestal regions, P1 and P2, and hexon has been widely used in virus detection and vaccine development (Roberts et al. 2006).

The present detection methods for FAdV-4 include TaqMan-based real-time PCR (Wang et al. 2017), droplet digital PCR (Shen et al. 2019), LAMP real-time turbidity (Yuan et al. 2019), and CRISPR/Cas13a-based lateral flow (Yin et al. 2023). By combining RAA with the CRISPR/Cas12a system, a diagnostic method was developed for the rapid and precise detection of FAdV-4. This method completes the detection process in 40 min, resulting in high specificity and sensitivity without the need for specialized equipment. Based on this diagnostic method, epidemiological investigations have been conducted in broiler chickens, laying hens, ducks, geese, pigeons, and wild geese in central China.

Results

Optimization of RAA/Cas12a-based cleavage for FAdV-4 detection

Figure 1 illustrates the workflow of RAA/Cas12a-mediated FAdV-4 detection. First, the RAA produced exponential amplification of the FAdV-4 target double-stranded DNA (dsDNA) in the presence of two primers at a relatively low temperature (37 °C). Next, the Cas12a protein, which specifically recognizes and cleaves the target dsDNA with the aid of crRNA, breaks the dsDNA to generate sticky ends. Finally, with the activation of LbCas12a nuclease trans-cleavage activity, the FAM- and BHQ1-labeled ssDNA reporters are cleaved, releasing signals that can be visually detected under UV or blue light.

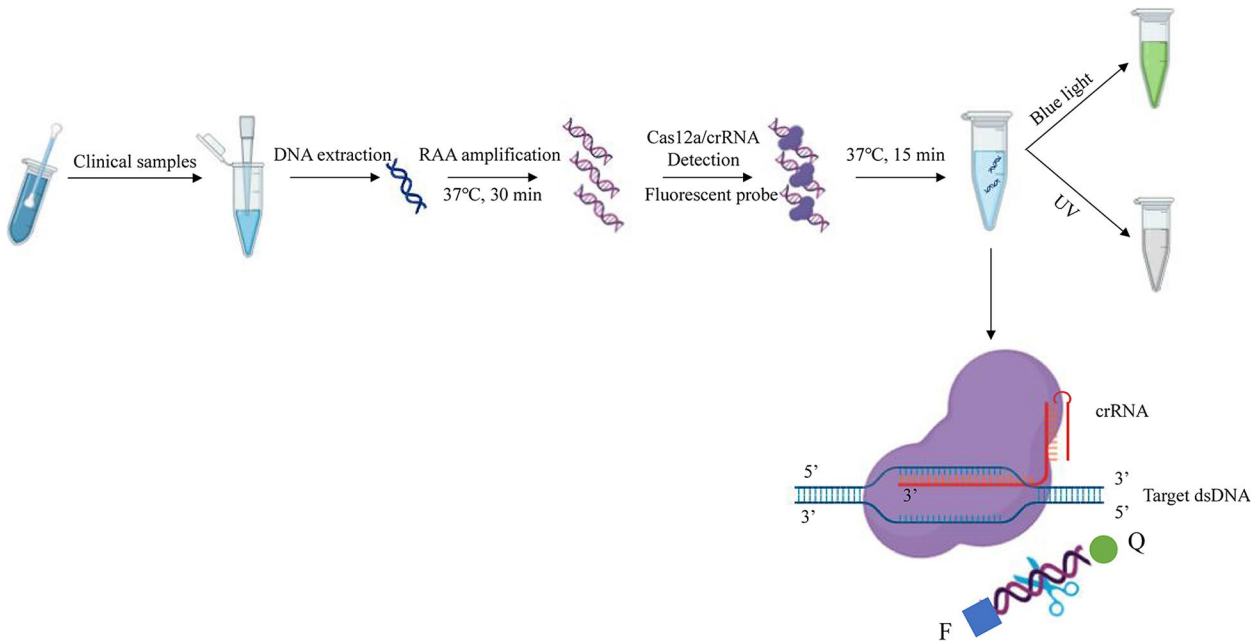


Fig. 1 Schematic diagram of RAA preamplification and Cas12a/crRNA cleavage for the detection of FAdV-4 nucleic acids

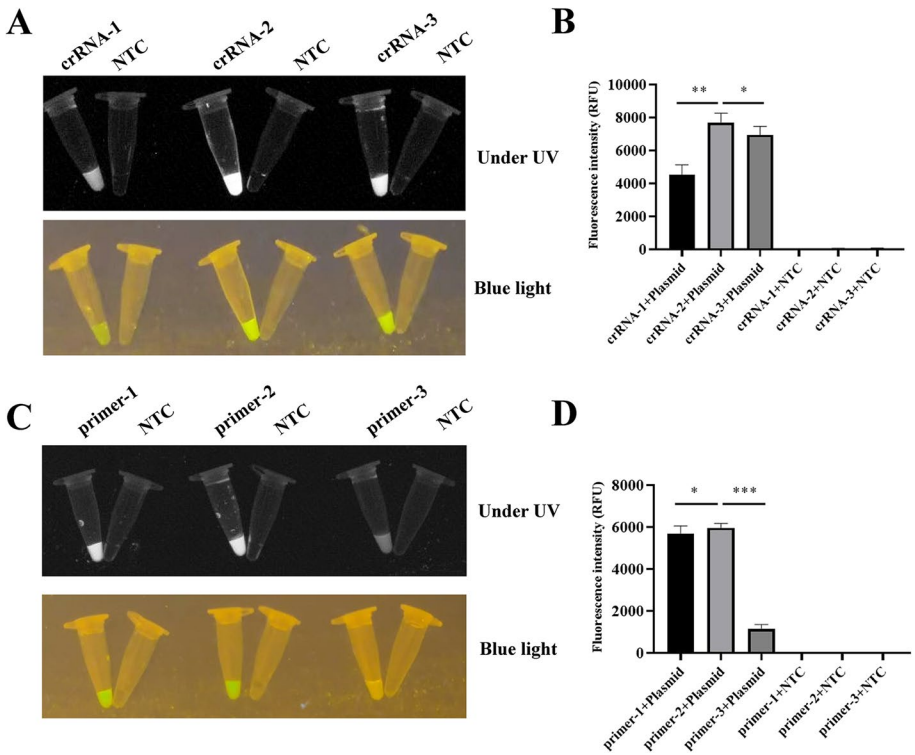


Fig. 2 Optimization and validation of crRNA screening and RAA amplification. **A** Different crRNA screening and visual observation under UV and blue light. **B** The fluorescence intensity of three pairs of crRNAs cleaved. **C** Three pairs of RAA primers were used to amplify the FAdV-4 gene, Cas12a/crRNA cleavage was performed, and the results were subsequently observed under UV and blue light. **D** Fluorescence intensity of three pairs of RAA primers. NTC, no template control. The error bars represent the SEMs; $n = 3$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$,

To assess the efficiency of crRNA, three pairs of crRNAs were designed to target the *hexon* gene, with pMD-19 T-hexon used as the template. Fluorescence intensity was measured across reactions containing different crRNAs, and crRNA-2 presented the highest fluorescence intensity, indicating its use in further experiments (Fig. 2A and 2B).

To validate the efficiency of the RAA primers, three pairs of RAA primers were designed for the amplification of the target *hexon* gene. The RAA products underwent Cas12a-mediated cleavage reactions, and the fluorescence intensity of these reactions was calculated. RAA primer two presented the highest fluorescence intensity (Fig. 2C and 2D).

Optimization of RAA/Cas12a-based cleavage reactions

To confirm the suitable reaction temperature and time for the Cas12a-mediated cleavage assay, 5 μl of the RAA amplification products were used in the Cas12a/crRNA assay at temperatures of 31, 33, 35, 37, 39, and 41 °C for 30 min. The highest fluorescence intensity was observed at 37 °C (Fig. 3A and 3B). Additionally, 5 μll of the RAA amplification products were used in the Cas12a/crRNA assay for reaction times of 0, 5, 10, 15, and 20 min. The optimal reaction time was determined by assessing the fluorescence intensity (Fig. 3C and 3D). The fluorescence intensity gradually increased with increasing reaction time, with the highest intensity observed at 15 min, after which it stabilized. All the results indicated that the optimal conditions for Cas12a-based cleavage reactions were 37 °C for 15 min.

Specificity and sensitivity analysis of the RAA/Cas12a-based diagnostic method

The specificity of the RAA/Cas12a-based method was confirmed by testing against several other common avian viruses, including FAdV-1, FAdV-7, FAdV-8a, FAdV-8b, FAdV-9, FAdV-10, IBDV, MDV, ILTV, ALV-J, IBV, NDV, H5 AIV, and H7 AIV. Fluorescence or brightness was observed only in samples containing FAdV-4 (Fig. 4A and 4B). To assess sensitivity, tenfold serial dilutions of pMD19-T-hexon were used as templates for RAA/Cas12a-based detection. The limit of detection (LOD) was determined to be one copy for visual observation under LED blue light (Fig. 4C and 4D).

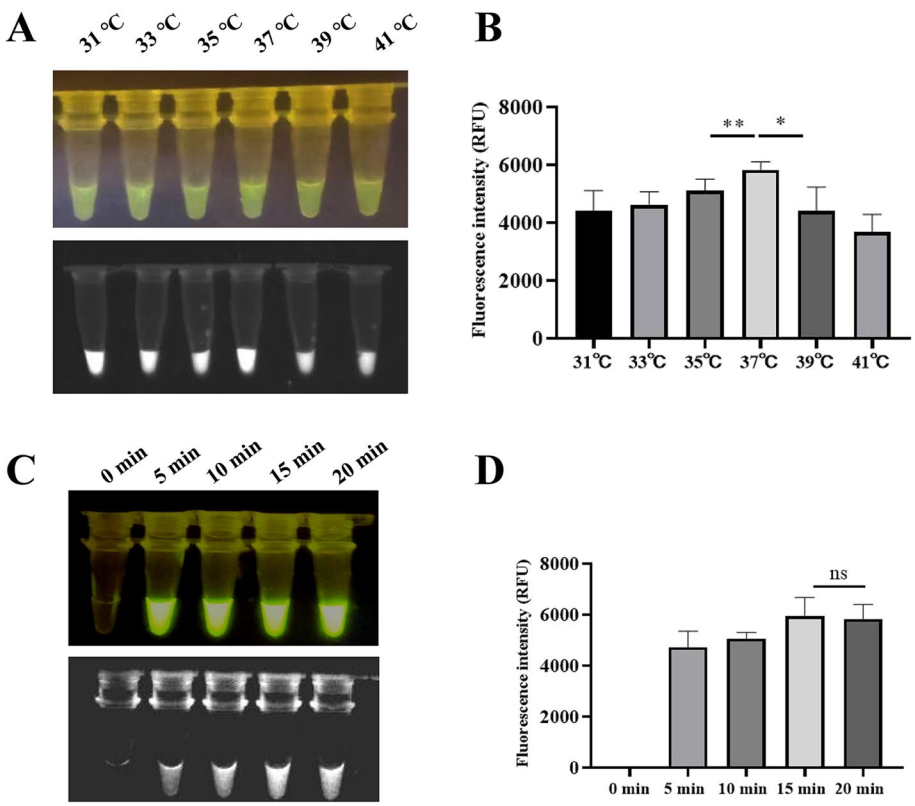


Fig. 3 Optimization of the RAA-CRISPR/Cas12a system. **A** Images under UV and blue light at different reaction times. **B** The fluorescence intensity at different reaction times. **C** Images under UV and blue light at different RAA reaction times. **D** The fluorescence intensity of different RAA reaction times. The error bars represent the SEMs; n = 3. *, $p < 0.05$; **, $p < 0.01$. ns, no significant difference

Inspection of the RAA/Cas12a-based diagnostic method in bird clinical samples

A total of 30 clinical samples collected from different birds were screened via both qPCR assays and the the proposed method, with positive samples further confirmed via DNA sequencing. The results from the RAA/Cas12a method indicated that 11 samples were positive and 19 were negative, perfectly matching the results of the qPCR assay (Fig. 5A and 5B). The RAA/Cas12a method proved to be accurate for the detection of FAdV-4 in clinical samples (Fig. 5C).

Epidemiological investigation of FAdV-4 in birds in China

FAdV-4 infects various host species, including farm poultry and wild birds. We detected 640 samples, with an overall positive rate of 30.78%. The detection rates of FAdV-4 were further categorized according to species. Among the farm poultry samples, 66 out of 236 broiler chicken samples, 41 out of 197 laying hen samples, one out of 23 duck samples, and three out of 16 geese samples tested positive. Among the wild bird samples, 57 out of 84 pigeon samples, 28 out of 59 wild goose samples, and one out of 25 crow samples were positive. Among poultry farms, broiler chickens had the highest positive rates. In wild birds, the highest positive rate was 67.86% in pigeons, which deserves special attention (Table 1).

Discussion

HPS, which is caused primarily by FAdV-4, has led to large-scale outbreaks in China since 2015. FAdV-4 exhibits high transmissibility to infect various host species (Xue et al. 2023). In farm poultry, FAdV-4 can spread horizontally among chickens within a short period through both vertical and horizontal transmission. Wild birds commonly have a wide range of movements, with some foraging for farm feed. These characteristics expose wild birds directly to a broad range of potential pathogens (Wang et al. 2022). FAdV-4 has been detected and isolated in multiple wild bird species, including wild black kites, pigeons, wild geese, crows, and others (Kumar et al. 2010; Zhuang et al. 2023). The control and prevention of FAdV-4 spread still rely on laboratory diagnostic methods (Shao et al. 2019). However, in some tiny farms and fields, the lack of sophisticated instruments and specialized technicians for timely diagnosis leads to disease transmission. Therefore, there is an urgent need

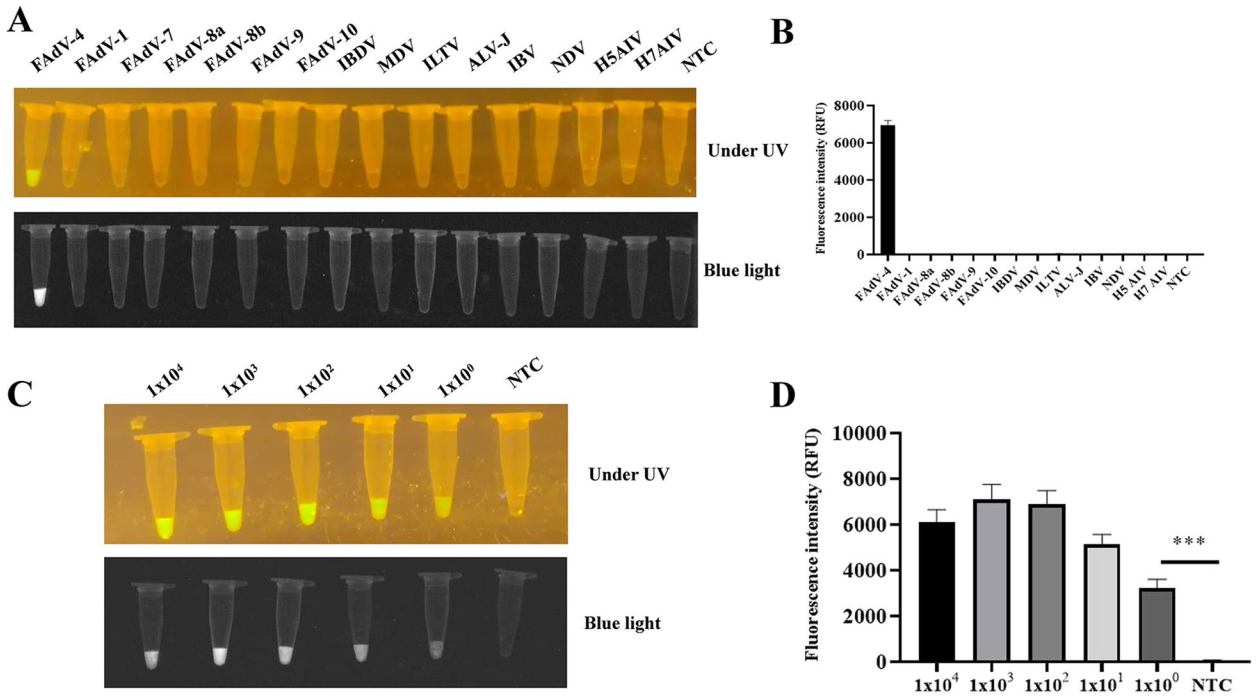


Fig. 4 Specificity and sensitivity of the RAA/Cas12a-based diagnostic method. **A** The specificity of the the proposed method for FAdV-4 was measured relative to that of 15 species of bird virus. **B** The fluorescence intensity was used to determine specificity. **C** Various concentrations of pMD-19 T-hexo were used as templates to assess sensitivity. **D** End-point fluorescence intensity was used to determine sensitivity. NTC, no template control. The error bars represent the SEMs; $n = 3$. ***, $p < 0.001$

to develop an accurate, rapid, portable diagnostic method to detect FAdV-4 effectively, which may contribute to preventing and controlling FAdV-4 transmission.

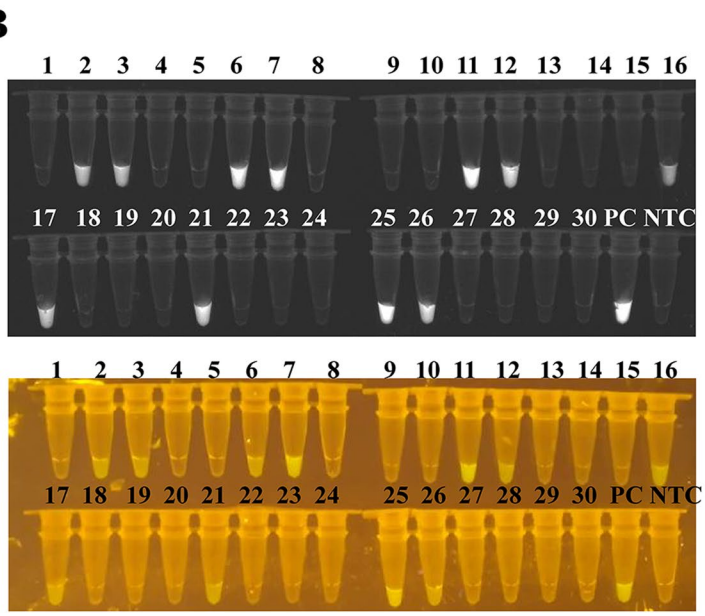
Although several FAdV-4 detection methods are currently in use, there are limitations in their clinical application. Serological detection methods, such as enzyme-linked immunosorbent assays, are unable to detect positive samples in the early stages of FAdV-4 infection because of their low specificity and sensitivity (Tabatabaei and Ahmed 2022). Other room-temperature amplification methods, such as loop-mediated isothermal amplification and recombinase polymerase amplification, can occasionally yield false positives, greatly affecting detection accuracy (Jaroenram and Owens 2014). Traditional PCR and qPCR require sophisticated instruments, which are not suitable for field use (Chen et al. 2023). The combined use of CRISPR/Cas12a technology addresses these issues, as the ssDNA reporter generates a positive signal, whereas Cas12a recognizes the intended target and activates the nonspecific cleavage properties of the ssDNA reporter, thereby overcoming the possibility of false positives (Tanny et al. 2023). The CRISPR/Cas12a system was used to detect hepatitis B virus with a high sensitivity of 1 copy/μl (Ding et al. 2021). Wuyin Zhang et al. used the CRISPR/Cas12a system to detect porcine circovirus type 3, and the method has high sensitivity for detection down to seven copies and no cross-reactivity with other types of porcine circovirus (Yu et al. 2024). The CRISPR/Cas12a system was used to generate the pseudorabies virus. By targeting the gB, gE and TK genes of pseudorabies viruses, the method can distinguish infected, uninfected, and vaccinated swine (Wang et al. 2024). The *hexon* gene is divided into conserved pedestal regions (P regions), which express the conserved protein located inside the virion, and variable regions (L regions), which express the loop protein located on the outer surfaces between subtypes to form type-specific epitopes (Ganesh et al. 2001). *Hexion* genes are widely used to analyze the genetic evolutionary relationships and subtype classification of FAdV (Zhang et al. 2018). In this study, the highly conserved regions of the *hexon* gene were chosen as targets to design RAA primers and crRNAs. The method is highly specific, and it does not cross-react with common FAdVs (1, 4, 7, 8a, 8b, 9 and 10 subtypes) or other pathogens, such as AIV. RAA/Cas12a is as sensitive as qPCR but does not require sophisticated instruments and takes less than 1 h. The lowest detection limit of RAA/Cas12a was only 1 copy. All of these advantages contribute to clinical diagnosis and wide application.

FAdV-4 can be transmitted from farm poultry to wild birds, and wild birds may also serve as reservoirs of

A

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
qPCR CT value	-	27.4	31.4	-	-	32.6	28.1	-	-	-	24.3	28.6	-	-	-	25.2
RAA-Cas12a FI value (x1000)	-	4.2	4.9	-	-	3.7	3.6	-	-	-	5.8	4.3	-	-	-	4.9

	17	18	19	20	21	22	23	24	25	26	27	28	29	30	PC	NTC
qPCR CT value	26.4	-	-	-	27.3	-	-	-	23.6	24.1	-	-	-	-	21.7	-
RAA-Cas12a FI value (x1000)	4.5	-	-		3.9	-	-		5.4	4.1	-	-			5.9	-



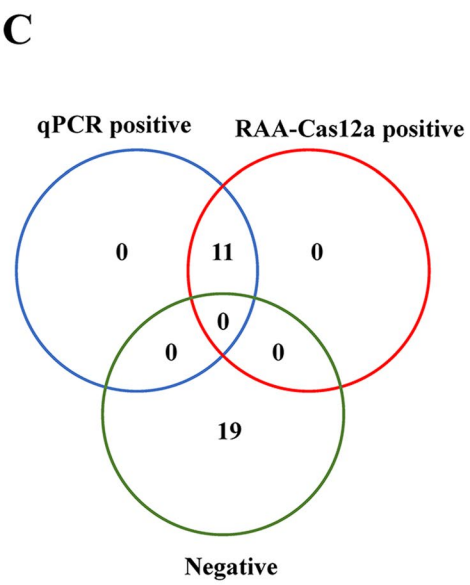


Fig. 5 accuracy calculation of the RAA/Cas12a-based diagnostic method via qPCR. **A** Results of the qPCR assay and RAA/Cas12a method for clinical samples 1–30. The results of qPCR are shown as the ct value. A ct value less than 30 was considered a positive sample. The fluorescence intensity of RAA/Cas12a is shown. **B** Results of the RAA/Cas12a method for clinical samples. The images were captured under UV and blue light. **C** Venn diagram of the results of qPCR and RAA/Cas12a, which were used for the detection of FAdV-4 in clinical samples. NTC, no template control. FI value, fluorescence intensity value

FAdV-4 (Mo 2021). Previous reports have shown that the positive rate of FAdV-4 infection was 16.87% in duck and chicken clinical samples (Andavar et al. 2023). Another report revealed that 80% of serum samples from crows were positive for FAdV-4 (Manzoor et al. 2013). Here, we found that farm poultry are sensitive and maintain high infection rates of FAdV-4, with broiler chicken samples collected from China showing a positive rate of 27.97%. In wild birds, we report for the first time that wild geese have a high infection rate of FAdV-4, as detected by the RAA/Cas12a method. The percentage of nucleic acid-positive individuals was 67.86% in pigeons and 4% in crows. The FAdV-4 strains isolated from chickens and waterfowl presented minimal genetic variation, suggesting the potential for cross-species transmission among chickens, ducks, and other avian species. Considering the bidirectional viral transmission pathways of FAdV-4 between domestic poultry and wild birds, continuous global surveillance for fowl adenovirus is imperative (Pan et al. 2017b).

Table 1 Positive rates (%) of FAdV-4 detected by RAA/Cas12a

Background	Farm poultry				Wild birds			Totol
	Broiler chickens	Laying hens	Duck	Geese	Pigeon	Wild goose	Crow	
FAdV-4 positive rate	27.97 66/236	20.81 41/197	4.35 1/23	18.75 3/16	67.86 57/84	47.46 28/59	4.0 1/25	30.78 197/640

Conclusion

We reported an RAA-CRISPR/Cas12a method for the convenient and sensitive detection of FAdV-4. It offers significant advantages, including speed, simplicity, and low cost, making it suitable for the establishment of mobile testing stations in the field. Using this method, we investigated the positive rates of FAdV-4 in common farm poultry and wild birds. Our results indicated that some wild birds, such as pigeons and wild geese, can be infected with FAdV-4, suggesting that wild birds may serve as specific reservoirs for the virus.

Methods

Viral strains

The viral strains FAdV-1, FAdV-4, FAdV-7, FAdV-8a, FAdV-8b, FAdV-9, FAdV-10, infectious bursal disease virus (IBDV), Marek's disease virus (MDV), avian infectious laryngotracheitis virus (ILTV), subgroup J avian leukosis virus (ALV-J), infectious bronchitis virus (IBV), Newcastle disease vaccine (NDV), and H5 and H7 subtype avian influenza viruss (H5 AIV and H7 AIV) were stored in our laboratory.

Clinical samples

Tracheal and cloacal swabs, as well as fecal samples, were collected from broiler chickens, laying hens, ducks, geese, pigeons, wild geese, and crows in central China from 2020–2024. The samples were categorized according to the species of birds. The farm poultry samples included 236 from broiler chickens, 197 from laying hens, 23 from ducks, and 16 from geese. The wild bird samples included 84 samples from pigeons, 59 from wild geese, and 25 from crows. The swabs or 0.5 g of fecal samples were mixed with 0.5 ml of PBS. After thorough mixing by vortexing, the supernatant was centrifuged at 10,000 × *g*. A commercial TIANamp Virus DNA/RNA Kit (Tiangen, Beijing, China) was used to extract DNA from the samples for further detection.

Standard plasmid construction

The *hexon* sequence of FAdV-4 was chemically synthesized (Sangon Biotech, Wuhan, China). The *hexon* sequence was subsequently fused into the pMD19-T plasmid (Takara, Dalian, China), which was subsequently named pMD19-T-hexon. The copy number of pMD19-T-hexon was calculated via the following formula: pMD19-T-hexon copy number = $(6.02 \times 10^{23} \times$ plasmid concentration (ng/μl))/(660 Da/base pair × plasmid length × 10^{9}).

Recombinase-aided amplification (RAA) primers, crRNA and DNA reporter design

The *hexon* sequences of the representative and classic and emerging isolated FAdV-4 strains (accession numbers: KU587519.1, KU991797.1, KP295475.1 KX364099.1, LC628937.1, KX364099.1, and KY636400.1) were analyzed via MegAlign software. The highly conserved regions of the *hexon* gene were chosen as targets to design RAA primers and crRNAs. The website https://ezassay.com/primer was used to design RAA primers, and the website http://www.rgenome.net was used to design crRNAs.

The real-time fluorescent probes used included FAM (fluorophore) and BHQ1 (quencher). FAM-BHQ1-labeled single-stranded DNA (ssDNA) reporters, RT-RAA primers, and crRNAs are listed in Table 2 and were synthesized by Sangon Biotech.

RAA reaction of viral DNA

The amplification of the FAdV-4 *hexon* genes was performed with commercial RAA basic (Cat. No. HP80201, HuicH Gene Biotechnology Co., Ltd., Shanghai, China). Briefly, 15.0 μl of master mixture, 8.0 μl of water, and 5 μl of RAA primers (10 μM) were added to an RAA pellet to dissolve the enzyme, and then, 1 μl of extracted sample DNA was added and vortexed to mix thoroughly. The reactions were incubated at the appointed temperature in a water bath for 20–30 min.

CRISPR/Cas12a-based detection of RAA-amplified products

The RAA-amplified products were used for Cas12a-mediated cleavage. Briefly, 5 μl of RAA product was transferred to 20 μl of CRISPR/Cas12a-mediated cleavage reaction mixture with 500 nM Cas12a protein (kindly provided by Prof. Xueyan Sun, Wuhan Institute of

Table 2 Primers and probes

Primers	Sequences (5′–3′)
crRNA-1	UAAUUUCUACUAAGUGUAGAUACAAGTTCAGACAGACGGTCGT
crRNA-2	UAAUUUCUACUAAGUGUAGAUGCCCACCCGAAATGTCACGACAGAA
crRNA-3	UAAUUUCUACUAAGUGUAGAUGAAAAACAAGTTCAGACAGA
RAA-1F	ATCGCGGGCCCCGGGACGCGCG
RAA-1R	CGTTGAGCCTTTTCTGTCGTGACATTTCGGG
RAA-2F	GCGAATACCTCTCTGAGGACCTCCAACAGT
RAA-2R	GATTTGCAGCCGTTGAGCCTTTTCTGTCGTG
RAA-3F	CGGCTCCAGTATTTTCACATCGCGGGC
RAA-3R	CGTTGAGCCTTTTCTGTCGTGACATTTCGGG
ssDNA reporter	FAM-TTTTTT-BHQ1

Technology), 500 nM crRNA, 500 nM ssDNA reporter, and 1×NEB buffer 2.1 (New England Biolabs, Ipswich, MA). This mixture was incubated for 20 min at 37 °C. The emitted fluorescence signal was detected by a Light-Cycler 96 (Roche, USA) through direct visual observation with 800-ms UV light exposure or under blue light.

Sensitivity and specificity analysis of the RAA/Cas12a-based detection method

Tenfold serial dilutions of pMD19-T-hexon (original concentration of 1×10^6) were used as samples to evaluate the sensitivity of this diagnostic method.

A total of 6 species of FAdV, including 1, 7, 8a, 8b, 9 l and 10 subtypes and the IBDV, MDV, ILTV, ALV-J, IBV, NDV, H5 AIVl and H7 AIV strains, were used as samples to perform RAA/Cas12a-based detection. FAdV-4-positive clinical samples were used as positive controls.

FAdV-4 detection by quantitative real-time PCR

DNA was extracted from clinical samples. The qPCRs were prepared in a final volume of 20 µl as follows: 10 µl of 2×AceQ qPCR Probe Master Mix, primers at a final concentration of 0.25 µM, a TaqMan probe at a final concentration of 0.25 µM, 2 µl of DNA template and ddH_2O to 20 µl. The cycle threshold (CT) value of each sample was observed. A standard curve was generated, and the correlation coefficient was calculated via Light Cycler software.

Abbreviations

PBS Phosphate-buffered saline
crRNA CRISPR RNA

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Authors' contributions

Conceptualization, X.Z.; Methodology, Y.Z.; Software, Y.Z.; Validation, L.Y.; Formal analysis, Y.F.; Investigation, X.Z., H.W. and Y.F.; Writing—original draft, L.Y. and P.Z.; Project administration, L.Y. and D.F.; Funding acquisition, X.Z. All the authors have read and agreed to the published version of the manuscript.

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Data availability

The datasets used and/or analyzed during this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All the authors approved the final manuscript and its submission to this journal.

Competing interests

The authors declare that they have no conflicts of interest.

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