Journal of Virological Methods 273 (2019) 113687

Contents lists available at ScienceDirect

Journal of Virological Methods

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

Development of a colorimetric loop-mediated isothermal amplification assay for rapid and specific detection of *Aves polyomavirus 1* from psittacine birds

Min-Ji Park[a], Hye-Ryung Kim[a], Ha-Gyeong Chae[a], Da-Rae Lim[a], Oh-Deog Kwon[a], Kwang-Hyun Cho[b], Choi-Kyu Park[a,*]

[a] College of Veterinary Medicine & Animal Disease Intervention Center, Kyungpook National University, Daegu, 41566, Republic of Korea
[b] Gyeongbuk Veterinary Service Laboratory, Daegu, 41405, Republic of Korea

ARTICLE INFO

Keywords:
Aves polyomavirus 1
Budgerigar fledgling disease
Loop-mediated isothermal amplification
Psittacine birds

ABSTRACT

A colorimetric loop-mediated isothermal amplification (LAMP) assay was developed for the rapid and specific detection of the T gene of *Aves polyomavirus 1* (APyV), a causative agent of budgerigar fledgling disease (BFD) in psittacine birds. The amplification can be completed in 40 min at 60 °C, and the results can be visually detected by the naked eye using hydroxyl naphthol blue as a colorimetric indicator. The assay specifically amplified APyV DNA but not other viral and bacterial nucleic acids. The limit of detection of the assay was 5×10^2 DNA copies/reaction, which was comparable to those of previously reported conventional polymerase chain reaction assays. In the clinical evaluation, the LAMP results showed 100% concordance with those of the previously reported PCR assays with regard to specificity, sensitivity, and percentage of overall agreement, with a kappa value of 1.0. These results indicate that the developed LAMP assay will be a valuable tool for the rapid, sensitive and specific detection of APyV from BFD-suspected psittacine bird samples even in resource-limited laboratories.

1. Introduction

Budgerigar fledgling disease (BFD) was first identified in fledgling and young budgerigars (*Melopsittacus undulatus*) in the United States (Davis et al., 1981) and Canada (Bemier et al., 1981), and has subsequently been confirmed in various avian species such as non-budgerigar *Psittaciformes, Falconiformes*, domestic geese, finches and gallinaceous birds in other countries (Katoh et al., 2009, 2010). The causative agent of BFD was originally designated as BFD polyomavirus (Lehn and Müller, 1986). However, it has since been renamed as *Aves polyomavirus 1* (APyV) within the genus *Gammapolyomavirus* of the family *Polyomaviridae* by the International Committee on Taxonomy of Viruses based on its genomic sequences and broad avian host range (Calvignac-Spencer et al., 2016). APyV is a small icosahedral and non-enveloped virus with a diameter of 45–50 nm, and contains a circular double-stranded DNA genome, which is 4981 bp in size. The genome can be divided into early and late gene-encoding regions; the early region encodes both large and small tumor antigens, whereas the late region encodes a major structural protein, VP1, and three minor structural proteins, VP2, VP3, and VP4 (Johne and Müller, 1998).

Previous reports have indicated that the degree of susceptibility to and severity of the diseases vary depending on the avian species infected as well as on the bird's age at the time of infection. Fatal infection resulting in mortality without any premonitory sign of the disease was reported in fledgling budgerigars at a mortality rate of nearly 100% (Bozeman et al., 1981; Davis et al., 1981). Acute infections with polyuria, subcutaneous hemorrhages, dyspnea and depression as well as chronic infections of adult psittacine birds have also been reported (Ritchie et al., 1991; Stoll et al., 1993). Persistently or subclinically infected birds can intermittently shed the virus through feces and feather, and can serve as a viral reservoir for transmitting the virus to susceptible psittacine birds (Rahaus and Wolff, 2005). Early diagnosis and isolation of infected birds that shed or will shed virus are key elements for preventing viral transmission to healthy birds. Therefore, there is an urgent need to develop a diagnostic method for rapid and sensitive detection of the virus from suspected birds (Katoh et al., 2008).

Various assays have been used for the diagnosis of APyV infection, including immunofluorescent antibody staining (Graham and Calnek, 1987; Phalen et al., 1996), *in situ* hybridization (Ramis et al., 1994), electron microscopy (Bernier et al., 1981; Bozeman et al., 1981; Davis et al., 1981), virus-neutralization tests (Phalen et al., 1993), enzyme-

* Corresponding author at: College of Veterinary Medicine & Animal Disease Intervention Center, Kyungpook National University, 80 Daehakro, Bukgu, Daegu, 41566, Republic of Korea.
E-mail address: parkck@knu.ac.kr (C.-K. Park).

https://doi.org/10.1016/j.jviromet.2019.113687
Received 5 April 2019; Received in revised form 24 June 2019; Accepted 1 July 2019
Available online 02 July 2019

linked immunosorbent assay (Khan et al., 2000), polymerase chain reaction (PCR) (Johne and Müller, 1998; Ogawa et al., 2005; Phalen et al., 1991; Tomasek et al., 2007), and real-time quantitative PCR (qPCR) (Katoh et al., 2008). Among these diagnostic assays, PCR and qPCR are currently used for the routine diagnosis of APyV infection due to their higher specificity and sensitivity, rapidity, and multiplexing capability for concurrently detecting and differentiating multiple targets (Johne and Müller, 1998; Katoh et al., 2008; Ogawa et al., 2005; Phalen et al., 1991; Tomasek et al., 2007). However, these PCR-based assays require sophisticated equipment, specialized labor, and complicated procedures for the detection of amplified products, making them unsuitable for under-equipped laboratories in developing countries. Therefore, the development of a simple, rapid, and cost-effective assay with desirable specificity and sensitivity is imperative for the detection of APyV from BFD-suspected bird samples.

Since its development in 2000 (Notomi et al., 2000), loop-mediated isothermal amplification (LAMP) assay has been widely applied in animal pathogen detection owing to its rapidity, simplicity, and high amplification efficiency (Dhama et al., 2014; Mori and Notomi, 2009). However, there has been little report on the use of LAMP assays for the diagnosis of parrot pathogens (Jelocnik et al., 2017; Kuo et al., 2015). Recently, a LAMP assay was successfully developed for rapid, highly sensitive and specific detection of psittacine beak and feather disease virus (PBFDV) (Kuo et al., 2015), but no LAMP assay is currently available for the detection of APyV. Considering that the clinical symptoms of PBFDV and APyV infections are similar, and that both viruses may be highly prevalent in global psittacine bird population, a LAMP assay also needs to be developed for the rapid and simple diagnosis of APyV infection. In this study, therefore, a colorimetric LAMP assay was developed for the rapid and visual detection of APyV DNA, and its diagnostic efficacy was evaluated using clinical samples of BFD-suspected psittacine birds.

2. Materials and methods

2.1. Samples and nucleic acid extraction

A Korean APyV strain (KAPV-1443; GenBank Accession No. MK516256) that was identified in Alexandrine parakeet (*Psittacula eupatria*) was used for the construction of DNA standard (Kim et al., 2014c). Clinical samples confirmed as positive for PBFDV (Kim et al., 2014b), avian bornavirus (ABV) (Kim et al., 2014a), or *Chlamydia psittaci*(not published) at our laboratory were selected for the specificity test of the LAMP assay. For clinical evaluation of the developed LAMP assay, 74 clinical samples were collected from BFD-suspected or asymptomatic psittacine birds, which were brought from animal hospitals or pet shops to the diagnostic laboratory of our veterinary hospital, Kyungpook National University for routine microbiology diagnostic procedures during 2018–2019 (Table 1). Total nucleic acid was extracted from each sample using a commercial nucleic acid extraction kit (GeneAll Biotechnology, Korea) as previously described (Johne and Müller, 1998; Ogawa et al., 2005), and used as a template for the developed LAMP and conventional PCR assays. All samples and extracted nucleic acids were stored at −80 °C until use.

2.2. Construction of APyV DNA standard

Viral DNA was extracted from the Korean APyV strain KAPV-1443 using a commercial nucleic acid extraction Kit (GeneAll biotechnology). The partial T gene of APyV was amplified with PCR using the forward primer 5′-TCGAGGTTTACGGGTACACT-3′ (Katoh et al., 2009) and reverse primer 5′-ATGGCTTCTTTGCGCAGAT-3′ (the present study). PCR was performed with a 50 μL reaction mixture containing 25 μL of 2× reaction buffer, 0.4 μM of each primer, and 5 μL of template DNA using a commercial PCR kit (Inclone Biotech, Korea). Amplification was carried out in a thermal cycler (Applied Biosystems, Foster City, CA, USA) under the following cycling conditions: initial denaturation at 95 °C for 2 min, followed by 35 cycles at 95 °C for 20 s, 60 °C for 40 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The 1581-bp amplified product was purified and cloned into the pTOP TA V2 vector (TOPcloner™ TA core Kit; Enzynomics, Korea), which was transformed into *Escherichia coli* competent cells according to the manufacturer's instructions (DH5α Chemically Competent *E. coli*; Enzynomics). Plasmids containing the APyV T gene were purified using a commercial kit (GeneAll Expin™ Combo GP 200 miniprep kit, GeneAll Biotechnology), and the sequence of the inserted DNA was confirmed by nucleotide sequencing. The concentration and purity of the purified plasmid constructs were measured using a NanoDrop Lite (Thermo Fisher Scientific, USA), and the copy numbers of the target DNA were quantified using a previously described method (Park et al., 2018). Ten-fold dilutions of this DNA sample (from 10^6 to 10^0 copies/μL) were stored at -80 ℃ and used as the DNA standard.

2.3. Primers for the LAMP assay

Based on previous reports (Henriques et al., 2018; Katoh et al., 2009; Zhuang et al., 2012), 29 T gene sequences of APyV that originated from various species of psittacine birds were selected and retrieved from GenBank. Conserved nucleotide sequences within the T gene were identified by multiple alignments using the BioEdit Sequence Alignment Editor program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Based on the conserved sequences, a set of six primers, including two outer primers (forward, F3; reverse, B3), two inner primers (forward, FIP; reverse, BIP), and two loop primers (forward, LF; reverse, LB), were manually designed for the LAMP assay aided by Primer Explorer V4 (Eiken Chemical, Japan). All primers were synthesized by a commercial company (Bionics, Korea). Sequences or primer binding sites are shown in Fig. 1.

2.4. LAMP conditions

The LAMP reaction was carried out in a 25 μL reaction volume using a Mmiso DNA amplification kit (Mmonitor, Korea) and a thermal cycler (Applied Biosystems). The reaction mixture contained 5 μL of viral DNA, 12.5 μL of premixed 2× buffer with 240 μM of hydroxyl naphthol blue (HNB), 8 U of *Bst* DNA polymerase, 1.6 μM of inner primers (FIP and BIP), 0.2 μM of outer primers (F3 and B3), and 0.8 μM of loop primers (LF and LB). To optimize the reaction conditions, LAMP was first performed at a temperature range of 52–68 °C for 40 min to find the optimal reaction temperature and then with three dilutions of the APyV T gene DNA (10^5, 10^4, and 10^3 copies/μL) for different reaction times (20–50 min) at the optimized reaction temperature. The reactions were terminated by heating at 80 °C for 5 min. All experiments were repeated three times The assay was interpreted by visual detection of a color change from purple to sky blue due to the presence of the metal ion indicator HNB (Goto et al., 2009). Amplicons were also detected by observing LAMP-specific ladder-like DNA bands with an ultraviolet light transilluminator (Bio-Rad, USA) after 1.5% agarose gel electrophoresis and staining with NEO green dye (Neoscience, Korea).

2.5. PCR assays

To perform a comparative analysis of LAMP, two conventional PCR assays using T gene specific primers were performed as previously described by Johne and Müller (1998) and Ogawa et al. (2005), which have been designated as J-PCR and O-PCR in this study, respectively. The PCR assays were carried out using a commercial PCR kit (Excel TB 2X Taq premix; Inclone, Korea) and a thermal cycler (Applied Biosystems) with previously described primers (Table 2) in a 50 μL reaction mixture containing 25 μL of 2× premix, 0.4 μM (J-PCR) or 0.15 μM (O-PCR) of each primer, and 5 μL of template, according to the manufacturer's instructions. The J-PCR program consisted of initial

Table 1
APyV DNA detection from clinical samples of psittacine birds by different diagnostic assays.

No.	Scientific name	Common name	Samples[a]	Detection of APyV DNA[c]		
				J-PCR	O-PCR	LAMP
1	*Agapornis lilianae*	Nyasa lovebird	B	–	–	–
2	*Agapornis lilianae*	Nyasa lovebird	B	–	–	–
3	*Agapornis lilianae*	Nyasa lovebird	B	–	–	–
4	*Amazona*	Amazon parrot	C	–	–	–
5	*Amazona*	Amazon parrot	B	–	–	–
6	*Amazona*	Amazon parrot	B	–	–	–
7	*Amazona*	Amazon parrot	B	–	–	–
8	*Amazona aestiva*	Blue-fronted amazon	B	–	–	–
9	*Ara ararauna*	Blue and yellow macaw	B	–	–	–
10	*Ara ararauna*	Blue and yellow macaw	F	–	–	–
11	*Ara ararauna*	Blue and yellow macaw	B	–	–	–
12	*Ara ararauna*	Blue and yellow macaw	B	–	–	–
13	*Ara chloropterus*	Green-winged macaw	B	–	–	–
14	*Ara chloropterus*	Green-winged macaw	B	–	–	–
15	*Ara chloropterus*	Green-winged macaw	B	–	–	–
16	*Aratinga solstitialis*	Sun conure	T	+	+	+
17	*Aratinga solstitialis*	Sun conure	T[b]	+	+	+
18	*Aratinga solstitialis*	Sun conure	B	+	+	+
19	*Diopsittaca nobilis*	Hahn's macaw	B	–	–	–
20	*Diopsittaca nobilis*	Hahn's macaw	B	–	–	–
21	*Diopsittaca nobilis*	Hahn's macaw	B	–	–	–
22	*Diopsittaca nobilis*	Hahn's macaw	B	–	–	–
23	*Diopsittaca nobilis*	Hahn's macaw	B	–	–	–
24	*Eclectus Parrot*	Eclectus roratus	B	–	–	–
25	*Eclectus Parrot*	Eclectus roratus	F	–	–	–
26	*Eclectus Parrot*	Eclectus roratus	B	–	–	–
27	*Eclectus Parrot*	Eclectus roratus	B	–	–	–
28	*Melopsittacus undulatus*	Budgerigar	B	–	–	–
29	*Myiopsitta monachus*	Quaker parrot	C[b]	+	+	+
30	*Myiopsitta monachus*	Quaker parrot	C[b]	+	+	+
31	*Myiopsitta monachus*	Quaker parrot	C[b]	+	+	+
32	*Myiopsitta monachus*	Quaker parrot	C[b]	+	+	+
33	*Myiopsitta monachus*	Quaker parrot	B[b]	+	+	+
34	*Myiopsitta monachus*	Quaker parrot	T[b]	+	+	+
35	*Myiopsitta monachus*	Quaker parrot	T[b]	+	+	+
36	*Myiopsitta monachus*	Quaker parrot	T[b]	+	+	+
37	*Myiopsitta monachus*	Quaker parrot	F	–	–	–
38	*Myiopsitta monachus*	Quaker parrot	B	–	–	–
39	*Nymphicus hollandicus*	Cockatiel	C	–	–	–
40	*Nymphicus hollandicus*	Cockatiel	B	–	–	–
41	*Nymphicus hollandicus*	Cockatiel	B	–	–	–
42	*Nymphicus hollandicus*	Cockatiel	B	+	+	+
43	*Nymphicus hollandicus*	Cockatiel	B	–	–	–
44	*Nymphicus hollandicus*	Cockatiel	B	–	–	–
45	*Nymphicus hollandicus*	Cockatiel	B	–	–	–
46	*Nymphicus hollandicus*	Cockatiel	B	–	–	–
47	*Pionites melanocephalus*	Black-headed caique	B	–	–	–
48	*Pionites melanocephalus*	Black-headed caique	B	–	–	–
49	*Poicephalus senegalus*	Senegal parrot	B	+	+	+
50	*Poicephalus senegalus*	Senegal parrot	B	–	–	–
51	*Psittacula eupatria*	Alexandrine parakeet	T	+	+	+
52	*Psittacula krameri*	Ring-necked parakeet	F	–	–	–
53	*Psittacula krameri*	Ring-necked parakeet	F	–	–	–
54	*Psittacus erithacus*	Grey parrot	B	–	–	–
55	*Psittacus erithacus*	Grey parrot	F	–	–	–
56	*Psittacus erithacus*	Grey parrot	B	–	–	–
57	*Psittacus erithacus*	Grey parrot	B	–	–	–
58	*Psittacus erithacus*	Grey parrot	B	+	+	+
59	*Pyrrhura molinae*	Green-cheeked conure	B	–	–	–
60	*Pyrrhura molinae*	Green-cheeked conure	T[b]	+	+	+
61	*Pyrrhura molinae*	Green-cheeked conure	T[b]	+	+	+
62	*Pyrrhura molinae*	Green-cheeked conure	T[b]	+	+	+
63	*Pyrrhura molinae*	Green-cheeked conure	T[b]	+	+	+
64	*Pyrrhura molinae*	Green-cheeked conure	T[b]	+	+	+
65	*Pyrrhura molinae*	Green-cheeked conure	T[b]	+	+	+
66	*Pyrrhura molinae*	Green-cheeked conure	C[b]	+	+	+
67	*Pyrrhura molinae*	Green-cheeked conure	C[b]	+	+	+
68	*Pyrrhura molinae*	Green-cheeked conure	C[b]	+	+	+
69	*Pyrrhura molinae*	Green-cheeked conure	C[b]	+	+	+
70	*Pyrrhura molinae*	Green-cheeked conure	C[b]	+	+	+
71	*Pyrrhura molinae*	Green-cheeked conure	C[b]	+	+	+
72	*Pyrrhura molinae*	Green-cheeked conure	C[b]	+	+	+

(*continued on next page*)

Table 1 (*continued*)

No.	Scientific name	Common name	Samples[a]	Detection of APyV DNA[c]		
				J-PCR	O-PCR	LAMP
73	*Pyrrhura molinae*	Green-cheeked conure	B	–	–	–
74	*Pyrrhura molinae*	Green-cheeked conure	B	–	–	–
No. of positive/tested samples				28/74	28/74	28/74

[a] B, blood; C, cloacal swab; F, feather; T, tissue.
[b] These samples of 1 sun conure, 8 quaker parrots, and 13 green-cheeked conures were collected from the same aviary located at Gyeongnam Province in Korea.
[c] + or – indicates positive or negative results by each diagnostic assay.

denaturation at 95 °C for 5 min, followed by 35 cycles of PCR (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s) amplification, and a final extension at 72 °C for 5 min. The O-PCR program consisted of initial denaturation at 95 °C for 5 min, followed by 35 cycles of PCR (94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s) amplification, and a final extension at 72 °C for 5 min. The expected 310-bp amplicons for J-PCR or 298-bp amplicons for O-PCR were visualized with an ultraviolet light transilluminator (Bio-Rad) after 1.5% agarose gel electrophoresis and staining with NEO green dye (Neoscience).

2.6. Specificity and sensitivity of the LAMP assay

To test the specificity of the LAMP assay, the assay was performed using psittacine pathogen nucleic acid extracted from an APyV- positive or -negative samples, including PBFDV-, ABV-, and *C. psittaci*- positive samples, five avian pathogens including Newcastle disease virus (NDV, La sota vaccine strain), Marek's disease virus (MDV, SB-1 vaccine strain), infectious bronchitis virus (IBV, K2 vaccine strain), fowl adenovirus (FAdV, Kr-Changnyeong Korean isolate), subtype H9N2 avian influenza virus (AIV, A/Chicken/Korea/01310/2001 strain), a negative psittacine tissue sample confirmed by previously reported PCR assays, and negative control (nuclease free water). The limit of detection (LOD) of LAMP was determined using 10-fold serial dilutions of DNA standard, ranging from 10^6 to 10^0 copies/μL. Subsequently, the LOD of the LAMP assay was compared with those of the J-PCR and O-PCR assays using the same DNA templates as described above. The sensitivity of the LAMP assay was repeated three times and compared with PCR assays.

2.7. Comparative evaluation of LAMP and PCR assays

For clinical evaluation of the LAMP assay, 74 clinical samples (12 tissue, 43 blood, 13 cloacal swab, and 6 feather samples) were collected from different species of psittacine birds and tested using the LAMP, J-PCR or O-PCR assays. The LAMP results were compared with those of the PCR assays, and the concordance was analyzed using Cohen's kappa statistics at 95% confidence interval (CI) (Kwiecien et al., 2011). When the calculated Kappa coefficient value (κ) was 0.81 or more, the agreement between the LAMP and each PCR assay was considered to be almost perfect.

3. Results

3.1. Optimization of the LAMP assay

In the LAMP reaction with APyV DNA templates, a positive color change, from purple to sky blue was visually detected in the reaction tubes at temperatures ranging from 52 °C to 68 °C (Fig. 2A), and all color-positive reactions produced a characteristic ladder of bands on an agarose gel (Fig. 2B). However, the color change and electrophoretic band were clearer at 60, 62, and 65 °C than at 52, 55, 58, and 68 °C. Subsequently, the LAMP assay was performed with three dilutions of DNA samples from the cloned T gene plasmid with different copy numbers (10^5, 10^4, and 10^3 copies/μL) for various reaction times (20–50 min). The color change and electrophoresis results indicated that amplification was initiated within 20 min. however, the optimal reaction time was 40 min, since a definite positive color change and strong specific DNA bands were observed at that time point for reactions with DNA samples containing 10^3 copies/μL of the DNA standard (Fig. 2C and 2D). Based on these results, the optimal reaction temperature and time for the LAMP assay were confirmed to be 60 °C and 40 min, respectively. Therefore, all subsequent experiments were carried out using these optimized reaction conditions.

3.2. Specificity of the LAMP assay

The specificity of the LAMP assay for detecting APyV was determined by assessing the cross-reactivity of the assay with an APyV-positive sample, other psittacine pathogen-positive samples (PBFDV, ABV, and *C. psittaci*), five avian pathogens (NDV, MDV, IBV, FAdV and AIV), a negative psittacine tissue sample, and a negative control. The LAMP assay yielded positive results for the APyV-positive sample, but negative results for all other pathogens and negative samples. Therefore, the primer set used for this assay shows a high degree of specificity for the T gene of APyV (Fig. 3).

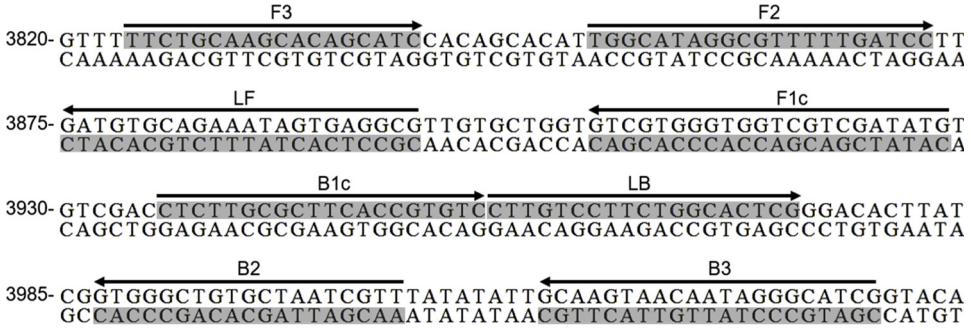

Fig. 1. Primer-binding sites and orientations of the LAMP primers. Locations of the primer sequences were derived from the NCBI reference sequence of *Aves polyomavirus 1* (GenBank Accession No. NC-004764).

Table 2
Primers used in this study.

Method	Primer	Sequence (5′-3′) set	Genome position [a]	Reference
LAMP	F3	TTCTGCAAGCACAGCATC	3824-3841	This study
	B3	CGATGCCCTATTGTTACTTGC	4034-4014	
	FIP	CATATCGACGACCACCCACGAC	3928-3907	
	(F1C + F2)	+ TGGCATAGGCGTTTTTGATCC	3852-3872	
	BIP	CTCTTGCGCTTCACCGTGTC	3936-3955	
	(B1C + B2)	+AACGATTAGCACAGCCCAC	4005-3987	
	LF	CGCCTCACTATTTCTGCACATC	3896-3875	
	LB	CTTGTCCTTCTGGCACTCG	3956-3974	
J-PCR	Forward	CAAGCATATGTCCCTTTATCCC	4303-4324	Johne and Müller (1998)
	Reverse	CTGTTTAAGGCCTTCCAAGATG	4612-4591	
O-PCR	BFDdupF	CAGGCCTTATATCCTGTTTGCGTC	4624-4647	Ogawa et al. (2005)
	BFDdupR	GATATCAAGACTGCCTATCGTCGC	4921-4898	

[a] Locations of all primer sequences for the LAMP, J-PCR, and O-PCR assays were derived from the NCBI reference sequence of *Aves polyomavirus 1* (GenBank Accession No. NC-004764).

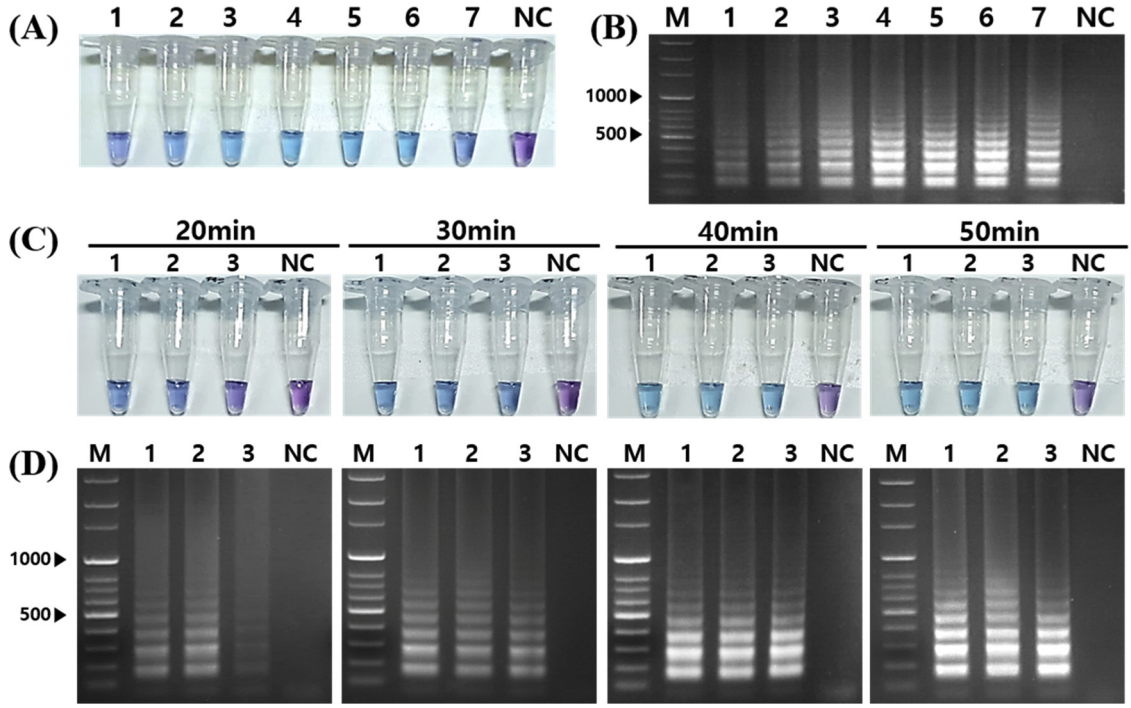

Fig. 2. Optimization of LAMP conditions for the amplification of APyV DNA at different reaction temperatures and times. (A and B) Visual (A) and electrophoretic detection (B) of the LAMP results at different reaction temperatures. Tubes and lanes 1–7, different reaction temperatures of 52 °C, 55 °C, 58 °C, 60 °C, 62 °C, 65 °C, and 68 °C, respectively; tube and lane NC, negative control (nuclease free water); lane M, 100-bp DNA marker. (C and D) Visual (C) and electrophoretic detection (D) of the LAMP results in different times. Tubes and lanes 1–3, 10-fold dilutions of APyV DNA standard with viral DNA copies of 10^5, 10^4, and 10^3 copies/μL, respectively; tube and lane NC, negative control (nuclease free water); lane M, 100-bp DNA marker.

3.3. Comparison of sensitivity between the LAMP and PCR assays

To compare the sensitivities of different molecular assays, the LAMP, J-PCR, and O-PCR assays were performed using serial dilutions of DNA standard, and the LODs of the assays were compared with each other. The LODs of the LAMP, J-PCR, and O-PCR assays were determined as 5×10^2 copies/reaction, showing that the analytical sensitivity of LAMP was comparable to those of PCR assays (Fig. 4).

3.4. Diagnostic performance of the LAMP assay

Out of 74 samples from different species of psittacine birds, 28 were confirmed as APyV DNA-positive by the LAMP, J-PCR, and O-PCR assays (Table 2). The percentages of positive, negative, and overall agreement of the developed LAMP assay compared with the two conventional PCR (J-PCR and O-PCR) assays were 100% (28/28), 100% (46/46), and 100% (74/74) for the detection of APyV DNA from the clinical samples, with a kappa value (95% CI) of 1.0 (1.000 – 1.000). These results demonstrated that the LAMP assay was specific and sensitive and could be an alternative to conventional PCR assays for the detection of APyV in clinical samples. The 28 APyV-positive samples were obtained from four species of psittacine birds, namely three sun conures (*Aratinga olstitialis*), eight quaker parrots (*Myiopsitta monachus*), one cockatiel (*Nymphicus hollandicus*), one senegal parrot (*Poicephalus senegalus*), one grey parrot (*Psittacus erithacus*), one Alexandrine parakeet (*P. eupatria*) and 13 green-cheeked conures (*Pyrrhura molinae*). Among them, 22 samples (one sun conure, eight quaker parrots, and 13 green-cheeked conures) were from the same aviary located in Gyeongnam Province, and the other six samples (two sun conures, one cockatiel, one senegal parrot, one grey parrot, and one Alexandrine parakeet) were from individuals of parrot lovers, respectively (Table 2).

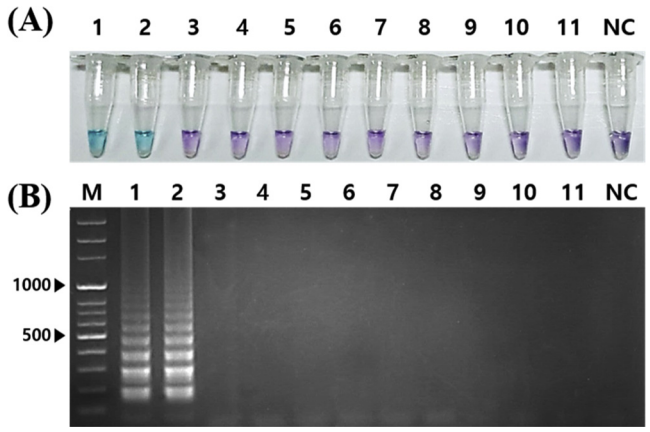

Fig. 3. Specificity of the LAMP assay for the detection of APyV. Visual detection (A) and electrophoretic patterns (B) of the LAMP assay results. Tube and lane 1, DNA standard of the APyV T gene; tube and lane 2, APyV positive clinical sample; tubes and lanes 3–5, PBFDV-, ABV-, *C. psittaci*-positive clinical samples; tubes and lanes 6–10, Newcastle disease virus (La sota vaccine strain), Marek's disease virus (SB-1 vaccine strain), infectious bronchitis virus (K2 vaccine strain), fowl adenovirus (Kr-Changnyeong Korean isolate), subtype H9N2 avian influenza virus (A/Chicken/Korea/01310/2001 strain); tube and lane 11, negative psittacine tissue sample; tube and lane NC, negative control (nuclease free water), respectively.

4. Discussion

APyV is an important viral pathogen that causes a lethal multisystemic disease in different species of psittacine birds around the world. Since clinically or subclinically infected birds can shed the virus for a long time, early detection and isolation of infected birds are of the utmost importance in the prevention of disease transmission and control (Tomasek et al., 2007). The LAMP assay has been recognized as a valuable tool for the detection of various animal and human pathogens due to its high sensitivity and specificity, rapidity, simplicity, and field-applicability (Dhama et al., 2014; Mori and Notomi, 2009). Although some LAMP assays have been reported for psittacine pathogens, no method of LAMP assay has yet been developed for the detection of APyV in psittacine birds (Jelocnik et al., 2017; Kuo et al., 2015). Therefore, a LAMP assay was first developed and evaluated for the rapid and specific detection of APyV DNA from clinical samples of psittacine birds in the present study.

The LAMP assay in our study is advantageous as it has high specificity because it uses six primers that recognize eight regions of the target gene sequences. However, the difficulty in designing primers that specifically target the desired gene has been a limiting factor in the development of this assay (Dhama et al., 2014). Previous researchers have successfully designed specific primers for APyV PCR assays from the T antigen region, which is the most conserved region of the APyV genome (Johne and Müller, 1998; Katoh et al., 2009; Ogawa et al., 2005). Therefore, in this study, three pairs of LAMP primers (F3 and B3, FIP and BIP, and LF and LB) recognizing 6 regions of the target T gene in the most conserved region of the APyV T gene sequences were successfully designed (Table 2 and Fig. 1). The LAMP assay results showed that the newly designed primers specifically amplified the APyV T gene, but not the other psittacine and avian pathogens, negative tissue sample and the negative control, suggesting that the primers are highly specific to the APyV T gene (Fig. 2).

The LAMP reaction was completed in 40 min at 60 °C, and the results were directly detected with the naked eye using HNB as a colorimetric indicator, without any additional detection process (Fig. 2). HNB can be added to the reaction mixture before the LAMP reaction, and the results can be monitored immediately after the reaction is completed without opening the reaction tube, which prevents DNA cross-contamination by pre-amplified products (Goto et al., 2009; Park et al., 2018). Therefore, the developed LAMP assay is more convenient and useful as a field diagnostic method compared with previously reported PCR assays, which require an additional electrophoresis step for amplicon detection.

The LODs of previously reported J-PCR or O-PCR assays were originally reported as 0.01 pg or 0.01 fg of the total template DNA, respectively (Johne and Müller, 1998; Ogawa et al., 2005), but they used different DNA samples total extracted DNA from organs of infected birds for J-PCR or purified target DNA for O-PCR as template for APyV, respectively. Therefore, it is not possible to compare the sensitivities of the assays with each other. To objectively compare the sensitivities of diagnostic methods, a DNA standard containing all the target gene regions to be amplified by each diagnostic method was cloned in this study, and the LOD of each diagnostic method was comparatively evaluated using this DNA standard as the common template. The results showed that the LODs of the LAMP and the two conventional PCR assays were equal at 5×10^2 copies/reaction, suggesting that the developed LAMP assay was sufficiently sensitive to replace the previously reported PCR assays (Fig. 4).

To evaluate the usefulness of the LAMP assay, the assay was performed with 74 clinical samples obtained from various species of

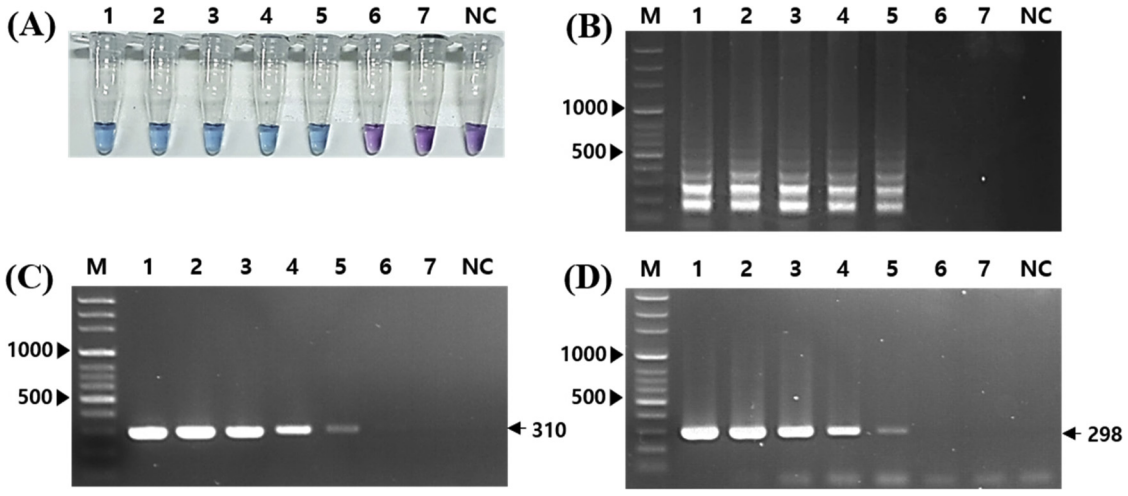

Fig. 4. Comparative sensitivity of the LAMP and PCR assays for APyV. Limit of detection of the LAMP (A and B), J-PCR (C), and O-PCR (D) assays for the amplification of APyV DNA. Tubes and lanes 1–7, 10-fold serial dilutions (from 5×10^6 copies to 5 copies/reaction) of the APyV T gene; lane M, 100-bp DNA marker; NC, negative control (nuclease free water).

psittacine birds, and the obtained results were compared with those of the J-PCR and O-PCR assays. The percentages of positive, negative, and overall agreement of the LAMP assay compared with the PCR assays were all 100%. (Table 1), showing that the newly developed LAMP assay can be used as an alternative to the PCR assays for the diagnosis of APyV infection from various clinical samples. APyV infection was first identified from Alexandrine parakeet in 2014 in Korea (Kim et al., 2014c). In this study, APyV infections were further identified in sun conures, quaker parrots, cockatiel, senegal parrot, grey parrot, and green-cheeked conures. These results demonstrated that APyV was widely distributed in the Korean psittacine bird population and can be transmitted from bird to bird. Therefore, further epidemiological and etiological studies on APyV are needed to prevent APyV infection in Korea. Clinical samples for evaluation of the developed LAMP assay were limited in this study. Therefore, further validation using additional clinical samples is required to determine the utility of the LAMP assay. Nevertheless, comparative evaluation of the LAMP assay with previously reported PCR assays showed that the results of the LAMP assay were 100% agreed with those of the PCR assays, suggesting that the LAMP assay can serve as useful diagnostic tool for APyVs infection in psittacine birds.

The established LAMP assay was specific and sensitive for the detection of APyV DNA, and the amplification can be accomplished with a conventional, constant-temperature water bath without a specialized thermocycler. The LAMP results can be rapidly confirmed within 40 min, whereas PCR or qPCR requires 2–3 h. Furthermore, the LAMP results can be observed with the naked eye as soon as the reaction is complete, allowing rapid and easy monitoring of the test results while avoiding the need for electrophoresis or other monitoring apparatus. Taken together, these advantages indicate that the LAMP assay developed in this study will be a valuable tool for the rapid and specific diagnosis of APyV in psittacine bird disease diagnostic laboratories as well as under-equipped veterinary hospitals.

Funding

This work was supported by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, and Forestry (IPET) through the Golden Seed Project (213010-05-3-SB610 and PJ012818012019) funded by the Ministry of Agriculture, Food and Rural Affairs (MAFRA), Ministry of Oceans and Fisheries (MOF), Rural Development Administration (RDA) and Korea Forest Services (KFS), Republic of Korea.

References

Bernier, G., Morin, M., Marsolais, G., 1981. A generalized inclusion body disease in the budgerigar (*Melopsittacus undulatus*) caused by a papovavirus-like agent. Avian Dis. 25, 1083–1092.

Bozeman, L.H., Davis, R.B., Gaudry, D., Lukert, P.D., Fletcher, O.J., Dykstra, M.J., 1981. Characterization of a papovavirus isolated from fledgling budgerigars. Avian Dis. 25, 972–980.

Calvignac-Spencer, S., Feltkamp, M.C., Daugherty, M.D., Moens, U., Ramqvist, T., Johne, R., Ehlers, B., 2016. A taxonomy update for the family Polyomaviridae. Arch. Virol. 161, 1739–1750.

Davis, R.B., Bozeman, L.H., Gaudry, D., Fletcher, O.J., Lukert, P.D., Dykstra, M.J., 1981. A viral disease of fledgling budgerigars. Avian Dis. 25, 179–183.

Dhama, K., Karthik, K., Chakraborty, S., Tiwari, R., Kapoor, S., Kumar, A., Thomas, P., 2014. Loop-mediated isothermal amplification of DNA (LAMP): a new diagnostic tool lights the world of diagnosis of animal and human pathogens: a review. Pak. J. Biol. Sci. 17, 151–166.

Goto, M., Honda, E., Ogura, A., Nomoto, A., Hanaki, K., 2009. Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. Biotechniques. 46, 167–172.

Graham, D.L., Calnek, B.W., 1987. Papovavirus infection in hand-fed parrots: virus isolation and pathology. Avian Dis. 31, 398–410.

Henriques, A.M., Fagulha, T., Barros, S.C., Ramos, F., Duarte, M.D., Luís, T., Carvalho, P., Mendonça, P., Monteiro, M., Craveiro, H., Fevereiro, M., 2018. Aves polyomavirus 1 in *Ara chloroptera* and *Eclectus roratus* with disclosure of full genomic sequences. J. Exo. Pet Med. 27, 4–10.

Jelocnik, M., Islam, M.M., Madden, D., Jenkins, C., Branley, J., Carver, S., Polkinghorne, A., 2017. Development and evaluation of rapid novel isothermal amplification assays for important veterinary pathogens: chlamydia psittaci and Chlamydia pecorum. Peer J. 8 (5), e3799.

Johne, R., Müller, H., 1998. Avian polyomavirus in wild birds: genome analysis of isolates from Falconiformes and Psittaciformes. Arch. Virol. 143, 1501–1512.

Katoh, H., Ogawa, H., Ohya, K., Fukushi, H., 2010. A review of DNA viral infection in psittacine birds. J. Vet. Med. Sci. 72, 1099–1106.

Katoh, H., Ohya, K., Fukushi, H., 2008. Development of novel real-time PCR assays for detecting DNA virus infections in psittaciform birds. J. Virol. Methods 154, 92–98.

Katoh, H., Ohya, K., Une, Y., Yamaguchi, T., Fukushi, H., 2009. Molecular characterization of avian polyomavirus isolated from psittacine birds based on the whole genome sequence analysis. Vet. Microbiol. 138, 69–77.

Khan, M.S., Johne, R., Beck, I., Pawlita, M., Kaleta, E.F., Muller, H., 2000. Development of a blocking enzyme-linked immunosorbent assay for the detection of avian polyomavirus-specific antibodies. J. Virol. Methods 89, 39–48.

Kim, J.H., Lee, B.H., Cho, J.K., Yoon, W.K., Kim, W., Kim, H.J., Kim, E.M., Kim, K.S., Park, C.K., 2014a. First detection of avian bornavirus by RT-PCR in proventricular dilatation disease-suspected Hahns Macaw (*Ara nobilis nobilis*) in Korea. Korean J. Vet. Serv. 37, 79–84.

Kim, H.J., Kang, D.Y., Kim, E.M., Kim, E.G., Lee, B.H., Yeo, S.G., Park, C.K., 2014b. Detection of psittacine beak and feather disease virus from a caged blue and yellow macaw (*Ara ararauna*) in Korea. Korean J. Vet. Serv. 37, 219–224.

Kim, H.J., Lee, S.R., Park, C.K., 2014c. First detection of avian polyomavirus by PCR from Alexandrine Parakeet (*Psittacula eupatria*) in Korea. Korean J. Vet. Serv. 37, 213–218.

Kuo, Y.H., Tsai, S.S., Liu, H.J., Chuang, K.P., 2015. Development of a loop-mediated isothermal amplification method for rapid detection of beak and feather disease virus in parrots. Arch. Clin. Microbiol. 7 (1), 1–8.

Kwiecien, R., Kopp-Schneicier, A., Blettner, M., 2011. Concordance analysis. Arztebl. Int. 108, 515–521.

Lehn, H., Muller, H., 1986. Cloning and characterisation of budgerigar fledgling disease virus, an avian polyomavirus. Virology 151, 362–370.

Mori, Y., Notomi, T., 2009. Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. J. Infect. Chemother. 15, 62–69.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28, e63.

Ogawa, H., Yamaguchi, T., Fukushi, H., 2005. Duplex shuttle PCR for differential diagnosis of budgerigar fledgling disease and psittacine beak and feather disease. Microbiol. Immunol. 49, 227–237.

Park, Y.R., Kim, H.R., Kim, S.H., Lee, K.K., Lyoo, Y.S., Yeo, S.G., Park, C.K., 2018. Loop-mediated isothermal amplification assay for the rapid and visual detection of novel porcine circovirus 3. J. Virol. Methods 53, 26–30.

Phalen, D.N., Wilson, V.G., Graham, D.L., 1991. Polymerase chain reaction assay for avian polyomavirus. J. Clin. Microbiol. 29, 1030–1037.

Phalen, D.N., Wilson, V.G., Graham, D.L., 1993. Organ distribution of avian polyomavirus DNA and virus-neutralizing antibody titers in healthy adult budgerigars. Am. J. Vet. Res. 54, 2040–2047.

Phalen, D.N., Wilson, V.G., Graham, D.L., 1996. Characterization of the avian polyomavirus-associated glomerulopathy of nestling parrots. Avian Dis. 40, 140–149.

Ramis, A., Latimer, K.S., Niagro, F.D., Campagnoli, R.P., Rithchie, B.W., Pesti, D., 1994. Diagnosis of psittacine beak and feather disease (PBFD) viral infection, avian polyomavirus infection, adenovirus infection and herpesvirus infection in psittacine tissues using DNA *in situ* hybridization. Avian Pathol. 23, 643–657.

Rahaus, M., Wolff, M.H., 2005. A survey to detect subclinical polyomavirus infections of captive psittacine birds in Germany. Vet. Microbiol. 105, 73–76.

Ritchie, B.W., Niagro, F.D., Latimer, K.S., Vernot, J., Pesti, D., Campagnoli, R.P., Lukert, P.D., 1991. Polyoma virus infections in adult psittacine birds. J. Assoc. Avian. Vet. 5, 202–206.

Stoll, R., Luo, D., Kouwenhoven, B., Hobom, G., Müller, H., 1993. Molecular and biological characteristics of avian polyomaviruses: isolates from different species of birds indicate that avian polyomaviruses form a distinct subgenus within the Polyomavirus genus. J. Gen. Virol. 74, 229–237.

Tomasek, O., Kubicek, O., Tukac, V., 2007. Unusual fatal avian polyomavirus infection in nestling cockatiels (*Nymphicus hollandicus*) detected by nested polymerase chain reaction. Vet. Med. 52, 193–201.

Zhuang, Q., Chen, J., Mushtaq, M.H., Chen, J., Liu, S., Hou, G., Li, J., Huang, B., Jiang, W., 2012. Prevalence and genetic characterization of avian polyomavirus and psittacine beak and feather disease virus isolated from budgerigars in Mainland China. Arch. Virol. 157, 53–61.