Research Note: Simultaneous detection of infectious laryngotracheitis virus, fowlpox virus, and reticuloendotheliosis virus in chicken specimens

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ABSTRACT Infectious laryngotracheitis (**ILT**), fowlpox (**FP**), and reticuloendotheliosis are important poultry diseases caused by gallid herpesvirus 1 (**ILTV**), fowlpox virus (**FWPV**), and reticuloendotheliosis virus (**REV**), respectively. Coinfections with ILTV and FWPV occur naturally in chickens, and FP in its more virulent wet form is characterized by diphtheritic lesions and easily confused with ILT. Moreover, the insertion of only partial REV-LTR or a nearly full-length REV into the FWPV genome, located between the ORF 201 and ORF 203, has increased recently in wild-type field FWPV isolates. Therefore, it is critical to detect ILTV, FWPV, REV-integrated FWPV, and REV early and accurately. In this study, we successfully developed a multiplex PCR assay for the simultaneous detection of ILTV, FWPV, REV-integrated FWPV, and REV, and the detection limits was 1×5^4 copies/tube. When used to test clinical samples, the results of the multiplex PCR were in 100% agreement with singleplex PCRs and sequencing. This new multiplex PCR is a simple, rapid, sensitive, specific, and cost-effective method for detection of 4 viruses in clinical specimens.

Key words: infectious laryngotracheitis virus, fowl pox virus, fowl pox virus integrated, reticuloendotheliosis virus, multiplex PCR

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INTRODUCTION

Infectious laryngotracheitis (**ILT**) is an acute, contagious, and upper respiratory disease caused by *Gallid herpesvirus type I* (**ILTV**) in chicken, pheasants, and peafowl. ILTV is a linear 155-kb double-stranded DNA virus belonging to the genus *Iltovirus*, subfamily *Alphaherpesvirinae*, and family *Herpesviridae* (Bagust et al., 2000; Ou and Giambrone, 2012). Sources of ILTV infection are clinically infected chickens, latent infected carriers, contaminated dust, litter, beetles, drinking water, and fomites (Ouand Giambrone, 2012). In ILTV infection, there are 2 clinical forms from severe signs to mild signs. The severe form of ILT includes dyspnoea, gasping, coughing, and bloody mucus, which can reach high morbidity and mortality up to 70% depending on the virulence of the circulating strain (Bagust et al., 2000; Ou and Giambrone, 2012). The mild form of ILT includes depression, decreased egg production and weight gain, conjunctivitis, swelling of the infraorbital sinuses (almond-shape eye), and nasal discharge (Bagust et al., 2000). ILT has been controlled with the chicken embryo origin vaccine such as SA2 vaccine and the tissue culture origin vaccine such as A20 vaccine in some countries (Diallo et al., 2010; Ou and Giambrone, 2012).

Fowlpox (**FP**) is a common viral disease caused by *Fowlpox virus* (**FWPV**) that can affect egg production in commercial chickens and turkeys (Singh et al., 2003). FWPV is a large and double-stranded DNA virus (260–309 kbp) of the genus *Avipoxvirus* of the family *Poxviridae*. FWPV infection is mostly associated with a low mortality rate in chickens; however, during some FWPV outbreaks, high mortality rates of up to 65 to 100% have been reported (Abdallah and Hassanin, 2013). FWPV is transmitted by direct contact between affected and susceptible chickens or through biting insects such as mosquitoes and mites (Weli and Tryland, 2011). Scabs containing viral particle also can be sloughed from infected birds and serve as a source of infection. Two forms of the disease are associated with different routes of infection, a "wet" form and a "dry" form, and both types can occur in the same bird (Singh

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et al., 2003). The most common cutaneous form (dry pox) is usually mild and characterized by multifocal cutaneous lesions on unfeathered areas of the skin, such as eyelids, beak, comb, and thighs. The second or diphtheric form (wet pox) is characterized by fibrous necrotic proliferative lesions on the mucous membranes of the mouth, pharynx, larynx, and trachea and often cause death by asphyxiation (Weli and Tryland, 2011). In addition, the diptheritic tracheal lesions associated with wet poxvirus infection are similar to lesions caused by ILTV.

Reticuloendotheliosis virus (**REV**) is a *gammaretrovirus* of the family *retrovirus* consisting of single-stranded RNA with positive polarity. There are 2 types that can be distinguished based on genetic structure and phenotype, REV-A and REV-T (Woźniakowski et al., 2018). The genome of nondefective (replication competent) REV-A strain is 9.0 kbp encoding a group-specific antigen (**gag**), protease (**pro**), polymerase (**pol**), and envelope (**env**) regions flanked by long-terminal repeats (**LTRs**), while the replication defective (needs help for replication and is associated with tumors) REV-T is 3.3 kbp shorter because of the deletion between env, gag, and pol junctions (Woźniakowski et al., 2018). REV can cause an oncogenic, immunosuppressive, and runting syndrome in multiple avian hosts, including chickens, turkeys, and ducks and is a potential contamination hazard in the use of chicken embryos and cells for preparation of vaccines (Awad et al., 2010; Song et al., 2018). In the 1970s, REV was isolated from a commercial Marek's disease vaccine by serial passage of the REV-contaminated vaccine on chicken embryo fibroblast in Australia (Bagust and ILT and FP are characterized by similar respiratory signs such as sneezing, gasping, coughing, reduced egg production, and head-shaking.). Moreover, simultaneous infection with both ILTV and FWPV has been reported in commercial chickens, and the outbreaks of FP have continued to occur worldwide despite regular vaccination with FWPV live vaccines (Singh et al., 2003; Diallo et al., 2010). The failure of vaccination against FP is associated with REV contamination in the commercial vaccines, and FWPV field isolates containing nearly full-length REV or REV LTRs of various lengths are becoming more pathogenic in poultry (Singh et al., 2003; Awad et al., 2010). In order to establish prevention and control strategies for ILTV, FWPV, REV-integrated FWPV, or REV, it is necessary for a sensitive and convenient method to simultaneously detect the pathogens in field isolates (Bagust and Dennett, 1977). Moreover, numerous outbreaks of FP have been reported among vaccinated flocks in Australia and the United States, and lymphoma has been shown in broiler chickens after the use of commercial live FWPV vaccines contaminated with REV (Weli and Tryland, 2011).

Coinfection and the integration of the partial or total genome of REV into FWPV open reading frame (**ORF**) and Marek's disease virus have been reported continuously over the past few decades (Singh et al., 2003; Diallo et al., 2010). Although the integration region is constant, the size of the integrated fragments differs between field isolates and vaccine strains. Most FWPV field isolates carry the near full-length provirus, while most vaccine strains include a LTRs with various sizes of approximately 200 to 600 bp, with the exception of the Australian vaccine strain FWPV-S, which carries a nearly full-length REV provirus. Hence, the presence of REV sequences with various lengths in FWPV genome may enhance disease progression (Awad et al., 2010; Weli, and Tryland, 2011).

Previous studies have been reported that the conventional laboratory diagnosis of ILTV, FWPV, and REV is carried out by histopathological examination, electron microscopy, virus isolation, serologic methods, and nucleic acid detection assays (Ou and Giambrone, 2012; Song et al., 2018). The traditional method of virus isolation and identification is the most reliable; however, it is time-consuming and labor intensive. Although a commercial ELISA for detection of antibodies against ILTV is available, the test is not used for routine laboratory diagnosis. It is not possible to identify different strains of ILTV by serological methods because of the close immunodominant domain. In addition, an immunodiffusion test is often used for detection of FWPV antigens; however, it is not sensitive because of variable specificity and requires a high level of precipitating antibodies in the sera (Ou and Giambrone, 2012). Compared with electron microscopy, histologic test, viral isolation, and PCR-based methods for FP, ILT, and RE diagnosis, nucleic acid amplification with conventional PCR, nested PCR, real-time PCR, or loop-mediated isothermal amplification was the most sensitive method for ILTV, FWPV, and REV detection (Ou and Giambrone, 2012; Song et al., 2018). In the present study, by targeting highly conserved genes of ILTV, FWPV, REV-integrated FWPV, and REV, we successfully developed a multiplex PCR assay for the simultaneous detection of ILTV, FWPV, REV-integrated FWPV, and REV.

MATERIAL AND METHODS

Samples and Virus Isolation

The scab and trachea samples, from 5 layers (18R076, 25 wk old) coinfected with ILTV and FWPV, were grounded with sterilized pestles in the presence of phosphate buffered saline. The artificial air sac was made over the chorioallantoic membrane (**CAM**) of 10 specific pathogen-free 9-day-old developing embryos, and the suspension 200 μL was inoculated into the CAM. At 5 d after inoculation, the embryos were chilled at 4°C for 2 h and broken with a pair of sterile forceps. The thickened CAM was harvested and examined for pock lesion. Subsequent passages were required for adaptation of ILTV and FWPV in CAM. To verify the identity of ILTV and FWPV, pocks on the CAMs were subjected to histological examination. The 5 trachea samples were fixed in 10% neutral buffered formalin for 24 h. After fixation, the tissues were trimmed, dehydrated, and

embedded in paraffin. The sections of 3 to 4 μm were mounted on adhesive slides and stained with hematoxylin and eosin for histopathologic evaluation under a light microscope.

Oligonucleotide Primer Design

Specific 4 pairs of primers were designed based on the published envelope glycoprotein G (GenBank accession no. MK078556) for ILTV, transcription termination factor (GenBank accession no. AF198100) for FWPV, ORF201 and envelope glycoprotein for REV-integrated FWPV (GenBank accession no. MG711457), and surface envelope protein for REV (GenBank accession no. KC884561) using OligoAnalyzer (Integrated DNA Technologies, Coralville, IA). To screen for an association between FWPV and REV, 2 sets of primers were designed to amplify LTR region of REV-integrated FWPV or envelope gene (gp90) of REV, respectively.

Especially, for the detection of REV-integrated FWPV, one primer was designed to bind to the conserved fowl pox virus region flanking the constant/fixed integration site and the other primer within the integration site. Specificity of the primers was confirmed by the BLAST tool against the National Center for Biotechnology Information databases (https://www.ncbi.nlm.nih.gov/). The nucleotide sequences and amplicon sizes are listed in Table 1.

Standard Plasmid Preparation For Positive Controls

Total viral DNA was isolated from naturally coinfected ILTV and REV-integrated FWPV using a QIAamp DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was used in singleplex PCR amplification with the 4 pairs of primers and then the purified PCR products were cloned into the pGEM-T vector (Promega, San Luis Obispo, CA) to construct standard control plasmids. After transformation, the selected monoclonal *E. coli* (DH5α) strains were cultured, and the extracted plasmids were identified by DNA sequencing and analysis. The standard pGEM-ILTV, pGEM-FWPV, pGEM-REV-integrated FWPV, and pGEM-REV plasmids were stored at −70°C until use.

Establishment of Multiplex PCR

Multiplex PCR assays were carried out by varying the annealing temperature, each primer concentration, extension time, and cycle number to optimize the PCR conditions using 4 pairs of primers listed in Table 1. Increasing the primer concentrations for REV-integrated FWPV and REV genes facilitated the production of the expected PCR products at low DNA template concentrations. Multiplex and singleplex PCR assays were prepared in a total of 20-μL volume containing 2 μL of each DNA extract, 1 μL of each primer (12 pmoles/μL) for ILTV and FWPV, 1 μL of each primer (14 pmoles/μL) for REV-integrated FWPV, 1 μL of each primer (20 pmoles/μL) for REV, 2 × reaction buffer (Black PCR premix QM; Ventech Science, South Korea). Polymerase chain reaction amplification was carried out in a thermal cycler (Eppendorf, Hamburg, Germany) for 1 cycle of 5 min at 94°C, followed by 94°C for 45 s, 58°C for 45 s, and 72°C for 45 s for 38 cycles, and then final extension at 72°C for 5 min. Amplified PCR fragments were analyzed under UV light after separation by 1.5% agarose gel electrophoresis. To confirm the exact genes, all PCR products amplified by multiplex and singleplex PCR were purified using a QIAquick Gel Extraction Kit (Qiagen), and sanger sequencing was analyzed by Cosmo Genetech Co. (Seoul, South Korea).

Determining the Multiplex PCR Detection Limits

The sensitivity of the multiplex and singleplex PCR was determined using the recombinant plasmids (pGEM-ILTV, pGEM-FWPV, pGEM- REV-integrated FWPV, and pGEM-REV) as templates after a 5-fold serial dilution from 5^6 copies/tube to 5^0 copy/tube. All dilutions were repeated in triplicate for detection limits of the multiplex and singleplex PCR.

Table 1. Specific primers designed for the detection of ILTV, FWPV, FWPV integrated REV, and REV.

Target virus	Target gene	Primer	Sequence (5′–3′)	Concentration	Amplicon size (bp)
ILTV	US4	Forward primer	TGA GCG GCT TCA GTA ACA TAG	12 pmoles/μL	877
		Reverse primer	CTA CTG CTG GAG CGT AGA G	12 pmoles/μL	
FWPV	NPH-I	Forward primer	AGT TCT CTT CTA GGA CGA AGG AT	12 pmoles/μL	630
		Reverse primer	GTA GGC TTA TCG GGT AAT ACT GTG	12 pmoles/μL	
FWPV integrated REV	gag	Forward primer	ACT ACC TAT GCC TCT TAT TCC ACT	14 pmoles/μL	429
		Reverse primer	GGC TCA GTA TGA TAG TTC GAT CTC	14 pmoles/μL	
REV	gp90	Forward primer	GGA TTT AGA CAA CAG TGG GAG T	20 pmoles/μL	207
		Reverse primer	CGT CTT CAT ACG AAC CTA GCT T	20 pmoles/μL	

Abbreviations: FWPV, fowlpox virus; ILTV, gallid herpesvirus 1; REV, reticuloendotheliosis virus.

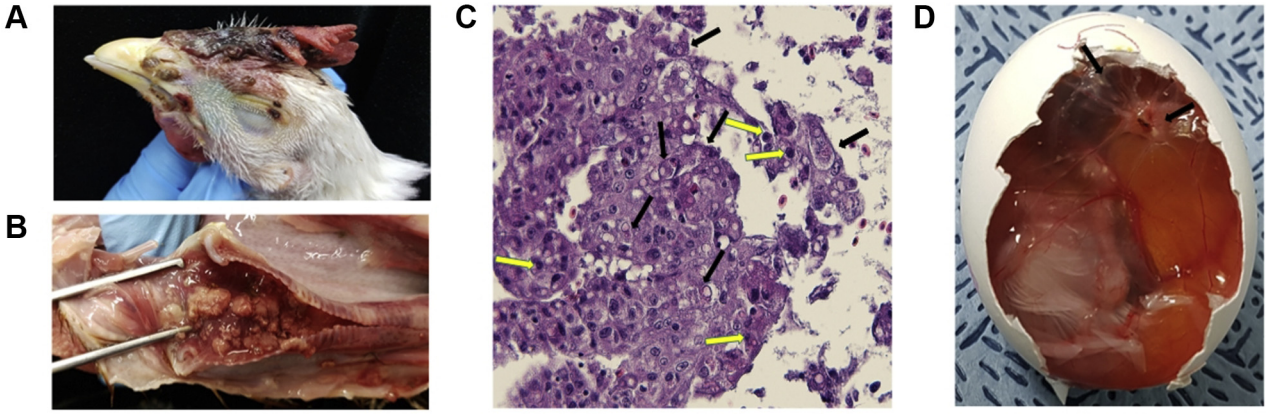

Figure 1. Clinical findings of ILTV and FWPV coinfection in commercial chicken with respiratory signs. (A) Gross skin lesions on the nonfeathered areas (comb, eyelid, and beak) of the head. (B) Gross trachea lesions. (C) HE staining of a longitudinal section of the trachea; multinucleated cells, and intranuclear inclusions bodies (yellow arrows), and acidophilic haloed intracytoplasmic inclusions (black arrows). (D) Small white necrotic pocks on CAM (black arrows). Abbreviations: CAM, chorioallantoic membrane; FWPV, fowlpox virus; HE, hematoxylin and eosin; ILTV, gallid herpesvirus 1.

Screening of ILTV, FWPV, and REV in Clinical Samples

The multiplex PCR was used to test clinical samples such as trachea, comb, and skin of the thirty-one bird with ILT or FP. The tissues were homogenized separately in 2 mℓ phosphate buffered saline, and DNA was extracted from 200 μL of suspension using a QIAamp DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) for use in the multiplex PCR.

RESULTS AND DISCUSSION

Gross lesions of the bird affected with ILTV and FWPV were observed in the multiple skin areas (eyelid, comb, and beak) and trachea, and the mucosa of the upper respiratory tract is covered with fibrinous exudate (Figures 1A and 1B). Histopathology on tissue section using the hematoxylin and eosin staining indicated typical intracytoplasmic inclusions, multinucleated cells, and intranuclear inclusions bodies (Figure 1C). The membrane had taken a cloudy and whitish appearance (Figure 1D).

The optimized multiplex PCR mixture was visualized in 4 bands on a 1.5% agarose gel: a 877-bp band for ILTV, a 630-bp band for FWPV, a 429-bp band for REV-integrated FWPV, and a 207-bp band for REV (Figure 2, lane 1). The 4 amplicons were successfully amplified in 4 singleplex PCRs containing individual gene-specific primer pair, and no primer dimers or nonspecific amplicons were yielded on 1.5% agarose gels (Figure 2, lanes 2–5). All PCR products amplified in multiplex and singleplex PCRs were analyzed by DNA sequencing, and the sequences of the multiplex and singleplex PCR products were identical in respective amplification regions (data not shown). To evaluate the detection limits of the optimized multiplex PCR, each standard plasmid DNA (pGEM-ILTV, pGEM-FWPV, pGEM-REV-integrated FWPV, and pGEM-REV) was prepared at the concentrations ranging from 5^6 copies/tube to 5^0copy/tube. The minimum simultaneous detection limit for the multiplex PCR was 1×5^4 copies/tube, and the sensitivity for the singleplex PCR using the individual gene-specific primer pairs was 1×5^2 copies/tube (Figure 3).

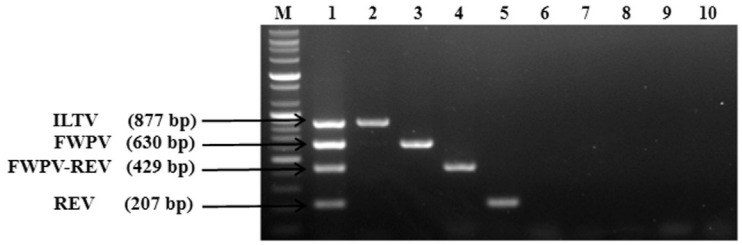

Figure 2. Validation of 4 primer pairs by multiplex and simplex PCR assays. To confirm whether primer pairs can correctly amplify each region, 4 kinds of primer pairs were evaluated in multiplex and simplex PCR assays using ILTV, FWPV, FWPV integrated REV, and REV genomic DNA. LaneM, 100-bp DNA ladder; lane 1, multiplex PCR with ILTV, FWPV, FWPV integrated REV, and REV genomic DNA; lane 2, simplex PCR with only ILTV primers; lane 3, simplex PCR with only FWPV primers; lane 4, simplex PCR with only FWPV integrated REV primers; lane 5, simplex PCR with only REV primers; lane 6, negative control using multiplex PCR with 4 kinds of primer pairs; lane 7, negative control using simplex PCR with only ILTV primers; lane 8, negative control using simplex PCR with only FWPV primers; lane 9, negative control using simplex PCR with only FWPV integrated REV primers; lane 10, negative control using simplex PCR with only REV primers. Abbreviations: FWPV, fowlpox virus; ILTV, gallid herpesvirus 1; PCR, polymerase chain reaction; REV, reticuloendotheliosis virus.

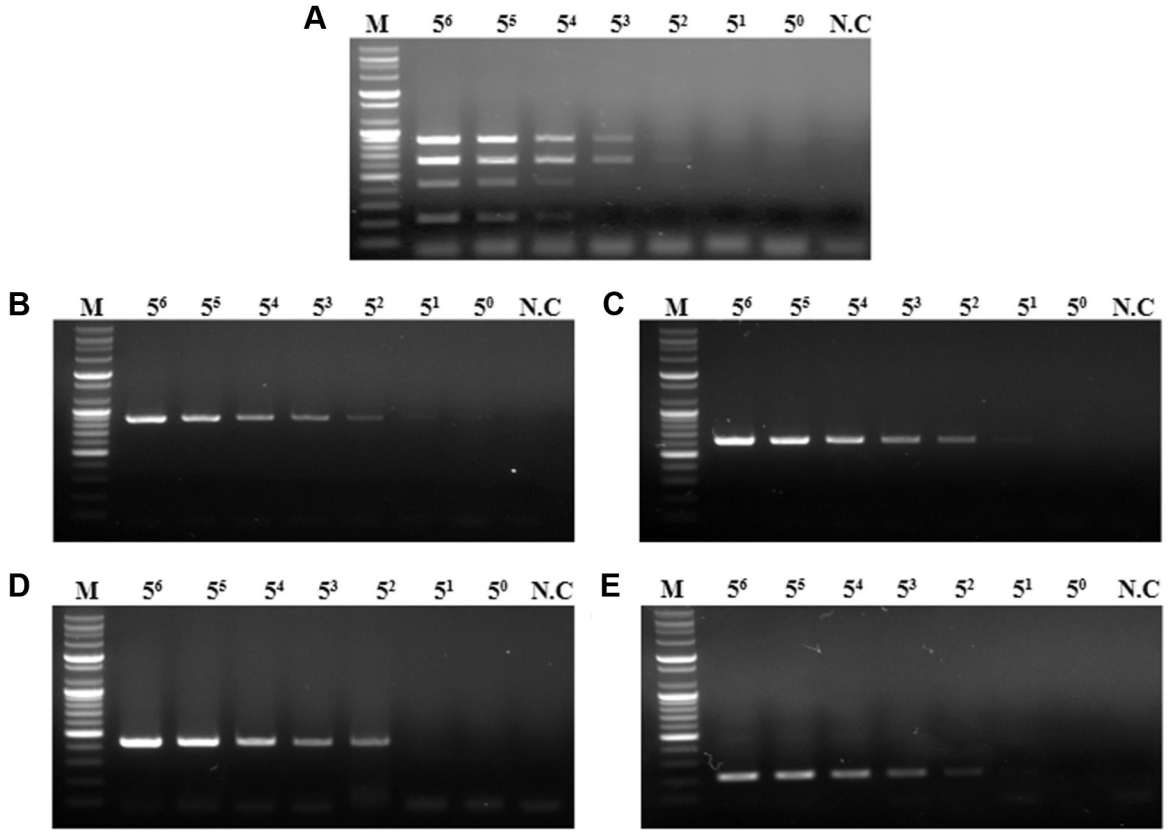

Figure 3. Detection limits of ILTV, FWPV, FWPV integrated REV and REVprimers using multiplex PCR. The 4 recombinant plasmids and plasmid mixtures were serially diluted from 5^6 to 5^0 DNA copies/tube. (A) The sensitivity of pGEM-ILTV, pGEM-FWPV, pGEM-FWPV-REV, and pGEM-REV plasmids for detection of 4 different viruses. (B) The sensitivity of the pGEM-ILTV plasmid for ILT. (C) The sensitivity of the pGEM-FWPV plasmid for FWPV. (D) The sensitivity of the pGEM-FWPV integrated REV plasmid for FWPV integrated REV. (E) The sensitivity of the pGEM-REV plasmid for REV; lane M, 100-bp DNA ladder; lane N.C, negative control; 877 bp for ILT, 630 bp for FWPV, 429 bp for FWPV integrated REVand 207 bp for REV. Abbreviations: FWPV, fowlpox virus; ILTV, gallid herpesvirus 1; PCR, polymerase chain reaction; REV, reticuloendotheliosis virus.

In total, 31 clinical samples collected from the Animal and Plant Quarantine Agency in Korea were tested to compare multiplex PCR and singleplex PCR, and the statistics are shown in Table 2. The multiplex PCR is consistent with conventional PCR, and the ratio of positive samples to total samples was 6.5% (2/31) for ILTV detection and 71.0% (22/31) for REV-integrated FWPV detection in single infection. In addition, the present study shows the natural occurrence of dual infection of ILTV and FWPV carrying the full-length REV provirus genome (22.6%). Notably, only FWPV was not detected in any of the clinical samples. From the results, we confirmed the multiplex PCR is a reliable method for detecting ILTV, FWPV, REV-integrated FWPV, and REV.

ILT and FP are characterized by similar respiratory signs such as sneezing, gasping, coughing, reduced egg

Table 2. Outcomes of multiplex PCR assay of clinical samples, compared with the diagnostic PCR assays.

	Multiplex PCR			Simplex PCR		
Target virus	P/T (%)	N/T (%)	F/T (%)	P/T (%)	N/T (%)	F/T (%)
Single infection						
ILTV	2/31 (6.5)	29/31 (93.5)	0/31 (0.0)	2/31 (6.5)	29/31 (93.5)	0/31 (0.0)
FWPV (not integrated REV)	0/31 (0.0)	31/31 (100.0)	0/31 (0.0)	0/31 (0.0)	31/31 (100.0)	0/31 (0.0)
FWPV integrated REV	22/31 (71.0)	9/31 (29.0)	0/31 (0.0)	22/31 (71.0)	9/31 (29.0)	0/31 (0.0)
REV	0/31 (0.0)	31/31 (100.0)	0/31 (0.0)	0/31 (0.0)	31/31 (100.0)	0/31 (0.0)
Dual infection						
ILTV + FWPV (not integrated REV)	0/31 (0.0)	31/31 (100.0)	0/31 (0.0)	0/31 (0.0)	31/31 (100.0)	0/31 (0.0)
ILTV + FWPV integrated REV	7/31 (22.6)	24/31 (77.4)	0/31 (0.0)	7/31 (22.6)	24/31 (77.4)	0/31 (0.0)
ILTV + REV	0/31 (0.0)	31/31 (100.0)	0/31 (0.0)	0/31 (0.0)	31/31 (100.0)	0/31 (0.0)
REV + FWPV (not integrated REV)	0/31 (0.0)	31/31 (100.0)	0/31 (0.0)	0/31 (0.0)	31/31 (100.0)	0/31 (0.0)
Triple infection						
ILTV + REV + FWPV (not integrated REV)	0/31 (0.0)	31/31 (100.0)	0/31 (0.0)	0/31 (0.0)	31/31 (100.0)	0/31 (0.0)

Abbreviations: F, number of false positives and false negatives; FWPV, fowlpox virus; ILTV, gallid herpesvirus 1; N, number of true negatives; P, number of true positives; PCR, polymerase chain recation; REV, reticuloendotheliosis virus; T, number of total samples.

production, and head-shaking. Moreover, simultaneous infection with both ILTV and FWPV has been reported in commercial chickens, and the outbreaks of FP have continued to occur worldwide despite regular vaccination with FWPV live vaccines (Singh et al., 2003; Diallo et al., 2010). The failure of vaccination against FP is associated with REV contamination in the commercial vaccines, and FWPV field isolates containing nearly full-length REV or REV LTRs of various lengths are becoming more pathogenic in poultry (Singh et al., 2003; Awad et al., 2010). In order to establish prevention and control strategies for ILTV, FWPV, REV-integrated FWPV, or REV, it is necessary for a sensitive and convenient method to simultaneously detect the pathogens in field isolates. Hence, a multiplex PCR assay was developed for monitoring and screening ILTV, FWPV, REV-integrated FWPV, or REV. Especially, this assay simultaneously amplified the FWPV junction in ORF201 with the fragment of the 5′ REV-LTR as well as a fragment of the REV *env* gene.

In this study, viral isolation sometimes failed to detect in some clinical samples, and some strains of field isolates did not produce characteristic white pock lesions on CAM of 9-day-old embryos. However, the multiplex PCR and singleplex PCR showed positive results in the absence of an isolate. This indicated that the nucleic acid released from the virus could be detected. Therefore, the multiplex PCR may be more sensitive than virus isolation in cell culture and electron microscopy.

In conclusion, this newly established multiplex PCR assay may have diagnostic value for the efficient, rapid, sensitive, specific, and cost-effective detection of the ILTV, FWPV, REV-integrated FWPV, and REV simultaneously using a single assay.

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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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