Development of a real-time quantitative RT-PCR to detect REV contamination in live vaccine

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ABSTRACT Based on the published Avian reticuloendotheliosis virus (REV) whole genome sequence, primers and TaqMan probes were designed and synthesized, and the TaqMan probe fluorescence real-time quantitative RT-PCR (qRT-PCR) method for detecting the REV *pol* gene was established by optimizing the reaction conditions. Sensitivity analysis showed that the qRT-PCR method had a sensitivity that was 1,000-fold higher than conventional PCR. Additionally, no amplification signals were obtained when we attempted to detect DNA or cDNA of ALV-A/B/J, MDV, CIAV, IBDV, ARV, NDV, AIV, or other viruses, suggesting a high specificity for our method. Various titers of REV were artificially "spiked" into the FPV and MDV vaccines to simulate REV contamination in attenuated vaccines to validate this qRT-PCR method. Our findings indicated that this qRT-PCR method could detect REV contamination at a dose of 1 $TCID_{50}$/1,000 feathers, which was 10,000-fold more sensitive than the regular RT-PCR detection (10^4 $TCID_{50}$/1000 feathers).

Key words: reticuloendotheliosis virus (REV), real-time RT-PCR, detection, contamination, live vaccine

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INTRODUCTION

Avian reticuloendotheliosis virus (**REV**) is an important pathogen that can cause avian tumor diseases. Epidemiological studies have detected REV infection in Chinese chicken flocks, most commonly in local strains (Cheng et al., 2007; Cui et al., 2009; Zhao et al., 2012). Because REV can be vertically transmitted through eggs (Witter and Salter, 1989), if chicken embryos used to produce attenuated vaccines are contaminated by REV, the attenuated vaccines also may be contaminated by REV, which represents an important transmission route (Fadly et al., 1996; Fadly and Garcia, 2006; Awad et al., 2010). Recently, the application of avian attenuated vaccines contaminated by REV has been considered to be one of the most important reasons for the epidemic of REV in China (Wang et al., 2010; Wei et al., 2012; Li et al., 2015).

Various methods can be used to detect REV, such as indirect immunofluorescence assay (**IFA**), enzyme linked immunosorbent assay (**ELISA**), and PCR (Cui et al., 1986; Cui et al., 1988; Aly et al., 1993; Reimann and Werner, 1996). To our knowledge, a series of real-time RT-PCR method for REV detection had been established that amplified the *env* gene or REV-LTR as the target region (Zhao et al., 2007; Li et al., 2012; Miao et al., 2015). However, our experience indicated that the anterior real-time RT-PCR method frequently led to a higher rate of false positive results when detecting clinical samples or attenuated vaccines, especially fowlpox virus (**FPV**) and Marek's disease virus (**MDV**) vaccines. In other words, no REV virion could be isolated from some of those clinical samples or attenuated vaccines that were judged positive by those methods. Previous research has shown that REV-LTR or partial REV *env* gene, but not the whole REV viral genome, was carried by several field or vaccine strains of DNA viruses, like FPV and MDV (Hertig et al., 1997; Garcia et al., 2003; Pratik et al., 2003; Su et al., 2015). Therefore, it could be concluded that REV-LTR or partial REV *env* gene carried by those recombinant viruses might contribute to the false positive rate in clinical samples and some attenuated FPV and MDV vaccines. In this present study, we developed a TaqMan probe real-time quantitative RT-PCR (qRT-PCR) assay to target the highly functioning conserved *pol* gene of REV, and we investigated the feasibility of this method in detecting REV contamination in attenuated vaccines by artificially "spiking" various doses of REV into MDV and FPV vaccines.

MATERIALS AND METHODS

Virus strains and Cell Lines

SPF chicken embryos were purchased from Jinan SPAFAS Poultry Co., and chicken embryo fibroblasts (**CEF**) were prepared using standard methods. The

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Table 1. Accession numbers of nucleotide sequences and origins of strains used for alignments.

Strain	Country	Origin of strain	Accession number
HLJR0901	China	Chicken	GQ415646
HLJ07I	China	Chicken	GQ375848
MD-2	China	Chicken	JX912710
HA9901	China	Chicken	AY842951
Chicken/3337/05	Taiwan, China	Chicken	FJ439120
Goose/3410/06	Taiwan, China	Goose	FJ439119
FA	US	Chicken	AF246698
APC-566	US	Chicken	DQ387450
SNV	US	Duck	DQ003591

REV HA9901 strain was isolated and identified by Shandong Agricultural University in 1999 and its whole genome sequencing was completed and made publically available (GenBank accession No. AY842951; Wang et al., 2005). Cell supernatants that had been frozen at −80°C were thawed and collected to calculate the $TCID_{50}$ using the Karber method. Each 0.1 mL of CEF cell supernatant contained $10^{4.5}$ $TCID_{50}$. A, B, and J subgroup avian leukemia virus (**ALV-A/B/J**), chicken infectious anemia virus (**CIAV**), avian reovirus (**ARV**), Marek's disease virus (**MDV**), avian influenza virus (**AIV**), Newcastle disease virus (**NDV**), and chicken infectious bronchitis virus (**IBV**) were all maintained at our lab.

Primer and Probe Design

Based on the published sequence of REV (Table 1), lasergene 7.0 was used to identify conserved regions in various virus sequences. Primer 5.0 was used to design primers P1/P2 and pol-F/pol-R/pol IN, which all targeted the *pol* gene of REV (Table 2). The first primer pair, P1/P2, was used to amplify a partial sequence of the REV *pol* gene. The amplified product was 1,434 bp and was used to construct a recombinant plasmid that served as a template for plotting a standard curve. The second primer pair, pol-F/pol-R, was used for qPCR and the amplified product was 163 bp. Primers and probes were synthesized by Shanghai Bioengineering Company.

Preparation of a Plasmid for a Standard Curve

CEF cells were collected after infection with the HA9901 strain for 7 d. Cellular RNA was extracted following the manufacturer's instructions (TaKaRa, Japan). A fragment of 1,434 bp was amplified according to the AMV 3.0 RT-PCR Kit manufacturer's instructions (TaKaRa, Dalian, China). Amplified products were identified by 1.0% agarose gel electrophoresis and were recovered and purified according to the manual provided with the E.Z.N.A Gel Extraction Kit (OMEGA, Norcross, GA). Purified DNA was ligated with a PMD-18T Vector (TaKaRa) and then used to transform DH5a competent *E. coli* cells. Bacterial colonies were picked and plasmids were extracted using a Plasmid Mini Kit (OMEGA). Plasmids were identified using an enzyme digestion method. Positive clones were selected and sent to Shanghai Boshang Biological Engineering Co. for sequencing. The concentration of positive plasmid was measured using a DNA quantitative instrument and the recombinant plasmid copy number was calculated.

Establishment and Optimization of a Florescence qPCR Assay

Using the positive plasmid standard as a template, forward and reverse primers and probe were added to the reaction mixture. The amplification reaction was conducted using an ABI 7500 Real-Time PCR System. Using a matrix method, 10, 25, and 50 nmol/mL primer concentrations and 5, 12.5, and 25 nmol/mL probe concentrations were screened to identify the optimal reaction system and conditions.

Plotting a Standard Curve for Florescence qPCR Assays

The concentration of recombinant plasmid was diluted in a 10-fold gradient from 10^9 copies to one copy per μl. Amplification reactions were conducted using plasmids at different copy concentrations as a template using reaction conditions obtained as described above. Triple duplicate reactions were set up for each dilution, and the standard curve was plotted.

Sensitivity Analysis of Florescence qPCR Assays

Sensitivity of qPCR was evaluated by 10-fold gradient dilution of recombinant plasmid from 1×10^9 to one copy per μl. Additionally, an equal amount of plasmid

Table 2. Primer and probe sequences used in this study.

Primer	Sequence	Function
P1	5′-AAGTAAGAAGACGCCTCCGGGTAA-3′	Preparation of recombinant plasmid
P2	5′-GTCTGCAGTACCCAATTGTACCTA-3′	
pol F	5′-CCCCATTCATGTCCAGCTAT-3′	Real-time PCR
pol R	5′-AGGGAGGAGAGGAGTGTTCC-3′	
pol IN	5′-FAM-TACCGCCCTACCTGTGAGGGTAAGACA-BHQ-3′	Probe

DNA as template was used to conduct regular PCR. Then, 5 μl amplification products were analyzed by 1% agarose gel electrophoresis. The lowest detectable template concentration of the 2 methods was calculated and the difference in sensitivity was compared.

Specificity of Florescence qPCR Assays

The specificity of the established qPCR method was evaluated using ALV-A/B/J, MDV, CAIV, IBDV, ARV, NDV, and AIV DNA or cDNA as templates (deionized water was used as a negative control).

Reproducibility of Fluorescence qPCR Assays

Florescence qPCR assays were carried out using plasmid standard as templates, which were diluted following gradients in different concentrations. A total of 3 reactions were set up for each concentration. Reproducibility was evaluated based on the coefficient of variation (**CV**) of CT values within the group (standard deviation/mean). Moreover, florescence qPCR were performed using tissue DNA, which was extracted from 3 REV-positive liver tissue samples as templates. Then, the intra- and inter-batch reproducibility of this method was analyzed. Intra-batch reproducibility: Triple duplicates were set up for each of the aforementioned three samples and qPCR was conducted under identical conditions; then, the intra-batch CV was calculated. Inter-batch reproducibility: Three independent fluorescence qPCR assays were conducted for the above 3 samples under identical conditions; then, the inter-batch CV was calculated.

Application of Fluorescence qPCR to Detect REV Contamination

The quantified HA9901 was diluted to 10,000 $TCID_{50}$/2 mL, 1,000 $TCID_{50}$/2 mL, 100 $TCID_{50}$/2 mL, 10 $TCID_{50}$/2 mL, and one $TCID_{50}$/2 mL with sterile PBS. A total of 5 bottles of fowlpox vaccine (1,000 feathers) of the same batch was diluted using the aforementioned PBS solution that contained HA9901 in different titers. In this system, it was considered as that 5 bottles of fowlpox vaccine were contaminated by 10,000 $TCID_{50}$/2 mL, 1,000 $TCID_{50}$/2 mL, 100 $TCID_{50}$/2 mL, 10$TCID_{50}$/2 mL, and 1$TCID_{50}$/2 mL of REV, respectively. Additionally, 2 mL sterile PBS (not containing HA9901) was used to dilute one bottle of fowlpox vaccine (1,000 feathers) of the same batch, which served as a negative control. The same process was conducted in Marek's disease vaccine. The negative vaccine samples were confirmed by virus isolation using CEF as described before (Li et al., 2015). REV viral stock was used as positive control.

RESULTS

PCR Amplification and Construction of A Recombinant Plasmid Encoding the Pol Gene of REV

Double-restriction enzyme digestion of recombinant plasmid showed that PMD-18-pol carried a ~1,500 bp fragment, which was consistent with its expected size. This finding indicated that the *pol* gene had been correctly inserted into the vector. For further confirmation, DNA sequencing was carried out, which indicated that the homology of the amplified fragment and reference sequence reached 99.9%. The plasmid standard was extracted by using a reagent kit. The concentration of DNA was 560 μg/mL, as measured by spectrophotometer, and the ratio of A260/A280 was 1.81.

Establishment and Optimization of a Real-time Fluorescent qPCR Assay

The real-time fluorescent qPCR conditions were optimized and the optimal concentrations of primer and probe were 10 and 25 nmol/mL, respectively. The total volume of the reaction system was 20 μl, which contained 10 μl 2 × Premix Ex Taq Mix buffer, 0.4 μl Rox reference dye II, one μl forward primer, one μl reverse primer, 0.4 μl probe, 2 μl template, and an appropriate amount of deionized water. The reaction procedure was as follows: 95°C 5 min; 95°C 10 s; 60°C 34 s (collect fluorescence signal), repeated for 45 cycles.

Establishment of a Standard Curve

Real-time fluorescence qPCR assays, in which templates were PMD-18-pol with different concentrations at a 10-fold dilution gradient, were carried out. The dynamic (Figure 1) and standard (Figure 2) curves were plotted. There was a good linear relationship when the template concentration was in the range of 10^9–1 copies/μl and R^2 was 0.998. The standard curve generated using SDS analytic software (AVI) is shown in Figure 2 (in which the horizontal coordinate represents log values of the copies of the plasmid standard [x] and longitudinal coordinates represent CT values [y]). The relationship between x and y was $y = -3.243x + 37.12$. Statistical analysis of CT values for each dilution of template suggested that the CV of CT values for the 9 dilutions of plasmid standards were 0.04 to 0.97% (Table 3).

Sensitivity, Specificity, and Reproducibility of Real-time Fluorescence qPCR Assays

The real-time fluorescence qPCR detection of various dilutions of recombinant plasmid PMD-18-pol is indicated, and this method could detect virus nucleic acid molecules that were as low as one copy. By contrast,

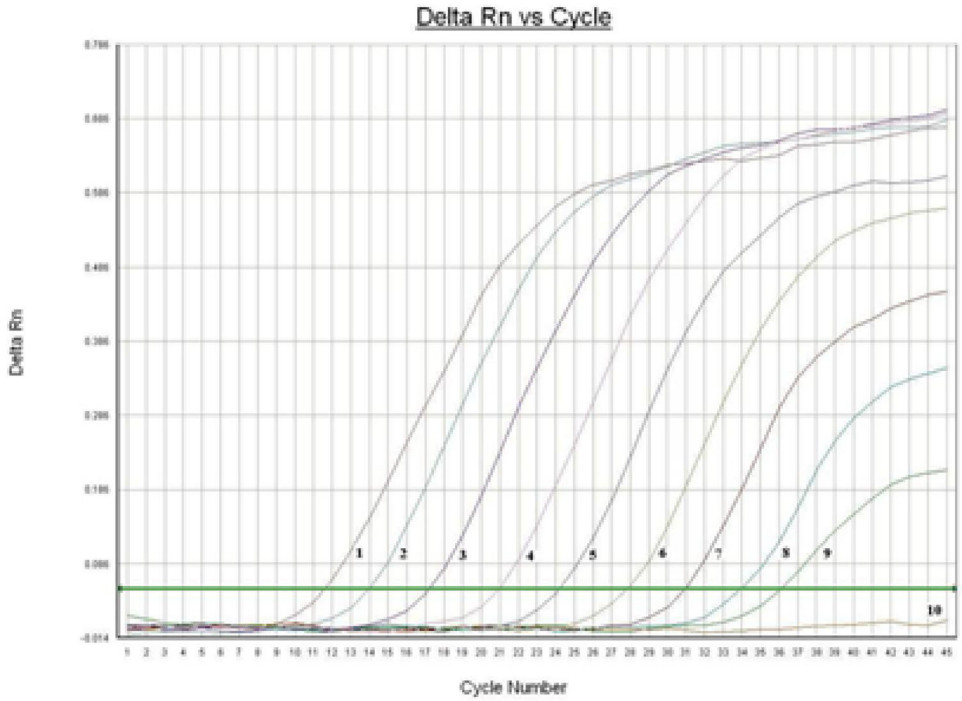

Figure 1. Amplification curve of the *pol* assay. Copy number for the plasmid pMD-18-pol was determined spectrophotometrically and was diluted serially from 10^9 to 10copies/μL (1 to 9), and deionized water was added into reaction as control (10).

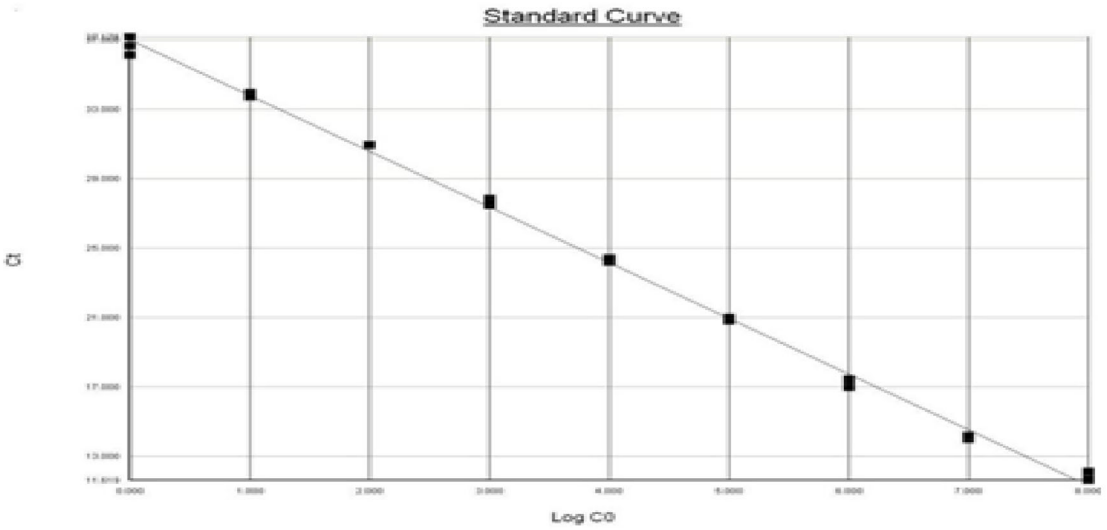

Figure 2. A standard curve used for real-time PCR assays. The spectrophotometrically quantitated standard plasmids were diluted serially and used as template to establish the standard curve for the qPCR assay. Standard curve ($y = -3.243x + 37.12$) for *pol* gene quantification ($R^2 = 0.998$) was analyzed with the ABI SDS software 1.4.

using a regular PCR method, the expected band could be scarcely observed when the copy number reached 10^3 (Figure 3). This finding suggested that the sensitivity of the established real-time fluorescence qPCR method was 1,000-fold higher than that of regular PCR. No amplification signals were detected using real-time fluorescence qPCR assays to detect DNA or cDNA of ALV-A/B/J, MDV, CAIV, IBDV, ARV, NDV, and AIV, whereas the plasmid standard showed good amplification. These findings supported the good specificity of this method. Moreover, real-time fluorescence qPCR detection of REV-positive liver tissue samples indicated that the intra- and inter-batch CV were both less than 2%, which suggested that the method was stable and reliable and, thus, that it had a good reproducibility (Table 4).

Detection of REV Contamination in FPV and MDV by Real-time Fluorescence qPCR Assays

The real-time fluorescence qPCR assays for detecting REV contamination in 5 different doses were all

Table 3. Reproducibility of the real-time PCR assay using standard plasmids as template.

Input copies of standard plasmid	Log CT values 1	2	3	Mean±SD	Coefficient of variation/%
10^8 copies	11.62	11.67	11.84	11.71 ± 0.11	0.97
10^7 copies	13.99	14.17	13.93	14.03 ± 0.13	0.89
10^6 copies	17.15	17.21	16.98	17.12 ± 0.12	0.71
10^5 copies	20.94	21.00	20.82	20.92 ± 0.09	0.45
10^4 copies	24.44	24.21	24.38	24.34 ± 0.12	0.47
10^3 copies	27.49	27.49	27.65	27.54 ± 0.09	0.33
10^2 copies	30.97	30.93	30.92	30.94 ± 0.03	0.08
10^1 copies	33.73	33.73	33.76	33.74 ± 0.01	0.04
10^0 copies	37.00	37.18	36.88	37.02 ± 0.15	0.40

Note: Different copies of standard plasmids were used as template to measure the reproducibility of the qPCR assay. The coefficients of variations (CV) in each group were below 1%, indicating a good reproducibility.

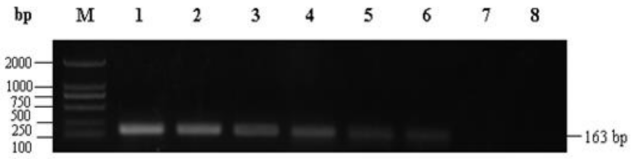

Figure 3. Sensitivity of the regular PCR method. The 10-fold diluted plasmid pMD-18-pol was amplified by conventional PCR. The PCR products were separated on a 1% agarose gel and stained with ethidium bromide. M, Marker 2000, 1 to 8 represent 10^8 copies/μl to 10 copies/μl.

positive and the test for the blank control was negative, which suggested that the established fluorescence qPCR method had both high sensitivity and good specificity (Table 5). It also showed high specificity for the FPV and MDV, for which genomic integration has been reported to occur relatively easily.

DISCUSSION

Recently, the positive rate of detecting anti-REV antibody in Chinese chicken flocks has gradually increased, particularly in certain local strains (Cheng et al., 2007; Cui et al., 2009; Zhao et al., 2012). This trend suggests that there is a high risk in Chinese local strains for REV infection. Whether in China or in other countries, REV contamination in avian attenuated vaccines has attracted significant attention. Many cases of REV infection have been suspected to be caused by attenuated vaccines that are contaminated by REV, in which the most common contaminated vaccines are FPV and MDV (Yuasa et al., 1976; Bagust et al., 1979; Fadly et al., 1996; Diallo et al., 1998; Fadly and Garcia, 2006; Awad et al., 2010; Wang et al., 2010; Wei et al., 2012; Li et al., 2015). Award et al. detected REV from contaminated FPV using various methods, including REV

Table 4. Reproducibility of the real-time PCR assay detecting samples.

Reproducibility tests	Results (Mean ± SD)	Coefficient of variation	Viral load (copies/μL)
Intra-assay reproducibility	20.61 ± 0.06	0.31	$1.24'10^5$
	23.61 ± 0.13	0.54	$1.46'10^4$
	21.46 ± 0.07	0.34	$6.74'10^4$
Inter-assay reproducibility	20.41 ± 0.19	0.95	$1.42'10^5$
	23.27 ± 0.34	1.44	$1.87'10^4$
	21.45 ± 0.32	1.59	$6.77'10^4$

Note: The positive clinical liver DNA samples of REV were detected in intra-assay and inter-assay tests. The mean intra-assay coefficients of variations (CV) were below 1% and inter-assay CV were below 2% in the method, indicating a good reproducibility.

Table 5. Results of real-time PCR detection of different doses of REV contamination in different vaccines.

Vaccine against	Different dose of REV contamination in different vaccine: 10^4TCID$_{50}$/1000 feather	10^3TCID$_{50}$/1000 feather	10^2TCID$_{50}$/1000 feather	10TCID$_{50}$/1000 feather	1TCID$_{50}$/1000 feather	Negative control[1]	Positive control[2]
FPV	+	+	+	+	+	–	+
MDV	+	+	+	+	+	–	+

[1]A commercial attenuated vaccine served as a negative sample in this experiment, and it was determined by virus isolation using chicken embryo fibroblast (CEF).

[2]REV viral stock was used as the positive control in this experiment.

inoculation, isolation, and identification, as well as PCR and antibody tests for SPF chickens inoculated by REV (Awad et al., 2010). It has also been found that REV contamination of attenuated vaccines may lead to reduced antibody levels after immunization using contaminated vaccines (Witter et al., 1979; Sun et al., 2009).

In China, the most often used methods for detecting exogenous viruses in vaccines include cell culture methods and SPF chicken tests. It is difficult and complicated to detect the REV contamination in MDV and FPV live vaccines. This is because, after inoculation with MDV and FPV, CEF cells could undergo pathological changes and REV replication of REV will be affected, thereby making the isolation of REV difficult. Currently, only SPF chicken tests can effectively detect REV contamination, and cell assays have a limited ability to detect REV. Therefore, more and more researchers attempt to detect REV contamination using molecular tests. Compared with regular PCR assays, fluorescent qPCR techniques have advantages such as rapid, accurate, high specificity, real-time monitoring, and quantification capacities. This technique has been widely used in the rapid diagnosis of viral diseases of animals. For low-dose REV contamination in attenuated vaccines, this technique has significant advantages in sensitivity. Several Chinese researchers have established a series of fluorescence qPCR methods that target different regions of REV genes, such as the *env* gene and REV-LTR region (Zhao et al., 2007; Li et al., 2012; Miao et al., 2015). However, even though these methods can show whether a vaccine has been contaminated by REV, the virus frequently cannot be isolated or shows a negative result using other methods. Therefore, some researchers suspect that the positive result ensues from REV gene recombination with other viruses, but not the free REV virus.

It has been established that genetic components of REV can be integrated into the genome of other viruses, which is most commonly reported for the MDV genome (Isfort et al., 1992; Jones et al., 1993; Kost et al., 1993; Jones et al., 1996; Davidson, 2001). In 2004, Chinese researchers identified the MDV recombinant street strain, which had integrated with a LTR fragment of REV (Zhang et al., 2005; Sun et al., 2009; Sun et al., 2010). Apart from MDV, the genetic components of REV are also often integrated into FPV (Hertig et al., 1997; Moore et al., 2000; Biswas et al., 2011). In China, it has been reported FPV can integrate with the genetic fragments of REV, even including the *env* gene and LTR (Ding et al., 2004). However, the recombination of the highly conserved *pol* gene has not been reported, except that Singh et al. isolated a FPV strain that had integrated with a nearly whole proviral genome (Singh et al., 2003). We established a TaqMan probe fluorescence qPCR method to target the conserved *pol* gene of REV, which showed good specificity. All tests of FPV and MDV, which had been proven to be REV-free using our newly developed method, yielded negative results.

Generally, the contaminating dose of REV in attenuated vaccines is very low. Regular RT-PCR assays may not effectively detect REV contamination. Herein, we artificially added different doses of REV into MDV and FPV vaccines and detected REV using an established TaqMan probe fluorescence qPCR method. This method showed high sensitivity; even a dose of $1TCID_{50}$/1,000 feathers was detectable. The sensitivity was 10,000-fold higher than that of the regular RT-PCR assay (10^4 $TCID_{50}$/1,000 feathers). In fact, the qRT-PCR method established in this study had been extensively used in our regular practice. Up to now, more than 100 batches of attenuated vaccines such as NDV, infectious bursal disease virus (**IBDV**), MDV, and FPV had been performed using the qRT-PCR method established in this study. A positive attenuated IBDV sample was detected during this process (CT = 19.34), and this result was confirmed by virus isolation using CEF. The genomic sequence feature and the pathogenicity of this isolated REV will be published elsewhere in the future. We believe that this qRT-PCR method will facilitate the detection of REV contamination of attenuated vaccines.

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