Development of a PCR-based dot blot assay for the detection of fowl adenovirus

Lidan Hou,[*,†,1] Qi Su,[‡,1] Yawen Zhang,[‡,1] Dan Liu,[†] Yaqing Mao,[†] and Peng Zhao[‡,2]

[*]*China Institute of Veterinary Drug Control, Beijing 100081, China;* [†]*College of Veterinary Medicine, China Agricultural University, Beijing 100193, China; and* [‡]*College of Veterinary Medicine, Shandong Agricultural University, Tai'an, Shandong 271001, China*

ABSTRACT Group-I Fowl adenovirus (**FAdV**) is still widespread in China's chicken farms, leading to huge economic losses. The traditional PCR method, which can detect all serotypes at the same time, is not sensitive enough to obtain accurate results, especially in some samples containing only a low titer of virus, such as contaminated live vaccine. In order to solve this problem, this study developed a dot blot assay based on the above PCR method. A total of 6 probes targeting the conserved region of FAdV were designed and systematically optimized through sensitivity, accuracy, and stability analyses. Results showed that it is not only suitable for 12 serotypes, but also effectively improve the sensitivity, which increased more than 100 times in comparison with PCR assay. Moreover, this sensitivity was increased 100 times when detecting contaminated live vaccine samples, showing the great prospect of this method in daily monitoring.

Key words: dot blot, fowl adenovirus, detection, PCR, serotypes

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INTRODUCTION

Fowl adenovirus (**FAdV**) is a conditional pathogen in poultry and wild birds, with complex serotypes and genotypes (Shah et al., 2017). Most FAdVs usually infect hosts without causing obvious clinical symptoms, and several strains have caused widespread epidemic without being noticed (Chen et al., 2019; Huang et al., 2019). However, when co-infected with other pathogens such as chicken infectious anemia virus (**CIAV**), it can cause serious clinical diseases and lead to huge economic losses (Su et al., 2018). Traditionally, all FAdV can be divided into 3 groups (Ⅰ−Ⅲ) according to the clinical symptoms and host range (Schachner et al., 2018). Among them, group-I FAdV was mainly isolated from chickens and turkeys, and can be further divided into 5 species (A-E) and then 12 serotypes (1-8a, 8b-11), while other groups only contain one strain or serotypes. Therefore, detection and control of group-I FAdV is the main challenge for chicken farms.

Since June 2013, hydropericardium-hepatitis syndrome (**HHS**) caused by FAdV-4 has appeared in many provinces of China, posing a huge threat to poultry industry (Jiang et al., 2019; Su et al., 2020). Several studies have demonstrated that the using of live vaccines contaminated with both FAdV-4 and CIAV maybe the main reason for the outbreak of HHS (Su et al., 2018; Su et al., 2019a; Su et al., 2019b). After that, a lot of attention has been paid to the detection of FAdV-4, and many detection methods for FAdV-4 have been established (Niczyporuk et al., 2015; Shao et al., 2019; Yao et al., 2019; Yuan et al., 2019; Lu et al., 2020; Pan et al., 2020). At present, the prevalence of FAdV-4 in China has gradually subsided, but accumulating evidences show that there are many other serotypes of FAdV circulating in China (Chen et al., 2019; Huang et al., 2019; Su et al., 2019c).

It is difficult to detect all these 12 serotypes of group-I FAdV at the same time because of the huge differences between their genomes (Li et al., 2017). Up to now, only one traditional PCR method can do this (Hess, 2000), but its sensitivity is far from meeting the needs, especially in some samples with only a small amount of virus, such as contaminated live vaccine (Su et al., 2020). More importantly, FAdV is a kind of vertical transmission virus, which can enter the live vaccine through chicken embryo used to produce vaccine, and then spread widely, thus the detection of exogenous FAdV contamination in live vaccines is also very important (Su et al., 2019b), but it needs an extremely high sensitivity, which puts forward higher requirements for the establishment of new methods.

To this end, this study developed a dot blot assay based on the above PCR method, which is not only

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[1]These authors contribute equally to this article.

[2]Corresponding author: zhaopeng@sdau.edu.cn

suitable for 12 serotypes, but also effectively improve the sensitivity, meeting the requirements of the above 2 aspects.

MATERIALS AND METHODS

Viral Strains Used in This Study

A total 12 group-I FAdV reference strains for 12 serotypes were used in this study, and all these strains were kept by the China Veterinary Drug Supervision Institute. Details about these strains can be found in Table 1. The virus titer of them were determined by Reed-Muench method using LMH cells and calculated as $TCID_{50}$ per 100 μL. Briefly, LMH cells were plated (at approximately 10^6 cells per dish) in 60-mm dishes 1 d before infection with the stored FAdV strains. The infections were carried out in the presence of 1% FBS at 37°C under 5% CO_2, and the mixture of aliquots and cells were harvested on d 7 postinfection. After 3 freeze-thaw cycles, the harvested samples were examined for FAdV by PCR to determine the virus titer. Other reference virus strains, including subgroup A strain of avian leukosis virus (**ALV-A**) SDAU14A1 (GenBank accession number: KU375453), ALV-J strain NX010 (GenBank accession number: DQ115805), reticuloendotheliosis virus strain (**REV**) HA9901 (GenBank accession number: AY842951), CIAV strain SDLY08 (GenBank accession number: FJ172347) were kindly provided by Peng Zhao (College of Veterinary Medicine, Shandong Agricultural University). The virus titer of these reference strains were determined before and presented as TCID50 or EID50 per 100 μL.

PCR Amplification

A pair of universal primers (F-H1, R-H2) that are suitable for all the 12 FAdV serotypes were employed in this study for the identification and amplification of the reference strains with reference to the published literature (Hess, 2000). Details about the primers used in this study can be found in Table 2. Briefly, DNA was isolated from 200 μL of each FAdV stock using a commercial kit (Bio-Tek, Norcross, GA), and total DNA was resuspended in 50 μL of DNase-, RNase- and proteinase-free water and then served as template. After then, the PCR was done in a 50-μL reaction containing 4 μL of dNTP mixture (TaKaRa, Dalian, China), 5 μL of 10 × PCR buffer (TaKaRa), 1 μL of Taq polymerase (TaKaRa), 2 μL of DNA solution, 1 μL of forward and reverse primers (10 μmol/L), and 36 μL of ddH_2O with the following PCR conditions: initial incubation at 94°C for 3 min, 48°C for 2 min, and 72°C for 40 min, followed by 31 cycles of denaturation at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 10 min.

Table 1. Reference group I FAdV strains used in this study.

Representative strains	Genbank No.	Abbreviation
Fowl adenovirus 1 (CELO)	U46933	FAdV-1
Fowl adenovirus 2 (SR-48)	KT862806	FAdV-2
Fowl adenovirus 3 (SR49)	KT862807	FAdV-3
Fowl adenovirus 4 (KR5)	HE608152	FAdV-4
Fowl adenovirus 5 (TR22-CK8)	AF508953	FAdV-5
Fowl adenovirus 6 (CR119)	KT862808	FAdV-6
Fowl adenovirus 7 (YR3)	KT862809	FAdV-7
Fowl adenovirus 8a (TR-59)	KT862810	FAdV-8a
Fowl adenovirus 8b (764)	KT862811	FAdV-8b
Fowl adenovirus 9 (/)	AF083975	FAdV-9
Fowl adenovirus 10 (C2B)	KT717889	FAdV-10
Fowl adenovirus 11 (380)	KT862812	FAdV-11

Table 2. Primers used in this study.

Primers	Sequence (5′ - 3′)	Targets
F-H1	TCAAGTGCCTCAGAAGTAT	12 serotypes
R-H1	TGACGACCTGGTTAGATT	
P-F-1	CACGCTTCAGCAGGTC	Serotype 1
P-R-1	GCAGGTAGTCGGCAAT	
P-F-2-11	CGTCGCCGCTCTTTCA	Serotypes 2, 11
P-R-2-11	AGTTACGCCGCTGGGAG	
P-F-3-9	TTGCGAAAGTTACAGAC	Serotypes 3, 9
P-R-3-9	CCCACGGTTAAGTATG	
P-F-4-10	TTTAACAACTGGTCGGAGAC	Serotypes 4, 10
P-R-4-10	CGATTTCGTAGGAGGGTA	
P-F-5	CCTCCTTCAAGCCCTAC	Serotype 5
P-R-5	GACCCGTTCTCCCACA	
P-F-6-7-8	ACGGCGGCACGGCTTA	Serotypes 6, 7, 8a, 8b
P-F-6-7-8	TCGGGCAGGTAGTCGG	

Note: P means primers used for synthesizing probes.

Plasmid Construction and Sequencing

The above PCR products were separated by 1% agarose gel electrophoresis and purified using the Omega Gel Extraction Kit (Omega Bio-tek). The purified PCR products were cloned into the pMD-18T vector (Transgen Biotech, China). The resulting construct was then used to transform *Escherichia coli* DH5α cells (TaRaKa). The plasmids from these positive clones were isolated using a commercial kit and then named as "pFAdV-Serotype". The copy numbers of the plasmids were calculated according to the conventional protocol. Then, the plasmids were diluted at 10−fold series as standards for further analysis.

Sequence Analysis

To verified the constructed plasmids, DNA from positive clone was sequenced directly (Shenggong, Shanghai, China), and each fragment was sequenced 3 times independently. The DNA sequences were assembled using DNAStar Lasergene (version 6.0, DNAStar inc., Madison, WI). Multiple sequence alignment was performed using the BioEdit (version 7.0, http://www.mbio.ncsu.edu/bioedit/bioedit.html).

Design and Synthesis of Probe

According to the above sequencing and comparison results, all the 12 serotypes of FAdV reference strains can be divided into 6 categories (Table 2). We selected a highly conserved region for each category by sequence comparison, and designed 6 pairs of primers to amplify

the corresponding region served as probe. Details about the location and information about the probes and primers can be found in Table 2. Finally, using the six pairs of primers designed in this study for probes and corresponding plasmids, the probe synthesis was carried out using Roche's PCR DIG Probe Synthesis Kit according to the instruction. The amplified PCR products were recovered by 1% agarose gel electrophoresis. The probes should be purified and quantified with OMEGA Nucleic Acid Gel Recovery Purification Kit, and then stored at −80°C in reserve.

Dot Blot Assay

The dot blot assay was carried out according to the literature (Meng et al., 2018). First, each probe was used to detect the corresponding FAdV serotype strains (the plasmids constructed above were used as the standard), and the minimum concentration and detection limit of each probe were determined by matrix analysis. At the same time, each probe is used to detect other common avian pathogens, including ALV-A, ALV-J, REV, and CIAV to determine their specificity (Meng et al., 2018). Second, 6 probes were mixed at the optimum concentration of each one to form a hybrid probe, called PM, which was then used to detect all these 12 serotypes FAdV strains. The sensitivity of it was also obtained by matrix analysis.

Application of the Method in Artificial Contaminated Live Vaccines

To test the performance of the dot blot assay designed in this study, a comparison analysis was employed using the artificial contaminated live vaccines carrying different concentrations of FAdV-4. Specifically, 5 bottles of Mukteswar vaccine (1,000 fowls) in the same batch were diluted into 2 mL with PBS, and 1000 $TCID_{50}$, 100 $TCID_{50}$, 10 $TCID_{50}$, 1 $TCID_{50}$, 0.1 $TCID_{50}$ of FAdV-4 were added into each bottle. Additionally, another 2ml sterile PBS was used to dilute 1 bottle of Mukteswar vaccine (1,000 fowls) as blank control. DNA of these artificial contaminated live vaccines was isolated from 200 μL diluent and then amplificated by the universal primers following the abovementioned process, and the PCR product were used as sample for dot blot assay. Also, the PCR products were analyzed by electrophoresis for comparing the sensitivity.

RESULTS

PCR Amplification and Construction of Plasmids

As shown in Figure 1, all 12 serotypes FAdV strains can be amplificated by the universal primers, F-H1 and R-H1, through the traditional PCR assay with high concentrations of DNA as samples. The fragments amplificated by the PCR were consistent with the expected results. All these PCR products were purified and then cloned into the pMD-18T vector, named as pFAdV-1, pFAdV-2, pFAdV-3, pFAdV-4, pFAdV-5, pFAdV-6, pFAdV-7, pFAdV-8a, pFAdV-8b, pFAdV-9, pFAdV-10, and pFAdV-11. The concentrations of these plasmids finally isolated were all around 10^9 copies/μL.

Sequence Analysis

The above constructed plasmids were verified by sequencing. Sequences analysis showed that all there 12 serotypes can be further divided into 6 categories according to the high identities (over 97.2%) between some serotypes (Table 3), suggesting the possibility of using the same probe to detect multiple serotypes. Briefly, they can be divided into category 1 (FAdV-1), category 2 (FAdV-2 and FAdV-11, 98.1%), category 3 (FAdV-3), category 4 (FAdV-4 and FAdV-10, 99.3%), category 5 (FAdV-5), category 6 (FAdV-6, FAdV-7, FAdV-8a and FAdV-8b, 97.2 to 99.8%).

Probe Design and Synthesis

According to the above sequence analysis, 6 pairs of primers were designed and then synthesized for amplification of corresponding probes used for the dot blot assay. The primers were named as "probe-F/R-serotype". Details of these primers and their targets can be found in Table 2. The probes were synthesized by Roche's PCR DIG Probe Synthesis Kit, and then purified and quantified for further analysis.

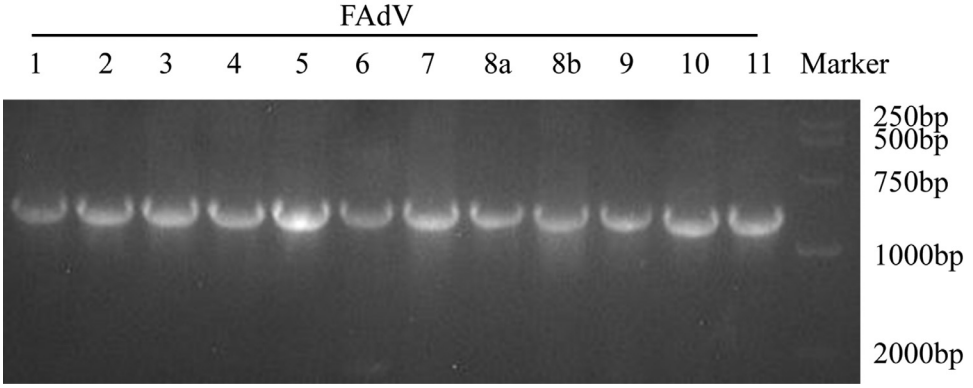

Figure 1. PCR amplification of 12 serotypes FAdV strains using a pair of universal primers.

Table 3. Homology analysis of the PCR products from 12 serotypes.

Serotypes	1	2	11	3	9	4	10	5	6	7	8a	8b
FAdV-1		71.5	71.5	70.5	70.2	75.4	75.3	73.8	75.1	76.2	75.7	75.7
FAdV-2			**98.1**	78.3	78.4	70.7	70.8	75.1	77	76.9	76.8	76.8
FAdV-11				78.5	78.6	70.5	70.6	75.2	77	77.1	76.9	76.9
FAdV-3					**98.8**	68.3	68.3	73.8	75.1	74.8	74.6	74.6
FAdV-9						67.9	67.9	73.8	75	75	74.7	74.7
FAdV-4							**99.3**	73.1	73.9	75	75.2	75.2
FAdV-10								73	73.9	74.9	75.2	75.2
FAdV-5									77.9	77.7	77.6	77.6
FAdV-6										**98.5**	**97.2**	**97.3**
FAdV-7											**99.4**	**98.8**
FAdV-8a												**99.8**
FAdV-8b												

Note: Higher homology is marked in bold.

Sensitivity, Specificity Analysis, and Condition Optimization of Each Probe

This study first analyzed the accuracy of the 6 probes, and the results showed that each probe could effectively detect its corresponding strains, and could not detect ALV-A, ALV-J, REV, CIAV and negative control (Figure 2). After that, matrix analysis of the concentration and detection limit of these probes showed that these probes had high sensitivity (Table 4). Subsequently, the 6 probes were mixed at their optimal concentrations to form a hybrid probe (named as PM) for the following analysis.

Table 4. Optimal concentration and detection limit of each probe.

Probes	Optimal concentration	Detection limit	Targets
P-1	10 pg	1 copies	Serotype 1
P-2-11	10 pg	1 copies	Serotypes 2, 11
P-3-9	5 pg	1 copies	Serotypes 3, 9
P-4-10	5 pg	1 copies	Serotypes 4, 10
P-5	25 pg	1 copies	Serotype 5
P-6-7-8	5 pg	1 copies	Serotypes 6, 7, 8a, 8b

Sensitivity and Specificity Analysis of the Hybrid Probe

Twelve plasmids for each serotype were diluted into 5 different concentrations, 10^0 to 10^4 copies/μL for dot blot assay using the hybrid probe. As shown in Figure 3, almost all serotypes can be detected by 10^1 copies, and even some serotypes can be detected by 10° copies (FAdV-5, FAdV-8b, FAdV-9, FAdV-10, and FAdV-11), which showed the high sensitivity of the hybrid probe. At the same time, the hybrid probe was also highly specific, and no control was positive. The same sample cohort was also analyzed by traditional PCR, using universal primers F-H1 and R-H1, but this method can detect at least 10^2 copies, which shows that this dot blot assay is more than 100 times more sensitive than traditional PCR.

Application of the Dot Blot Assay in Artificial Contaminated Live Vaccine

As vaccine contamination is an important way of transmission of FAdV, it is important to detect the contamination of exogenous FAdV in vaccine effectively. In

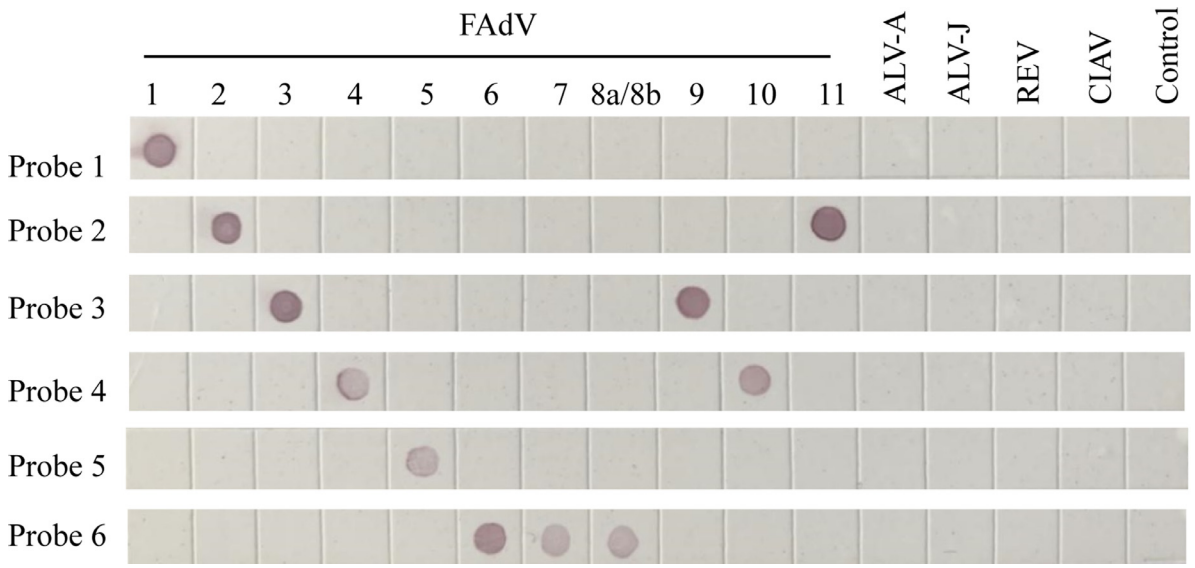

Figure 2. Specificity analysis of the six probes designed in this study.

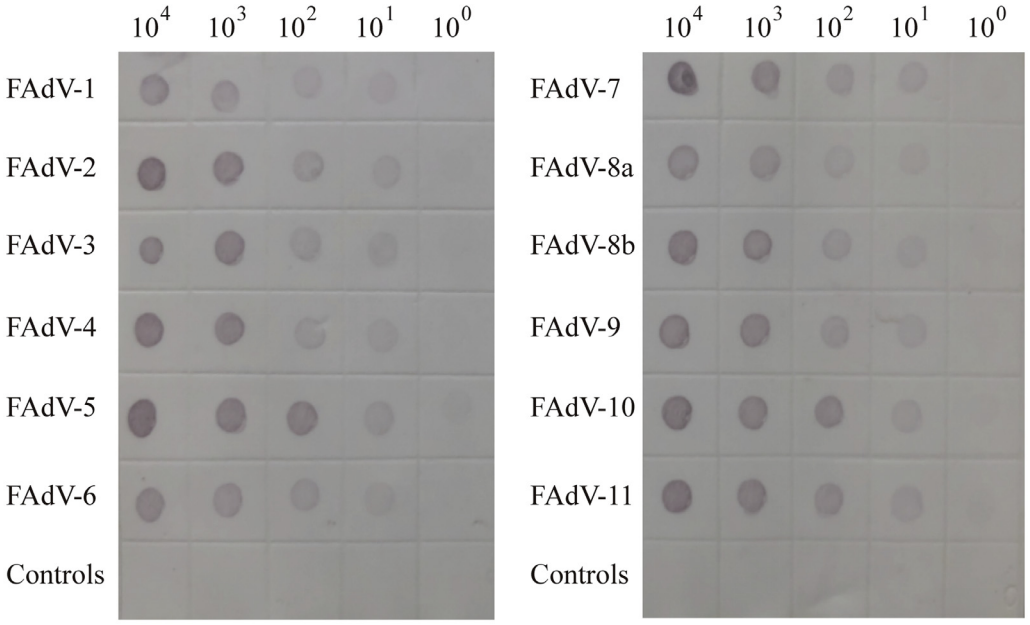

Figure 3. Sensitivity analysis of the mixed probes.

this study, FAdV-4 is taken as an example to analyze the application prospect of our dot blot assay in this aspect. Results showed that the dot blot assay can effectively identified the exogenous FAdV-4 with an extremely low dose (1 $TCID_{50}$) in a bottle of Mukteswar vaccine (1,000 fowls), while the traditional PCR can only detect the contaminated vaccine with a viral dose over 100 $TCID_{50}$ per bottle (Figures 4A and 4B), demonstrating that the sensitivity of this dot blot assay was 100 times higher than that of PCR. The same analysis was repeated using vaccines contaminated with mixed serotypes of FAdV and yielded the same conclusion.

DISCUSSION

FAdV infects chicken worldwide, resulting in global economic losses in the poultry industry (Shah et al., 2017). It can be divided into 3 groups, among which group I contains 12 genotypes and is widely distributed. Since 2013, FAdV-4, especially some highly pathogenic strains with novel gene mutations, has been prevalent in China (Ye et al., 2016; Jiang et al., 2019; Wang and Zhao, 2019). The HHS caused by FAdV-4 has seriously affected the health of poultry (Chen et al., 2019; Wang and Zhao, 2019). Fortunately, in the most recent

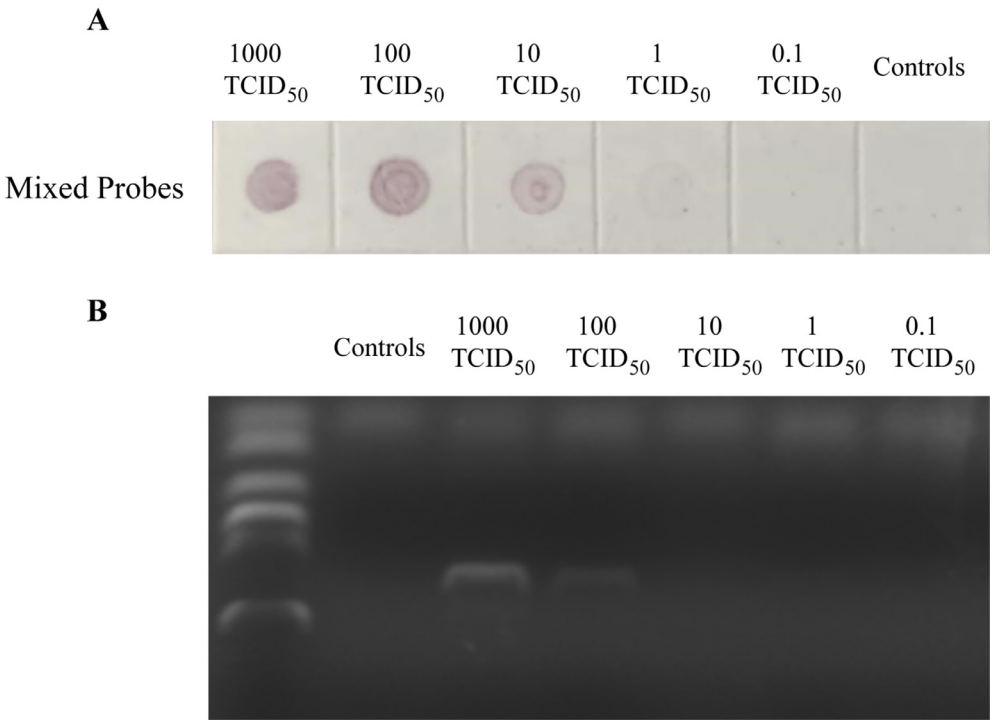

Figure 4. Sensitivity analysis of the mixed probes (A) and PCR assay (B) in detecting the artificially contaminated live vaccines.

3 yr, due to the increased awareness of prevention and control of FAdV-4, the epidemic gradually subsided, and the incidence of HHS gradually decreased throughout the Country. However, this does not mean that the epidemic of FAdV is under control, especially many serotypes are conditionally pathogenic, which can cause diseases only in some mixed infection or stress state, so it is easy to be ignored (Chen et al., 2019).

Recent studies showed that FAdV-4, FAdV-8a, FAdV-8b, FAdV-10, and FAdV-11 were prevalent in China (Chen et al., 2019; Su et al., 2019c). Also, the dominant serotypes may be different in different time periods. According to a 10-year epidemiological survey from 2007 to 2017, FAdV serotypes 11, 4, and 8b were the predominant serotypes in some regions of China between 2007 and June 2014, between June 2014 and 2016, and in the first half of 2017, respectively, indicating the dynamic situation of FAdV epidemic in China (Wang et al., 2018). Therefore, it is not enough to only rely on a single detection method for a certain serotype during the daily monitoring.

In the past few years, due to the epidemic of FAdV-4, many detection methods targeting FAdV-4 have been developed, including fluorescence quantitative PCR, loop-mediated isothermal amplification (**LAMP**) real-time turbidity method, droplet digital PCR, recombinase polymerase amplification assay, and the like (Niczyporuk et al., 2015; Shao et al., 2019; Yao et al., 2019; Yuan et al., 2019; Lu et al., 2020; Pan et al., 2020). Although some methods have confirmed that some other serotypes can also be detected at the same time, especially the serotype FAdV-10 which has a high homology with FAdV-4, many other serotypes are still difficult to detect using these methods (Dong et al., 2019). At present, there is only one very old PCR method that can effectively detect 12 serotypes in group I FAdV simultaneously (Hess, 2000). However, this method requires sufficient viral DNA concentration, so its sensitivity is poor, and it is easy to overlook samples with particularly low viral content.

The dot blot assay is a method similar to southern blot that can identify target DNA fragments with high sensitivity (Cheng et al., 2019). Some studies have combined dot blot with PCR to achieve a significant increase in sensitivity (El-Fadaly et al., 2016; Jung and Chae, 2005). Therefore, this research also hopes to establish a highly sensitive group I FAdV detection method through this method. First, this study analyzed the gene fragments of 12 serotypes of FAdV obtained using traditional PCR methods. The results showed that the homology between gene fragments of certain strains was very high, all of which were higher than 97.2%, meaning that we can design a probe to detect multiple strains at the same time, which can effectively reduce the experimental cost. Finally, the 12 serotypes were divided into 6 categories, and we designed 6 targeted probes based on the sequence of each category. The sensitivity, accuracy and specificity of these 6 probes were verified by matrix analysis. The results show that these probes can efficiently identify their corresponding serotypes, with very high sensitivity and specificity.

Subsequently, we mixed the 6 probes with the optimal concentration corresponding to the respective probes to form a tube of mixed probes and used it to detect 12 serotypes. Results showed that the mixed probes can effectively detect all 12 serotypes with an extremely high sensitivity. The lower detection limit of this method is 10° copies, while that of the traditional PCR is 10^2 copies, showing a 100-fold improvement by combining PCR and dot blot.

Finally, considering that a certain amount of interference may occur in practical applications, we took FAdV-4 as an example to test the detection efficient of the dot blot method in artificially contaminated live vaccines. The results were also exciting. The dot blot we established can effectively identify the 1 $TCID_{50}$ contamination of a bottle of vaccines (1,000 fowl), while the traditional PCR is far less effective and can only identify 100 $TCID_{50}$. Therefore, the method established in this research is reliable in analyzing the live vaccines. Considering that very low dose contamination in live vaccines can also cause infection, it is very promising to use this dot blot method to screen for exogenous FAdV in attenuated vaccines (Su et al., 2020).

In conclusion, a dot blot assay was designed on the basis of traditional PCR for group I FAdV, which can effectively improve the detection efficiency of traditional PCR and provide a reliable, simple, and low-cost way for simultaneous detection of 12 serotypes of FAdV in the future.

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Author contributions: LH, QS conceived and performed the experiments, analyzed the data, and drafted the manuscript. PZ supervised the project and edited the manuscript. YZ conducted part of the experiments, analyzed part of the data. DL and YM provided important suggestions.

DISCLOSURES

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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