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Original Article

Development of a Multiplex Polymerase Chain Reaction for Differential Diagnosis of Canary Pox Virus

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Abstract

Background and Aims: A multiplex transcription-polymerase chain reaction (m-PCR) was developed for direct detection and discrimination between canarypox virus (CPV) and other avian poxvirus (APV).

Materials and Methods: Three compatible primer sets were designed for m-PCR amplification of different loci; *fpv*126, *fpv*140, and *fpv*167 located at highly conserved APV genes.

Results: Results showed that m-PCR products of the expected sizes were obtained for all of the primer sets when they were tested either alone or in combination with an artificial mixture of positive controls. Based on the better amplification of *fpv*167 than other loci, the locus primer set was used to examine tissue samples from canaries clinically diagnosed as AVP-infected.

Conclusion: All canary samples were positive for CPV by the m-PCR and virus isolation. The results of the present study indicate the m-PCR assay holds potential to be versatile, rapid, and sensitive for detection of CPV and differentiation of the virus from the other APVs.

Keywords: Canary pox virus; Fragment-Length Polymorphism; Diagnosis

Introduction

Avian poxviruses (APVs), the large DNA viruses, which are found worldwide in many species of birds (1), are members of the genus Avipoxvirus subfamily Chordopoxvirinae falling into three major groups, representing fowlpox virus (FPV), canarypox virus (CPV), and psittacine poxviruses (2). Variable restriction enzyme profiles suggest significant genomic differences among avipox viruses (3-5). Canarypox virus (CPV) is an etiologic agent of canarypox which produces clinical signs including both cutaneous and diptheretic disease forms caused by the prototypical FPV and includes proliferative and necrotic changes in epithelial tissues of the dermis, notably around the eyes and commissures of the beak, feet, and respiratory tract (6-8). The first analysis of the complete genome of CPV was presented by Tulman et al. (9). CPV is most closely related to FPV, having overall synthetic genomic arrangement and similar gene complements. The linear 365-kbp CPV genome contains 328 potential genes in a central region and in 6.5-kbp inverted terminal repeats which is involved in virus-host interaction, viral virulence, and viral host range. These are expressed in three phases early, intermediate and late. Comparison of

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FPV and CPV genome sequences reveals a high level of divergence with significant differences between orthologous open reading frame (ORF) and the terminal, variable genomic regions. CPV lacks homologues of fifteen genes present in terminal genomic regions of FPV (9, 10). Phylogenetic analysis of Chordopoxviruses genomes has shown that the central region is highly conserved in gene content and arrangement, except for some gene inversions in FPV, while the termini region is the most divergent for FPV (11). The divergent may apply for differential diagnosis of AVPs by use of specific primers in PCR reaction. Some loci in the highly conserved genes have been investigated for discrimination between major clades in AVPs. The *fpv*126 locus of VLTF-1 gene encodes a late transcription factor, *fpv*167 locus of 4b gene encodes core protein, and *fpv*140 locus of H3L gene another envelope protein encodes an immunodominant antigen are interested (12). In this study we designed the multiplex polymerase chain reaction (m-PCR) in combination of fragment-length polymorphism for differential diagnosis of CPV in clinical samples.

Methods

Virus isolation and DNA extraction

The infected skin lesions including eyelids and toes from canaries, combs and wattles of fowls were screened for the presence of pox viruses. The tissues were removed and mixed with buffered saline containing antibiotics and clarified by centrifugation at 4000 RPM for 10 min. The supernatant was passed through a 0.45 nm membrane filter. 0.2 ml of each treated tissue suspension of lesions was inoculated on to the chorioallantoic membranes (CAMs) of 10–11-day-old chicken embryo eggs. These were incubated at 37°C for 5–7 days, and then examined for pock lesions on the CAMs. Furthermore, prepared tissue lesions from affected birds were used directly for DNA extraction.

Histopathology

Collected samples from twelve canaries and seven fowls with suspected pox lesions were examined for histopathology. The samples were fixed in 10% buffered neutral formalin, embedded in paraffin, sectioned at 5 micron, stained with haematoxylin, eosin and examined by bright-field microscopy.

Multiplex PCR amplification

Viral DNA was extracted using DNA Extraction Kit (Roche, Germany) according to the manufacturer's instruction. Based on multiple alignments of P4b, VLTF-1, and H3H genes of pox viruses deposited in GenBank, specific primers were designed for detection and discrimination of CPV from AVPs. The m-PCR was optimized using three primer sets (Table 1). The latter primer set was published by Lüschow *et al* (14). The PCR reaction in a 50µl volume contained 5 U of Taq DNA, 6µl of 20mM Mg2+, and 3µl of 2.5mM of each dNTP, appropriate concentrations of template cDNA, and 1µl of 10pM primer mixture. Each PCR product was amplified by the following conditions: denaturation step for 5 min at 94C, 30 cycles of denaturation at 94C for 30 s, annealing at 57C for 45s, and extension at 72C for 60s, followed by a final extension step at 72°C for 10min. The amplified products were analyzed by 1.0% agarose gel electrophoresis. The multiplex RT-PCR assays were also tested for specificity using positive and negative controls.

Results

Clinical signs of avian pox found in affected canaries and fowls. Typical appearance of the lesions of APVs including several pocks in CAM was observed following inoculation of the tissue suspensions (Figure 1).

As shown in figure 2 histopathological sections of the CAM of infected sample with APVs appeared cytoplasmic inclusion body (bolinger body) with balloning and membrane hyperplasia.

M-PCR assay

The results for detection of CPV and FPV from field cases with amplification via PCR with AP4b primer were in complete agreement with those for virus isolation in chicken embryonated eggs. Further identification and differentiation of CPV were carried out by the use of m-PCR analysis using AP4b and each of

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Table 1. The primer sets sequences used for development of multiplex transcription-polymerase chain reaction.

	Forward primer	Reverse primer	Size
CP167	5′-CTATACGCCCCCGTAACAGA-3′	5′-AAGCGCTTGGTATCTAGAAGG-3′	160 bp
CP140	5′-TCGGCAGTTTCTTTCTACCG-3′	5′-GTGACCCGATTCGAAATACC-3′	223 bp
CP126	5′-AAAGGTTACCTCTCTTTAGGTG-3′	5′-CCGTTACCATAATCAACGCTTA-3′	222 bp
AP4b	5′-CAGCAGGTGCTAAACAACAA-3′	5′-CGGTAGCTTAACGCCGAATA-3′	578 bp

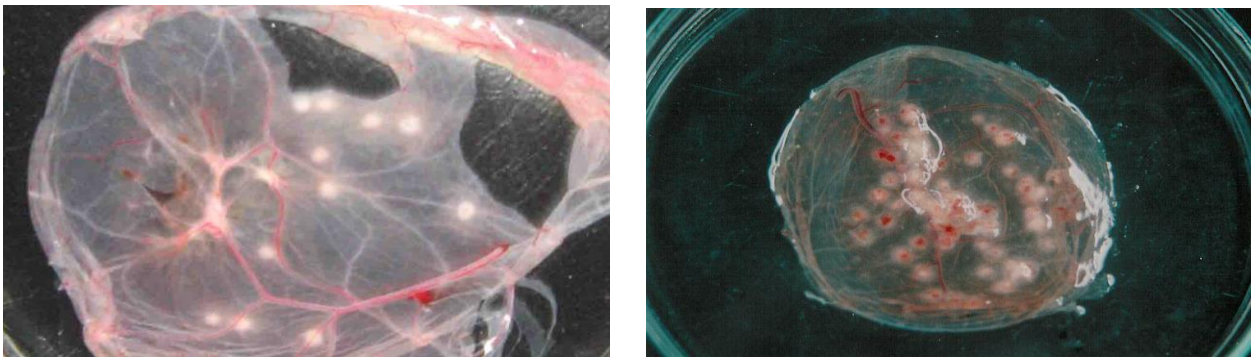

Fig. 1. Dispersion and typical appearance of of pock lesions throughout the CAM; left, CPV and right, FPV.

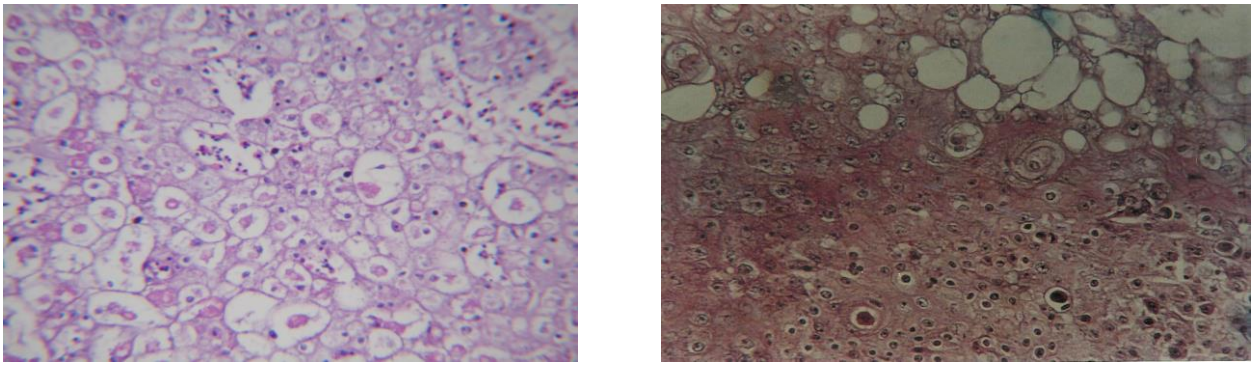

Fig. 2. Histopathological staining of infected samples; left, CPV and right, FPV (400 X).

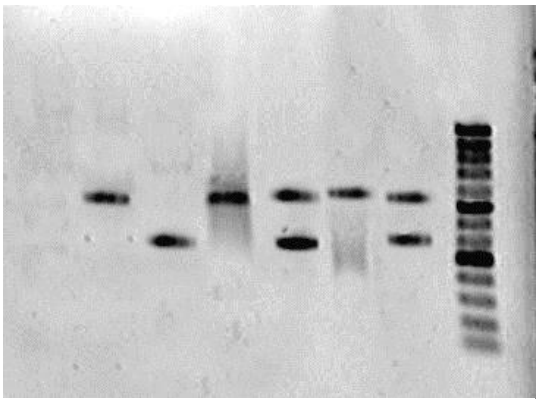

Fig. 3. Gel electrophoresis of m-PCR reaction. M: DNA marker; lane 1: APV positive control; lane 2: CPV positive control; lanes 3 and 5: APV detection in clinical samples; lanes 4 and 6: CPV detection in clinical samples.

CPV specific loci primer sets. The expected product sizes were obtained for positive control CPV isolate when they were tested either alone or in combination an artificial mixture with no interference. However, the amplification was relatively poor for *fpv*126 and *fpv*140 compared with the *fpv*167. Then we tested the field isolates with the designed m-PCR, which framed a region within the APV 4b core protein gene and also *fpv*167 locus of the gene. All of the canary tissue samples were positive for CAV by the m-PCR (Figure 3).

Discussion

Conventional diagnosis of APVs is applied by virus isolation, serological and molecular

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methods (15, 16). To date, most of the identification methods, concerning the APVs are based on PCR and restriction enzyme analysis of 4b gene of pox viruses, a highly conserved in all of Chordopoxvirinae. Differentiation of some APVs has been carried out by using *Eco*RV and *Nla*II restriction enzyme profiles on the amplified 4b gene fragment (4). In other study Luschow et al (14) have revealed that restriction digestion of a region between the gene with either *Mse*I or *Eco*RV enzymes resulted in differentiation of APVs that showed a nucleotide similarity of 72-100%. Despite applicability of the method, it was not able to differentiate CPV and FPV that share less identity. The genomes of APVs are extremely conserved (2, 12, 17) and require a technology that can offer flexibility in primers design to enable high level of specificity for differentiation. Thus having fragment-length polymorphism that could be detected directly by PCR is required. By alignment of the complete genome sequences of CPV and FPV we found that CPV showed a distinctive feature at three loci which had a less nucleotide identity to each other. According to the similarity and length of the nucleotide sequences, we designed primer sets for differentiation of CPV from other APVs without further restriction enzyme digestion. Initially, we choose primer sets for each *fpv*126, *fpv*140, and *fpv*167 loci and a specific primer for all of the APVs with known reference viruses then optimized the primers mixture based on the criteria of amplification efficiency, specificity, and the ability to distinguish products by size. Our results indicate that the designed primers for *fpv*167 locus and 578 bp fragment of 4b gene allows easy distinction between CPV and FPV based on the size of the PCR products. According to the polygenetic studies three major clades consist of the FPV-like group of viruses (clade A), the CPV-like group of viruses (clade B), and the Psittacine viruses (clade C) have been formed (2, 12). The *fpv* 167 locus differentiates AVPs to clade A as opposed to clade B or clade C with strong support by neighbor-joining bootstrap, parsimony and maximum likelihood. The analysis for *fpv*126 and *fpv*140 is the same and represent clades A and B and fail with clade C viruses. In this context, the *fpv*167 locus PCR product can be used as a differential marker of CPVs. Full-length of the locus has been amplified and subjected to restriction digestion (9) but this approach is slow to perform and may change by a nucleotide substitution in the restriction profile. Therefore, we selected partial conserved sequence of *fpv*167 locus and developed a m-PCR assay that could determine the presence of APV genome and specifically identify CPV in clinical samples. This method saves time and reagent costs compared with enzymatic digestions of PCR product which needs several reactions for the same number of tests.

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