Journal of Virological Methods 265 (2019) 59–65

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Development and evaluation of a droplet digital PCR assay for the detection of fowl adenovirus serotypes 4 and 10 in attenuated vaccines

Guiwei Dong[a,b,c,1], Fanfeng Meng[a,b,d,1], Yubiao Zhang[a,b,c], Zhizhong Cui[a,b,c], Hou Lidan[e,*], Shuang Chang[a,b,c,*], Peng Zhao[a,b,c,*]

[a] College of Veterinary Medicine, Shandong Agricultural University, Tai'an, Shandong, China
[b] Shandong Provincial Key Laboratory of Animal Biotechnology and Disease Control and Prevention, Tai'an, Shandong, China
[c] Shandong Provincial Engineering Technology Research Center of Animal Disease Control and Prevention, Tai'an, Shandong, China
[d] Beijing Dafaun Poultry Breeding Company Ltd., Beijing, China
[e] China Insititute of Veterinary Drug Control, Beijing, China

ARTICLE INFO

Keywords:
FAdV-4/10
Attenuated vaccine
ddPCR
Sensitivity
Specificity

ABSTRACT

In recent years, there has been an increase in reported cases of fowl adenovirus serotype 4 (FAdV-4) in chickens in China. The use of live attenuated vaccines contaminated with FAdV-4 has been proved to be one of the important causes of massive outbreaks of hydropericardium syndrome. To detect the contamination with FAdV-4 in attenuated vaccines more promptly and accurately, a droplet digital PCR (ddPCR) assay was developed for the rapid detection of FAdV-4 and FAdV-10. The ability of this assay to detect FAdV-4 contamination in attenuated Newcastle disease virus vaccines was assessed in comparison to a quantitative real-time PCR (qPCR) and a conventional PCR assay. The findings indicated that the ddPCR assay could detect FAdV-4 contamination at 0.1 EID_{50}/1,000 feathers, while the qPCR could detect FAdV-4 contamination at 1 EID_{50}/1,000 feathers with identical genomic targets, which was 1,000-fold more sensitive than conventional PCR detection with a sensitivity of 10^2 EID_{50}/1,000 feathers. The ddPCR assay also showed high specificity for FAdV-4/10 and no positive signals were detected for other FAdVs. Consequently, the intuitive and rapid results were especially suitable for the detection of FAdV-4 contamination in vaccines. In this study, a ddPCR assay was developed to effectively detect and quantify low-dose FAdV-4 contamination, providing a new method for rapid detection of FAdV-4 contamination in various samples, especially vaccines.

1. Introduction

Beginning in June 2015, an infectious disease characterized by pericardial effusion and enlarged liver broke out among chickens in Shandong, Henan, and Hebei provinces of China. The illness was referred to as inclusion body hepatitis (IBH) and hydro-pericardium hepatitis syndrome (HPS) (Liu et al., 2016; Chen et al., 2017; Pan et al., 2017). After a large-scale investigation, it was determined that the pathogen causing this disease was fowl adenovirus serotype 4 (FAdV-4). However, there was no definitive conclusion as to how FAdV4 was introduced into different flocks, especially among chickens with good biosecurity controls. In a previous investigation, FAdV-4 was detected in an attenuated Newcastle disease virus (NDV) vaccine, and comparison of the *hexon* gene of FAdV showed that it was highly consistent with a prevalent wild FAdV-4 strain (Li et al., 2017a,b Zhao et al., 2015). Therefore, the use of attenuated vaccines contaminated with FAdV-4 was suspected to be one of the important ways the disease has spread.

Over the past two decades, numerous attenuated vaccines for poultry have been confirmed to be contaminated with exogenous viruses (Fadly and Garcia, 2006; Barbosa et al., 2008; Awad et al., 2010; Li et al., 2017a,b). The main reason for this phenomenon is the use of vaccines made from specific pathogen-free chicken embryos infected by exogenous viruses. These viruses, such as avian leucosis virus (ALV) (Zavala and Cheng, 2006; Zhao et al., 2014), reticuloendotheliosis virus (REV) (Fadly and Garcia, 2006; Wei et al., 2012; Li et al., 2015, 2016), and chicken infectious anemia virus (CIAV) (Yuasa et al., 1979) are mainly transmitted vertically. However, at present, there are few reports of chicken vaccines being contaminated with FAdV-4. Generally, the exogenous virus only comprises a

---

* Corresponding authors.
E-mail addresses: houruif@163.com (H. Lidan), changshuang81@126.com (S. Chang), zhaopeng@sdau.edu.cn (P. Zhao).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.jviromet.2018.09.005
Received 16 August 2018; Accepted 12 September 2018

Table 1
Primer and probe sequences used in this study.

Application	Name	Sequences	Product size (bp)
Conventional PCR	FAdV-F	AACGTCAATCCCTTCAACCACC	1319
	FAdV-R	TTGCCTGTGGCGAAAGGCG	
qPCR/ ddPCR	hexon-F2	ATCAAAAACCTGCTGCTGCT	152
	hexon-R2	AAGTTGGCCATGAGGTTCAC	
	hexon-Probe	CAAAGACCCCAACATGATCCTCCAATC	

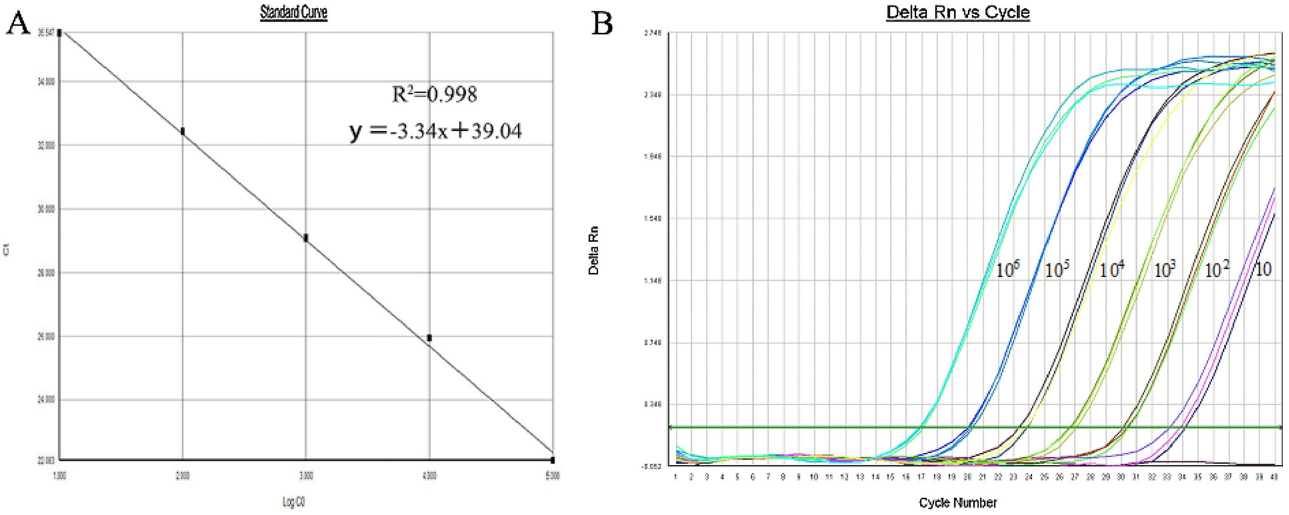

Fig. 1. Standard curve of FAdV-4 by qPCR. 073.

Table 2
Intra- and inter-assay reproducibility of real-time PCR.

Replication	Mean ± SD (C_T value)	Coefficient of variation (%)	Viral load (copies/μL)
Intra-assay reproducibility	22.99 ± 0.15	0.65	10^5
	25.99 ± 0.08	0.31	10^4
	29.03 ± 0.07	0.24	10^3
	32.35 ± 0.23	0.71	10^2
Inter-assay reproducibility	23.10 ± 0.18	0.78	10^5
	26.21 ± 0.23	0.98	10^4
	29.18 ± 0.21	0.70	10^3
	32.74 ± 0.32	0.89	10^2

Note: Clinical liver DNA samples that were positive for FAdV-4 were detected in intra-assay and inter-assay tests. The mean intra- and inter-assay coefficients of variations (CV) were < 2%, indicating good reproducibility.

relatively small proportion of the total nucleic acid. Thus, it is likely to escape detection and urgently requires a highly sensitive detection method. Droplet digital PCR (ddPCR) is a newly developed digital PCR method that facilitates the accurate and precise quantitation of nucleic acid targets without the need for calibration curves (Hindson et al., 2011). The analytical sensitivity of ddPCR is higher than that of real-time quantitative PCR. The ddPCR method established in the present study is an effective diagnostic technology that is suitable for both research and clinical use in the diagnosis of FAdV-4/10, especially for detecting FAdV-4 contamination in live attenuated vaccines.

2. Materials and methods

2.1. Virus strains and vaccines

The FAdV-4 N-22 strain (GenBank accession No. KU764777) was isolated from an attenuated NDV vaccine by the Poultry Phymatosis Laboratory in China (Li et al., 2016). The EID_{50} of the strain (10^5 EID_{50}/mL) was calculated according to the Reed-Muench method (Reed and Muench, 1938). Live attenuated vaccines commonly used in the poultry industry against NDV, Marek's disease (MD), infectious bursal disease (IBD), fowl pox (FP), and infectious bronchitis (IB) produced by local company were purchased. They have been identified by the China Institute of Veterinary Drug Control as qualified products that do not contain any exogenous virus and were used as negative controls. The DNA samples used for the specificity tests were ALV-A-SDAU09C1 (GenBank accession No. HM452339), ALV-J-NX0101 (GenBank accession No. DQ115805), REV-HA9901 (GenBank accession No. AY842951), CIAV-SDLY08 (GenBank accession No. FJ172347), and MDV-GX0101 (GenBank accession no. JX844666). DNA from FAdV genotypes 1, 2, 3, 4, 6, 7, 8a, 8b, 10, and 11 were obtained from the China Institute of Veterinary Drug Control.

2.2. Primers and probes

A pair of specific primers was designed to amplify fragments of the *hexon* gene based on the sequence of FAdV-4/10 in GenBank, with a target size of 1319 bp. Primers (hexon-F2/hexon-R2) and a probe (hexon-probe) were designed for droplet digital PCR (ddPCR) and real-time quantitative PCR (qPCR). The probe used FAM as the fluorescent reporter and BHQ was used as a fluorescence quencher (Table 1). All primers and probes were synthesized by Shanghai Bioengineering.

2.3. Preparation of plasmid for standard curve

Using nucleic acid from FAdV-4 N-22 as the template, a 152-bp fragment of the *hexon* gene was amplified by hexon-F2 and hexon-R2 primers. The PCR conditions were as follows: denaturation for 5 min at 95 °C, followed by 31 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 10 min. The PCR amplification products were analyzed by 1.0% agarose gel electrophoresis. The DNA fragments in the gel were extracted and recovered by using a gel extraction kit (OMEGA, Norcross, GA, USA). After purification, the DNA fragments were ligated to the pMD18-T vector (TaKaRa, Japan) and the

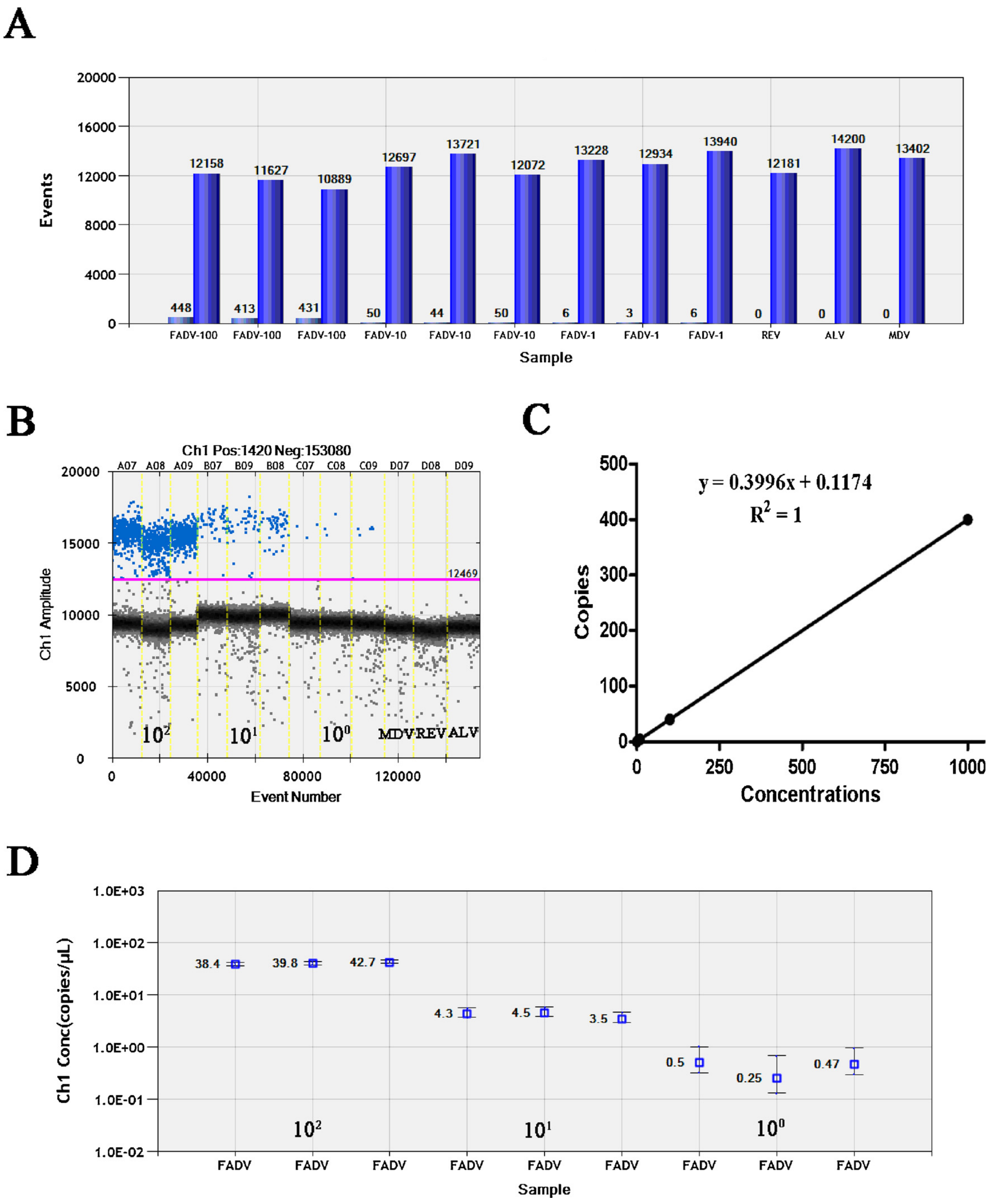

Fig. 2. The ddPCR results for the standard plasmid.
(A) Positive and negative droplets. The light blue bar is the number of positive droplets, and the dark blue is the number of negative droplets. **(B)** Results of droplet amplification at different standard concentrations. The blue ponits above the line represent the positive droplets and the black points below the line are the negative droplets. **(C)** The standard curve of ddPCR at different concentrations. **(D)** The copy numbers of samples with different concentrations.

ligation mix was transformed into E. coli DH5α competent cells. Bacterial colonies were picked and the plasmid DNA was extracted using a Plasmid Miniprep Kit (OMEGA, Norcross, GA, USA) and subsequently confirmed by enzyme digestion. Positive clones were selected and sent to Shanghai Sangon Biological Engineering Technology and Services Company for sequencing. The plasmid DNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the plasmid copy number was calculated. The recombinant plasmid was named p-Hexon and was stored at −80 °C.

2.4. Optimization of the TaqMan quantitative real-time PCR (qPCR) assay

An in-house qPCR assay was developed for detecting FAdV-4 with the primers hexon-F2 and hexon-R2 and the hexon-probe using an ABI 7500 Real-time PCR System. Using a matrix method, various concentrations of primer (10, 25, and 50 nmol/mL) and probe (5, 12.5, and 25 nmol/mL) were screened to determine the optimal reaction conditions. The constructed plasmid, p-Hexon, was serially diluted 10-fold (10^7–10^1 copies/μL) and qPCR was performed. Each dilution reaction was performed three times in duplicate and a standard curve was plotted.

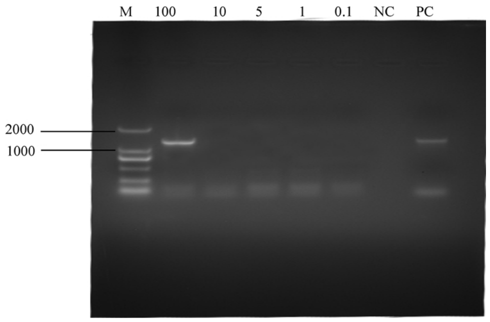

Fig. 3. The sensitivity of routine PCR for detecting FAdV-4 in contaminated vaccines.

2.5. Optimization of the ddPCR assay

The ddPCR consists of the following four steps: preparation of PCR, generation of droplets, PCR, and signal collection, as previously described (Morisset et al., 2013). Different concentrations of primers and probe as well as various annealing temperatures were tested in order to determine the optimal reaction conditions. The signal collection was conducted immediately after PCR. The optimized conditions were determined by comparing the number of droplets in the ddPCR, the droplet distribution status, and the intensity of the droplet fluorescence signal.

2.6. Specificity and reproducibility tests for qPCR and ddPCR

The DNA or RNA from viruses ALV-A (SDAU09C1), ALV-J (NX0101), REV (HA9901), CIAV (SDLY08), and MDV (GX0101), as well as viruses IBDV, NDV, and FPV from blank, control vaccines were extracted. Then, ddPCR and qPCR were performed to verify the specificity of these two methods. Next, to assess the repeatability and stability of the two methods, intra- and inter-assay replicates were performed. Each sample was assayed three times and the coefficient of variation (CV) of the intra- and inter-group differences was calculated.

2.7. Detection of contamination in attenuated vaccines with different doses of FAdV-4 by different methods

Quantified FAdV-4 N-22 was diluted to 100 EID$_{50}$/mL, 10 EID$_{50}$/mL, 5 EID$_{50}$/mL, 1 EID$_{50}$/mL, 0.1EID$_{50}$/mL, and 0.01EID$_{50}$/mL with sterile PBS. Six bottles of attenuated NDV vaccine from the same batch were diluted using PBS, which contained different amounts of FAdV-4 N-22. The six bottles were mixed with 100 EID$_{50}$/mL, 10 EID$_{50}$/mL, 5 EID$_{50}$/mL, 1 EID$_{50}$/mL, 0.1 EID$_{50}$/mL, and 0.01 EID$_{50}$/mL of FAdV-4, respectively. Additionally, 1 mL of sterile PBS, without FAdV-4 N-22, was used as a control. A 200 μL aliquot of the vaccine was used for DNA extraction with a DNA Extraction Kit (OMEGA), according to the manufacturer's instructions. Finally, the DNA was dissolved in 50 μL of TE buffer. Routine PCR, qPCR, and ddPCR were performed with DNA extracted from the six bottles of vaccine to determine and compare the sensitivities of these methods.

3. Results

3.1. The development of qPCR for FAdV-4/10

A band of approximately 152 bp was amplified using the hexon-F2 and hexon-R2 primers. The constructed recombinant plasmid, p-Hexon, was sequenced and BLAST analysis showed that the inserted fragment has 100% identity with the reference sequence, indicating the successful construction of a positive standard plasmid. Then, the concentration was determined by spectrophotometry to be 420 μg/mL, which was equivalent to 1.3×10^{11} copies/μL.

The optimal reaction conditions were as follows: a 20 μL total volume containing 10 μL of 2× Premix Ex Taq, 0.5 μL (10 μmol/L) of the F and R primer, 0.8 μL (25 μmol/L) of probe, 2 μL of template, and 6.2 μL of H$_2$O. Two-step PCR was used in the qPCR assay. The first step consisted of pre-denaturation at 95 °C for 30 s. The second step was comprised of 40 cycles of denaturation at 95 °C for 5 s and annealing at 60 °C for 34 s. The standard plasmid was serially diluted (1×10^7-1×10^1 copies/μL) and used as the template for amplification. Then, the kinetic and standard curves of FAdV-4 were obtained. The results showed that there was a linear relationship between 10^7 and 10^2 copies/μL (Fig. 1A), with an R^2 value of 0.997, which indicated that a minimum of 100 copies could be accurately and stably detected (Fig. 1B). The specificity of the qPCR was assessed using DNA samples from ten FAdV genotypes, showing that it could only detect FAdV-4 and -10, as only poor signals were obtained for the other genotypes and other viral nucleic acids. The CV for the intra- and inter-group replicates ranged from 0.24 to 0.98%, indicating that this method is accurate and has good reproducibility (Table 2).

3.2. Optimization of ddPCR

After optimization of the ddPCR conditions, the final ddPCR system consisted of a 20 μL reaction containing 10 μL of 2× mix, 1.8 μL (10 μmol/L) of the upstream and downstream primers, 0.8 μL (25 μmol/L) of probe, 2 μL of template, and 3.6 μL of H$_2$O. The ddPCR assay for FAdV-4 was optimized by varying the annealing temperatures, and slightly better efficiencies were observed at 60 °C when compared to lower annealing temperatures. The first step is a pre-denaturation step at 95 °C for 3 min, which is followed by 40 cycles of 95 °C for 10 s, 58 °C for 40 s, and 98 °C for 10 min, with a ramping speed of 2.5 °C/s. At least 10,000 droplets were generated per sample (Fig. 2A). The newly developed ddPCR showed high specificity for FAdV-4 and -10 and did not cross-react with other tested DNAs, such as ALV, REV, MDV, and other FAdVs (Fig. 2 B). Each standard plasmid was tested in four serial tenfold dilutions and the R^2 was calculated as one (Fig. 2C). The results showed that ddPCR has very high analytical sensitivity, and was able to consistently detect a single copy of the standard plasmid (Fig. 2D).

3.3. The sensitivity of different methods for detecting FAdV-4 in live attenuated NDV vaccine

Routine PCR tests of vaccines contaminated with different doses of FAdV-4 showed a positive result at 100 EID$_{50}$ for each vaccine and a negative result below this dose (Fig. 3). The qPCR assay was able to consistently detect more than 1 EID$_{50}$ of FAdV-4, while the DNA detection for different vaccines revealed no positive fluorescence signals. The reproducibility for detecting 0.1 EID$_{50}$ FAdV-4 per 1000 feathers in a vaccine by qPCR was poor and the cycle threshold (CT) value for the contaminated vaccines was very close to that of the blank vaccine.

Clinical samples contaminated with different doses of FAdV-4, which showed specific qPCR signals, were used to evaluate the efficiency of the ddPCR assay based on the standard curve analysis. Each DNA sample underwent five serial ten-fold dilutions. At least 10,000 droplets were generated from each sample (Fig. 4A). Only a single peak was observed in the negative sample (Fig. 4B), whereas two peaks were observed in the positive samples (Fig. 4C). In the blank vaccines, only a few positive droplets were detected (< one copy; Fig. 4D). However, when the dose of FAdV-4 was greater than 0.1 EID$_{50}$, the number of positive droplets was significantly higher than that of the blank vaccine ($p < 0.05$; Fig. 4E). The amount of the contaminant was linearly related to copy number (Fig. 4F). As the confidence level was not acceptable when the vaccine contamination was less than 0.01 EID$_{50}$ per bottle, the sensitivity of ddPCR was determined to be 0.1 EID$_{50}$ or 10 copies per bottle. Based on the above results, the observed sensitivity of the ddPCR assay is 1000 times greater than that of conventional PCR and more than 10 times greater than that of qPCR (Table 3).

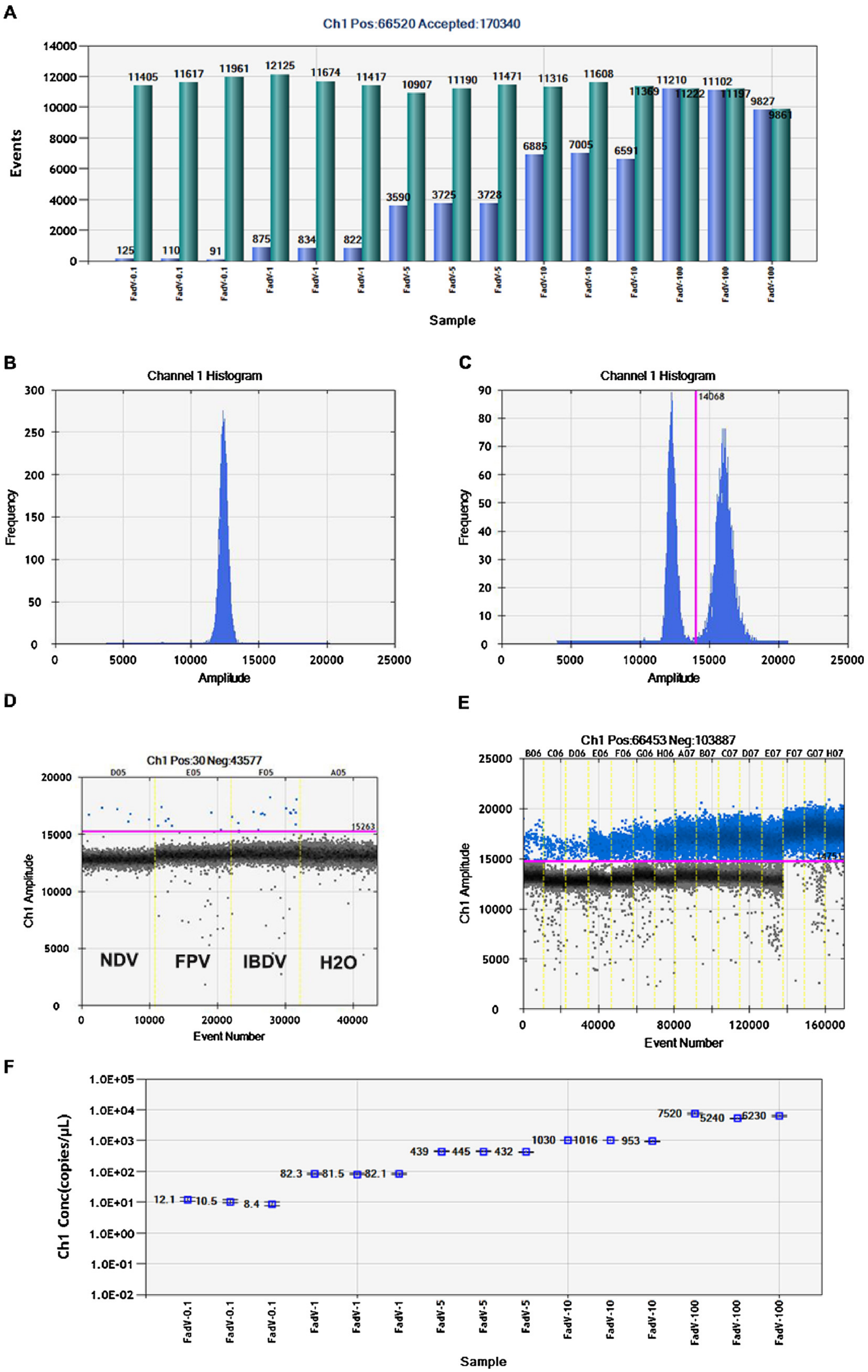

Fig. 4. The development and application of a ddPCR assay for FAdV-4 in vaccines.
(A) Positive and negative droplets. The light blue bar is the number of positive droplets and the dark blue bar is the number of negative droplets. **(B)** Peak frequency of negative droplets. **(C)** Peak frequency of positive droplets. **(D)** The ddPCR results for several blank vaccines. **(E)** The ddPCR results for vaccines contaminated with different concentrations of FAdV-4. **(F)** Copy numbers of vaccines contaminated with different concentrations of FAdV-4.

Table 3
Comparison of the sensitivity of different methods for detecting FAdV-4.

Detection	Vaccines with different concentrations of FAdV-4 (EID_{50})						NDV	FPV	IBDV	H_2O
	10^2	10^1	$10^{0.7}$	10°	10^{-1}	10^{-2}				
Conventional PCR	+	–	–	–	–	–	–	–	–	–
qPCR	+	+	+	+	–	–	–	–	–	–
ddPCR	+	+	+	+	+	–	–	–	–	–

4. Discussion

In recent years, many chicken flocks in different provinces of China have developed HPS caused by FAdV-4 (Liu et al., 2016; Chen et al., 2017; Pan et al., 2017). Initially, the infected flocks were mainly on small-scale farms with poor biosecurity measures. However, some flocks on large modern farms also experiencedthe sudden onset of pericardial effusion-hepatitis syndrome; what led to these outbreaks was not known. It was suspected that some vaccines may be contaminated with FAdV-4, causing morbidity. A large number of vaccines were tested and one strain of FAdV-4 was detected that had high similarity to wild-type FAdV-4 (Li et al., 2017a and b). This could trigger FAdV infection despite a series of strict biosafety measures, resulting in a large-scale outbreak of the disease. Contamination of vaccines with FAdV-4 may be an important cause of outbreaks of inclusion body hepatitis pericardial syndrome in China since 2013 (Su et al., 2018).

The methods presently used for detecting FAdV-4 in group I are mainly virus isolation and identification, electron microscopy, agar diffusion assay, neutralization assay, ELISA, PCR, and qPCR (Hess, 2000;Saifuddin and Wilks, 1990). The sensitivity of these methods varies greatly, with PCR and qPCR being the most commonly used methods. The *hexon* gene of FAdV is a unique gene to this virus. Therefore, primers and probes for routine PCR and qPCR were designed based on the *hexon* gene's conserved sequence in order to detect FAdV-4 and FAdV-10. The development and application of qPCR require the establishment of a standard curve, and quantification by qPCR, in this case, was based on relative quantification rather than absolute quantification. A qPCR specificity experiment using DNA from ten FAdV genotypes showed that this method could effectively detect FAdV-4/10. In practice, the CT value of the blank attenuated vaccines in qPCR was greater than 37, while the CT value of some vaccines with suspected exogenous virus contamination was 34–36. It was not possible to determine whether this was due to a mild operation error or a very low level of contamination. However, the poor reproducibility of the qPCR assay at lower analyte levels made positive versus negative judgment based on CT values difficult to determine.

ddPCR is a new method that allows absolute quantification of nucleic acid. Unlike qPCR, ddPCR does not rely on standard curves. Instead, the method relies on absolute quantification and the copy number is obtained directly. In ddPCR, the reaction mixture is distributed into a large number of water-in-oil droplets, such that most of the droplets contain zero or one template copy, which is then amplified by PCR. A positive reaction is determined by reading the fluorescent signal corresponding to the well, as well as the number of negative wells, and then the copy number in the sample is statistically calculated using the Poisson distribution. When a plasmid was used as the test sample, the detection sensitivity of ddPCR was 100 times higher than that of qPCR, indicating that the sensitivity of ddPCR was indeed much higher than that of qPCR. When a vaccine was contaminated with 0.1 EID_{50} FAdV-4, the CT value in the qPCR was not only variable, but was also close to the CT value of the uncontaminated blank vaccines. With ddPCR, only a few positive droplets were detected in randomly selected blank vaccines. Even at only 0.1 EID_{50}, a very significant difference in copy number was observed when compared with blank vaccines, allowing us to make highly intuitive and accurate judgments. Although ddPCR is a costly method, the high sensitivity and specificity may contribute to the acceptance of this method by biological companies.

5. Conclusions

In conclusion, the ddPCR assay established in this study demonstrated high sensitivity and specificity for the rapid detection of FAdV-4 contamination in vaccines, and the performce of this assay was superior to qPCR. Using this method could enhance the safety of vaccines that are on the market.

Authors' contributions

Experiments were performed by MFF and DGW. ZYB and TSB contributed through quantification and reproducibility studies. CZZ, HLD, CS, and ZP designed the study, provided general supervision, and prepared the manuscript. All authors have read and approved the submission of the manuscript.

Conflict of interest statement

The authors declare that they have no competing interests.

Acknowledgments

This work was supported by the National Key Research and Development Program of China (2016YFD0501606) and Funds of Shandong "Double Tops" Program (SYL2017YSTD11).

References

Awad, A.M., et al., 2010. Detection of reticuloendotheliosis virus as a contaminant of fowl pox vaccines. Poultr. Sci. 89, 2389–2395.

Barbosa, T., Zavala, G., Cheng, S., 2008. Molecular characterization of three recombinant isolates of avian leukosis virus obtained from contaminated Marek's disease vaccines. Avian Dis. 52, 245–252.

Chen, H., et al., 2017. Hydropericardium hepatitis syndrome emerged in cherry valley ducks in China. Transbound. Emerg. Dis. 64, 1262.

Fadly, A., Garcia, M.C., 2006. Detection of reticuloendotheliosis virus in live virus vaccines of poultry. Dev. Biol. 126, 301–305.

Hess, M., 2000. Detection and differentiation of avian adenoviruses: a review. Avian Pathol. 29, 195–206.

Hindson, B.J., et al., 2011. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal. Chem. 83, 8604.

Li, J., et al., 2015. Isolation, identification, and whole genome sequencing of reticuloendotheliosis virus from a vaccine against Marek's disease. Poultr. Sci. 94, 643–649.

Li, Y., et al., 2016. Genome analysis and pathogenicity of reticuloendotheliosis virus isolated from a contaminated vaccine seed against infectious bursal disease virus: first report in China. J. Gen. Virol. 97 (11), 2809–2815.

Li, Y., et al., 2017a. Isolation, identification, and hexon gene characterization of fowl adenoviruses from a contaminated live Newcastle disease virus vaccine. Poultr. Sci. 96, 1094.

Li, Y., et al., 2017b. Molecular characterization of chicken infectious anemia virus from contaminated live-virus vaccines. Poultr. Sci. 96, 1045–1051.

Liu, Y., et al., 2016. Genetic characterization of novel fowl aviadenovirus 4 isolates from outbreaks of hepatitis-hydropericardium syndrome in broiler chickens in China. Emerg. Microbes Infect. 5, e117.

Morisset, D., et al., 2013. Quantitative analysis of food and feed samples with droplet digital PCR. Plos One 8, e62583.

Pan, Q., et al., 2017. The first whole genome sequence and pathogenicity characterization of a fowl adenovirus 4 isolated from ducks associated with inclusion body hepatitis and hydropericardium syndrome. Avian Pathol. 46, 571.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoint. Am.

J. Hyg. 27, 493–497.

Saifuddin, M., Wilks, C.R., 1990. Development of an enzyme-linked immunosorbent assay to detect and quantify adenovirus in chicken tissues. Avian Dis. 34, 239.

Su, Q., et al., 2018. Newcastle disease virus-attenuated vaccine co-contaminated with fowl adenovirus and chicken infectious anemia virus results in inclusion body hepatitis-hydropericardium syndrome in poultry. Vet. Microbiol. 218, 52–59.

Wei, K., et al., 2012. Probable congenital transmission of reticuloendotheliosis virus caused by vaccination with contaminated vaccines. Plos One 7, e43422.

Yuasa, N., et al., 1979. Isolation and some characteristics of an agent inducing anemia in chicks. Avian Dis. 23, 366–385.

Zavala, G., Cheng, S., 2006. Detection and characterization of avian leukosis virus in Marek's disease vaccines. Avian Dis. 50, 209–215.

Zhao, P., et al., 2014. Isolation, identification, and gp85 characterization of a subgroup of avian leukosis virus from a contaminated live Newcastle disease virus vaccine: first report in China. Poultr. Sci. 93, 2168–2174.

Zhao, J., et al., 2015. Pathogenicity and complete genome characterization of fowl adenoviruses isolated from chickens associated with inclusion body hepatitis and hydropericardium syndrome in China. Plos One 10 e0133073.