Rapid Detection of Avian Leukosis Virus Subgroup J by Cross-priming Amplification

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Abstract

Avian leukosis virus subgroup J (ALV-J) causes oncogenic disease in chickens in China, resulting in great harm to poultry production. Herein, we employed a cross-priming amplification (CPA) approach and a nucleic acid detection device to establish a visual rapid detection method for ALV-J. When the amplification reaction was carried out at 60°C for just 60 min, the sensitivity of CPA was 10 times higher than conventional PCR, with high specificity, based on detection of 123 clinical plasma samples. The coincidence rate with real-time PCR was 97.3% (71/73). CPA detection of ALV-J does not require an expensive PCR instrument; a simple water bath or incubator is sufficient for complete DNA amplification, and the closed nucleic acid detection device avoids aerosol pollution, making judgment of results more intuitive and objective. The CPA assay would be a promising simple, rapid and sensitive method for identification of ALV-J.

Background

Avian Leukosis (AL) is an infectious tumour disease affecting poultry caused by avian leukosis viruses (ALVs) belonging to the Alpharetrovirus genus of the family Retroviridae [1]. Chickens infected with ALVs suffer from decreased performance and immunosuppression, leading to tumours and even death [2], and it is one of the most important diseases endangering the healthy development of the poultry industry in China [3]. Based on the envelope glycoprotein (gp85) associated with subgroup specificity, ALVs from naturally infected chicken flocks can be divided into seven subgroups (A, B, C, D, E, J and K) [4], among which endogenous subgroup E viruses with little to no pathogenicity are present in nearly all chicken lines, while the others are exogenous and pathogenic [5, 6]. Subgroup A and B viruses mainly cause lymphocytic leukemia and myeloid leukosis in field flocks [7, 8], while flocks naturally infected with subgroup C and D are rarely observed in the field [9, 10].

Avian leukosis virus subgroup J (ALV-J) was first isolated and identified in broiler chickens in 1988 [11]. Chickens infected with ALV-J can suffer from medulloblastoma and decreased production performance, causing huge economic losses to the poultry breeding industry across the world [12]. ALV-J was originally thought only to infect meat-type chickens [13], but its host range is now known to include layer chickens and local broiler breeders in most parts of China [14, 15], and the disease has even spread to waterfowl and wild birds [16]. Most large foreign breeder companies had eradicated classic exogenous ALVs by the end of the 1980s, but ALV-J remains widespread in China [17].

Effective vaccines and drugs have not yet been reported for AL. Obtaining a population of breeding poultry without exogenous ALV through continuous purification is therefore the main method for disease prevention and control, for which virus detection is crucial. A variety of methods for detecting and differentially diagnosing ALV have been established in various regions of the world, mainly involving virus isolation and identification [18], pathological diagnosis [19], serological detection methods such as enzyme-linked immunosorbent assay (ELISA) [20], and PCR-based molecular biological detection [21]. However, existing detection methods have some limitations and shortcomings. For example, traditional

virus isolation and identification is too time-consuming to meet the requirements of rapid diagnosis, and PCR and real-time PCR (RT-PCR) require expensive instruments and equipment which are not conducive to fieldwork. Additionally, while the loop-mediated isothermal amplification (LAMP) detection method does not require specific instruments or equipment [22], it is highly prone to false positives, weak positive results tend to be inaccurate, and contamination by aerosols can occur easily.

Cross-priming amplification (CPA) is a rapid detection method developed in recent years [23–25]. The CPA utilizes multiple primers and probes (4 s, 5a, 2a1s, 3a, and 2a) to amplify nucleotide sequence isothermally. By labelling primer 2a with biotin, primer 3a with FITC, then the amplified products were recognized by anti-biotin and anti-FITC monoclonal antibodies on the test line, where gold nanoparticles (AuNPs) were fixed, CPA amplified products could be visualized in nucleic acid test strip [26]. It benefits from high specificity and sensitivity, does not require expensive instruments, the operation process is relatively simple, amplification is rapid, and it can be used with disposable nucleic acid detection strip in closed tubes, thereby avoiding aerosol pollution, making results more intuitive and objective [27]. This method has been applied to the diagnosis of infectious diseases and the detection of multiple pathogens [24, 28]. In the present study, we developed a CPA assay for ALV-J, which would be a promising and user-friendly rapid detection method.

Methods

Main experimental materials and reagents

ALV-J (HN06), ALV-A (GD-13), ALV-B (CD08), ALV-K (GDFX0601), ALV-E (HN1301) and Salmonella enteritis (SE) were maintained in our laboratory. Escherichia coli ATCC15922 and Pseudomonas aeruginosa (PA)ATCC27853 were obtained from HuanKai Microbial (Guangzhou, China). Mycoplasma gallisepticum (MG) S6 was donated by Professor Ding (College of Veterinary Medicine, South China Agricultural University). Bst DNA polymerase, 10 × ThermoPol Reaction Buffer, dNTPs and MgSO₄ were products of New England Biolabs (Beverly, MA, USA). Betaine was acquired from Sigma (St. Louis, MO, USA). Disposable Nucleic Acid Detection Strips were purchased from Ustar (Hangzhou, China). The DNA Extraction Kit, Gel Extraction Kit, and Plasmid Mini Kit were produced from OMEGA (Norcross, GA, USA).

Primer design

Using ALV-J sequences published in the NCBI database (GeneBank ID: HQ900844.1, HM776937.1, JQ935966.1 and Z46390.1), online software PrimerExpoler (<http://primerexplorer.jp/e/>), Primer Premier 5.0 and Oligo 7 were used to design CPA primers and probes for the envelope glycoprotein gene of ALV-J. The primers set include two external primers (F3 and B3), one cross-amplification primer CPR consisting of two short primers (F2 and B2), two detection probes F1 (labelled at the 5' end with FAM) and F2 (labelled at the 5' end with biotin) (Table 1). The single primer B2 does not participate in the reaction system, it's only a part of the sequence of primer CPR. The amplified envelope glycoprotein gene fragment was 548 bp. The specific positions and sequence of primers and probes in the genome are

shown in Fig. 1 and Table 1. These primers and probes were synthesised by Sangon Biotech (Shanghai, China) and diluted to $10 \mu\text{mol L}^{-1}$ before use.

DNA extraction and quantification of recombinant plasmid standards

Total DNA was extracted from DF-1 cells (2.5×10^6 cells) infected with ALV-J ($2 \times 10^3 \text{ TCID}_{50}$) using an DNA Extraction kit (OMEGA, Norcross, GA) according to the manufacturer's instructions and stored at -20°C until use. The PCR amplification system and reaction conditions for the 548 bp target fragment amplified using primer F3 and B3 comprised $10 \mu\text{L}$ of $2 \times \text{Premix r Taq}$ (YEASE, Shanghai, China), $1 \mu\text{L}$ of primer F3 ($10 \mu\text{mol L}^{-1}$) and B3 ($10 \mu\text{mol L}^{-1}$), $1 \mu\text{L}$ of total DNA template ($100 \text{ ng}/\mu\text{L}$), and deionised water up to $20 \mu\text{L}$. An PCR System (Applied Biosystems, FosterCity, CA) was used for amplification with 1 cycle of 95°C for 5 min, 40 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, 1 cycle of 72°C 5 min. The PCR product was recovered and cloned into the pMD-18T vector according to the manufacturer's instructions (Takara, Japan), recombinants were transformed into *E. coli* DH5 α competent cells (Takara, Japan), monoclonal clones were selected on LB plates containing ampicillin, DNA was extracted from positive clones using a Plasmid Mini Kit (OMEGA, Norcross, GA), and sent to Sangon Biotech (Shanghai, China) for DNA sequencing. Plasmid concentration were measured by UV spectroscopy (Thermo, USA) and the recombinant plasmid copy number was calculated according to the formula: number of copies = (concentration in ng $\times 6.02 \times 10^{23}$) / (genome length $\times 10^9 \times 660$) [29], and the samples were stored at -20°C until use.

Establishment of the basic amplification system and optimisation of reaction conditions

The basic CPA reaction system was established as described previously [30]. Reactions were performed a $25 \mu\text{L}$ total volume in 0.2 mL centrifuge tube, which containing $1 \times \text{Thermo Pol Buffer}$, 0.4 mol L^{-1} of Betaine, 0.8 mmol L^{-1} of dNTPs, 4 mmol L^{-1} of Mg^{2+} , 8 units of Bst DNA polymerase, $0.8 \mu\text{mol L}^{-1}$ of CPR primer, $0.6 \mu\text{mol L}^{-1}$ of F1 primer, $0.6 \mu\text{mol L}^{-1}$ of F2 primer, $0.4 \mu\text{mol L}^{-1}$ of F3 primer, $0.4 \mu\text{mol L}^{-1}$ of B3 primer and $2.0 \mu\text{L}$ of template DNA. The reaction was carried out at 60°C for 60 min. The products were detected by 2% agarose gel electrophoresis in $1 \times \text{TAE}$ with EB staining and the Disposable Nucleic Acid Detection Strip (Ustar, Hangzhou, China) according to the manufacturer's instructions, when CPA amplification was completed. In brief, the 0.2 mL centrifuge tube after the reaction is embedded in the Disposable Nucleic Acid Detection Strip, then press the handle forcefully, and observe the results after 5–10 minutes. Recombinant plasmid standards and ddH_2O were used as positive template and negative controls, respectively. The basic reaction system could then be optimised if applicable. As shown in Table 2, different concentrations of primers were optimised, and each combination included a negative control. The reaction system was used to optimise the concentration of betaine, Mg^{2+} , dNTPs and Bst DNA polymerase by varying each one in turn. Finally, the reaction temperature and time were optimised. All these analyses were repeated three times.

Specificity and Sensitivity of the CPA assay

DNA was extracted from PA, SE, E. coli, MG and DF-1 cells infected with ALV-J, ALV-A, ALV-B, ALV-K and ALV-E using an SQ tissue DNA kit (OMEGA) according to the manufacturer's instructions. The specificity of the CPA method was determined using the above nucleic acids as templates. A 10-fold dilution series of ALV-J recombinant plasmid DNA was prepared using sterile ddH₂O, and different concentrations of plasmid DNA were amplified by the CPA assay, conventional PCR (F3 and B3 as primes, described above), and RT-PCR [31]. RT-PCR was carried out in a 20 µL reaction volume containing 10 µL 2 × iTaq Universal SYBR Green Supermix (BioRad, Hercules, CA), 1.0 µL each primer (Forward: 5'-TGTGTGCGTGGTTATTATTTC-3', Reverse: 5'-AATGGCGAGGTCGCTGA CTGC-3') (10 pmol µL⁻¹), 1 µL plasmid and 7 µL ddH₂O. An ABI 7500 Real-time PCR System (Applied Biosystems, FosterCity, CA) was used for amplification with 1 cycle of 95°C 3 min; 40 cycles of 95°C for 15 s and 60°C for 34 s. Fluorescence signals were collected after each amplification step. All these analyses were repeated three times.

Detection of ALV-J in clinical plasma samples

During the purification of exogenous avian leukosis, according to ELISA, positive ALV p27-positive plasma samples ($S/P > 0.2$) were collected to be screened using the ALV-J CPA method, routine PCR (F3 and B3 as primers), and RT-PCR [31] to measure coincidence rates for the three methods. Additionally, plasma samples with $0.1 \leq S/P \leq 0.2$ according to ELISA analysis of p27 were also tested using the above three methods, and the sensitivity of the three methods was compared. All these analyses were repeated three times.

Results

Calculation of plasmid DNA copy number

The extracted plasmid DNA was assessed by spectrophotometer at a concentration of 66 ng µL⁻¹. The purity was 1.94, the length of the pMD-18T vector was 2692 bp, the length of the insert was 548 bp, hence the total number of bases was 3240 bp, and the concentration was calculated to be 1.86×10^{10} copies µL⁻¹.

Establishment of the basic amplification system

The CPA product was observed under ultraviolet light following electrophoresis. The CPA products gave a ladder-like pattern on the agarose gel, result of their characteristic structure, but the control was negative (Fig. 2a). The results of Disposable Nucleic Acid Detection Strip showed that the positive control containing the recombinant plasmid as template yield red bands on both the control line (C) and the test line (T), while the negative control group only yielded a positive result for the C line (Fig. 2b), which suggests that the basic CPA reaction system could amplify ALV-J effectively.

Optimization of the reaction system and conditions

The optimal primers concentration was primer group 7 (Fig. 3), based on their clearest and brightest strips. Optimal concentrations for Mg^{2+} , betaine, dNTPs and Bst DNA polymerase in the amplification system were 4 mmol L^{-1} for Mg^{2+} (Fig. 4), 0.2 mol L^{-1} for Betaine (Fig. 5), 0.8 mmol L^{-1} for dNTPs (Fig. 6), and 8 U for Bst DNA polymerase (Fig. 7). The reaction system was tested at 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 and 65 °C for 60 min respectively, and the optimal temperature was 60 °C (Fig. 8).

Optimization of the ALV-J CPA assay reaction time

The ALV-J CPA reaction system was tested at 60 °C for 15, 30, 45, 60, 75, 90, 105 and 120 min, and production amplification was most effective at 60 min; when the amplification time was increased beyond this, the efficiency diminished. Therefore, 60 min was optimal reaction time (Fig. 9). The optimal reaction system and conditions for ALV-J CPA amplification are shown in Table 3.

Specificity of the ALV-J CPA assay

Specific experiments were carried out using the optimal reaction system and conditions for ALV-J CPA. The results showed that only recombinant plasmid standards and ALV-J cDNA yielded positive results in both test line and control line, while ALV-A, ALV-B, ALV-K, ALV-E, PA, E.coli, SE and MG yielded only positive results in control line (Fig. 10), indicating that the ALV-J CPA detection method is highly specific.

Evaluation of the Sensitivity of the CPA assay

Sensitivity tests were carried out using the optimal reaction system for CPA. The results showed that when the plasmid DNA concentration was between 1.86×10^1 and 1.86×10^{10} copies μL^{-1} , identification was feasible with nucleic acid test strips. By contrast, only the control line was positive at 1.86×10^{-1} – 1.86 copies μL^{-1} , hence the detection limit of the ALV-J CPA method was 1.86×10^1 copies. The results of conventional PCR amplification showed that the target band was amplified at 1.86×10^2 – 1.18×10^{10} copies μL^{-1} , equating to a detection limit of 1.86×10^2 copies (Fig. 11). Thus, the sensitivity of ALV-J CPA was up to 10 times higher than that of conventional PCR.

Detection of ALV-J in clinical plasma samples

A total of 48 ALV p27-positive plasma samples ($\text{S/P} > 0.2$) and 75 plasma samples with $0.1 \leq \text{S/P} \leq 0.2$ based on ELISA were tested for ALV-J using the CPA method, conventional PCR, and RT-PCR. The results showed that, among the 48 samples with $\text{S/P} > 0.2$, the positive rates for CPA, real-time PCR and PCR were 54.2% (26/48), 54.2% (26/48) and 52.1% (25/48), respectively. The results for CPA and RT-PCR were consistent, and the coincidence rate with the PCR method was 96.2% (25/26). Detailed results are shown in Table 4. Among the 75 plasma samples with $0.1 \leq \text{S/P} \leq 0.2$, the positive rates for CPA, RT-PCR and PCR were 60.0% (45/75), 62.6% (47/75) and 38.7% (29/75), respectively. Detailed results are shown in Table 5. The sensitivity of the ALV-J CPA assay was higher than that of conventional PCR, which was comparable with RT-PCR. The coincidence rate between CPA and RT-PCR was 97.3% (71/73) across all 123 plasma samples.

Discussion

ALV-J infection currently causes great economic losses in China [17], and its presence is associated with a variety of tumour types including myeloma, renal tumours and hemangioma [19, 32]. The high incidence of tumours reduces production, and virus elimination increases production costs. In order to meet the need for rapid diagnosis, it is necessary to develop a simple, rapid and sensitive diagnostic method for detecting ALV-J. The isolation and identification of viruses by cell culture is expensive and time-consuming. Conventional PCR and real-time PCR is an established method for detecting ALV-J [31], but it requires high-precision instruments, skilled operators, and the amplification time is 3 to 4 h. Additionally, it is difficult to adapt this method for rapid detection of ALV-J in the field. ELISA is a method that can potentially solve this problem since it can determine whether a sample contains ALV p27 antigen, but it cannot be used for the identification of ALV subgroups, nor can it distinguish exogenous from endogenous ALV.

In recent years, researchers have paid increasing attention to new detection methods based on molecular biology. Compared with traditional PCR, pathogen separation and similar methods, CPA is a new gene amplification approach with many advantages. One of the most important features of CPA is its high amplification efficiency and sensitivity. Even a small number of templates can produce a large number of amplification products [30]. The application of CPA technology not only overcomes the dependence on PCR and RT-PCR instruments, but also affords an operational procedure that is simple and accurate, and interpretation of results is more objective, convenient and intuitive than the case for loop-mediated isothermal amplification (LAMP), since it avoids subjectivity [22]. CPA technology facilitates molecular biology-based detection by non-specialist laboratories and economically underdeveloped areas, which is of great significance for the scientific research of nucleic acids and viral diseases [33].

In the process of developing a successful CPA assay, we found that primer design was crucial [25]. For accurate detection, the primers play a vital role in ensuring specificity and sensitivity in this assay and their design is one of the difficulties of CPA. Because the CPA assay makes use of five primers to recognize five distinct regions on the target DNA sequence, which makes the CPA assay more specific than conventional PCR using two primers [26]. The presence of multiple primers in the same tube can result in non-specific amplification leading to false positives and misleading detection results [34]. Before determining the final ALV-J CPA primers, we had made a lot of preliminary experiments to screen multiple primer sets and eliminated those giving non-specific amplification products.

In the present study, a rapid CPA detection method for ALV-J was established. Optimal reaction conditions were 60 °C for 60 min, and the sensitivity was 10 times that of conventional PCR. The assay proved negative for other ALV subgroups, and other virus, and bacteria. ALV p27-positive plasma samples ($S/P > 0.2$) were tested using the CPA method, and the results were consistent with those of RT-PCR; the coincidence rate with PCR was 96.2%. When ALV-J CPA was used to detect a non-positive plasma sample with $0.1 \leq S/P \leq 0.2$, the method exhibited the same excellent sensitivity as RT-PCR, and the detection rate was higher than that of conventional PCR. Statistical analysis of CPA and RT-PCR detection rates for

all 123 clinical plasma samples showed that the coincidence rate of the two methods was 97.3% (71/73), indicating that the CPA detection method for ALV-J established in this study can be applied for rapid on-site diagnosis of ALV-J and screening of samples.

Conclusions

In summary, the established ALV-J CPA method has significant advantages for the detection of ALV-J. It is convenient to execute, exhibits strong specificity, high sensitivity, and does not require complicated experimental equipment, making it suitable for analysis outdoors and in primary medical units. It is an ideal system for rapid detection of ALV-J with great potential for monitoring this viral disease.

Abbreviations

AL: Avian Leukosis; ALV: Avian leukosis virus; ALV-J: Avian leukosis virus subgroup J; CPA: Cross-priming amplification; ELISA: enzyme-linked immunosorbent assay; PCR: Polymerase Chain Reaction; RT-PCR: real-time PCR; LAMP: loop-mediated isothermal amplification.

Declarations

Ethics approval and consent to participate

The plasma samples were from yellow chickens of Guangdong province in China during an ALV epidemiological investigation conducted by our laboratory. And all the chicken plasma sampling procedures were approved by the Animal Care and Use Committee of Guangdong Province, China (2018B110). Our sampling processes were assisted by local authorities and veterinarians.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

WC and YX conceived and designed the study. YX performed the experiments. YX, LL and PL analyzed the data. YX and WC wrote the paper. All authors read and approved the final manuscript.

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Tables

Table 1. Primer and probe sequences

Primer	Sequence[5'-3']
F3	AGGATGAGGTGACTAAGAAA
B3	ACCGAACCAAAGGTAACACACG
B2	CAAGCAGGTGCGGAATGG
F2	Biotin-GGAAAGGTGAGCAAGAAGG
F1	FAM-CGCCAGCAACAAGCAAGAAA
CPR	GGAAAGGTGAGCAAGAAGGCAAGCAGGTGCGGAATGG

Table 2. Different primer concentration combinations

Primer groups	Working concentration ($\mu\text{mol}\cdot\text{L}^{-1}$)				
	F3	B3	F2	F1	CPR
1	0.4	0.4	0.6	0.6	0.8
2	0.4	0.4	0.8	0.8	1.0
3	0.4	0.4	0.6	0.6	1.0
4	0.4	0.4	0.6	0.6	1.2
5	0.2	0.2	0.8	0.8	1.2
6	0.2	0.2	0.4	0.4	0.6
7	0.2	0.2	0.8	0.8	1.0
8	0.2	0.2	0.6	0.6	1.0
9	0.6	0.6	0.8	0.8	1.0
10	0.6	0.6	1.0	1.0	1.2
11	0.6	0.6	0.8	0.8	1.2

Table 3. Optimal ALV-J CPA reaction system and conditions

Component	Volume (μL)	Working concentration
10 $\times$ ThermoPol Buffer	2.5	1 $\times$
5 mol L ⁻¹ Betaine	1.0	0.2 mol L ⁻¹
10 mmol L ⁻¹ dNTPs	1.5	0.6 mmol L ⁻¹
100 mmol L ⁻¹ Mg ²⁺	1.0	4.0 mmol L ⁻¹
8 U μL^{-1} <i>Bst</i> DNA polymerase	1.0	0.32 U μL^{-1}
10 $\mu\text{mol L}^{-1}$ CPR	2.5	1.0 $\mu\text{mol L}^{-1}$
10 $\mu\text{mol L}^{-1}$ F1	2.0	0.8 $\mu\text{mol L}^{-1}$
10 $\mu\text{mol L}^{-1}$ F2	2.0	0.8 $\mu\text{mol L}^{-1}$
10 $\mu\text{mol L}^{-1}$ B3	0.5	0.2 $\mu\text{mol L}^{-1}$
10 $\mu\text{mol L}^{-1}$ F3	0.5	0.2 $\mu\text{mol L}^{-1}$
DNA template	1.0	/
ddH ₂ O	Add to 25 μL	/
Amplification for 60 min at 60°C		

Table 4. Detection results for plasma samples with S/P >0.2

No.		RT-PCR		PCR		Total
Detected		Positive	Negative	Positive	Negative	
CPA	Positive	26	0	25	1	26
	Negative	0	22	0	22	22
Total		26	22	25	23	48

Table 5. Detection results for plasma samples with $0.1 \leq \text{S/P} \leq 0.2$

No.		RT-PCR		PCR		Total
Detected		Positive	Negative	Positive	Negative	
CPA	Positive	45	0	29	16	45
	Negative	2	28	0	30	30
Total		47	28	29	46	75

Figures

Figure 1

The specific positions and sequence of CPA primers and probes in the genome

Figure 2

The basic reaction system of ALV-J CPA. (a) Analysis of the CPA method by agarose gel electrophoresis. Lane M, DS2000 DNA markers; P, recombinant plasmid standards; N, negative control. (b) Analysis by the CPA method with nucleic acid test strips. Lane P, recombinant plasmid standards; N, negative control.

Figure 3

Analysis of ALV-J CPA at different concentration of primers (Table 2) by agarose gel electrophoresis. Lane M, DS2000 DNA markers; 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, were primers groups 1-11 (Table 2) respectively; 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22 were negative controls for the corresponding primers groups.

Figure 4

Analysis of ALV-J CPA at different concentration of Mg²⁺ by agarose gel electrophoresis. Lane M, DS2000 DNA markers; 1: 0 mmol L⁻¹; 2: 1 mmol L⁻¹; 3: 2 mmol L⁻¹; 4: 3 mmol L⁻¹; 5: 4 mmol L⁻¹; 6: 5 mmol L⁻¹.

Figure 5

Analysis of ALV-J CPA at different concentration of Betaine by agarose gel electrophoresis. Lane M, DS2000 DNA markers; 1: 0 mol L⁻¹; 2: 0.2 mol L⁻¹; 3: 0.4 mol L⁻¹; 4: 0.6 mol L⁻¹; 5: 0.8 mol L⁻¹; 6: 1.0 mol L⁻¹; 7: 1.2 mol L⁻¹; 8: 1.4 mol L⁻¹.

Figure 6

Analysis of ALV-J CPA at different concentration of dNTPs by agarose gel electrophoresis. Lane M, DS2000 DNA markers; 1: 0 mmol L⁻¹; 2: 0.2 mmol L⁻¹; 3: 0.4 mmol L⁻¹; 4: 0.6 mmol L⁻¹; 5: 0.8 mmol L⁻¹; 6: 1.0 mmol L⁻¹; 7: 1.2 mmol L⁻¹; 8: 1.4 mmol L⁻¹.

Figure 7

Analysis of ALV-J CPA at different units of Bst DNA polymerase (8 units μL^{-1}) by agarose gel electrophoresis. Lane M, DS2000 DNA markers; 1: 0 μL ; 2: 0.2 μL ; 3: 0.4 μL ; 4: 0.6 μL ; 5: 0.8 μL ; 6: 1.0 μL ; 7: 1.5 μL ; 8: 2.0 μL .

Figure 8

Analysis of ALV-J CPA at different temperatures by agarose gel electrophoresis. Lane M, DS2000 DNA markers; 1, 54°C; 3, 55°C; 5, 56°C; 7, 57°C; 9, 58°C; 11, 59°C; 13, 60°C; 15, 61°C; 17, 62°C; 19, 63°C; 21, 64°C;

23, 65°C; 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 were negative controls for the corresponding temperatures.

Figure 9

Analysis of ALV-J CPA with different reaction times by agarose gel electrophoresis. Lane M, DS2000 DNA markers; 1, 15 min; 2, 30 min; 3, 45 min; 4, 60 min; 5, 75 min; 6, 90 min; 7, 105 min; 8, 120 min; 9, Negative control amplification for 120 min.

Figure 10

Analysis of the specificity of the ALV-J CPA method.

Figure 11

Comparison of sensitivity between the CPA, PCR and RT-PCR of ALV-J detect methods based on recombinant plasmid standards at different copy numbers. (a) Sensitivity of CPA based on a nucleic acid detection device. N, negative control. (b) Sensitivity of conventional PCR. N, negative control. (c) Sensitivity of RT-PCR for ALV-J. N, negative control.