Polymerase chain reaction for the detection and differentiation of Marek's disease virus strains MDV-1 and HVT

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Summery

Marek's disease (MD) is a lymphoproliferative disease of chickens characterized by lymphocytic infiltration of various organs. The present study was an attempt to use polymerase chain reaction (PCR) to optimize a rapid and reliable assay for detection of MDV genome. Detection of serotype 1 of MDV (MDV-1) was confirmed by presence of a 200 bp DNA fragment as a PCR product. Differentiation of MDV-1 and herpesvirus of turkeys (HVT) was also conducted using specific primers from the glycoprotein A (gA) gene and a 388 bp DNA fragment was amplified from HVT genome. The specificity of the test was confirmed by sequencing of PCR products. Results indicate that MDV-1 can be diagnosed in clinical samples and inoculated cell cultures which is used for virus isolation. In addition, differentiation between MDV-1 and HVT viruses was confirmed based on the size of PCR products. The test proved to be rapid and reliable and can be performed as a robust diagnostic test in veterinary diagnostic laboratories.

Key words: Marek's disease virus, PCR, Detection

Introduction

Marek's disease virus (MDV) is a herpesvirus, which induces malignant lymphomas in its natural host, chickens, within a few weeks after infection (Bulow and Biggs, 1975b; Calnek and Witter, 1997). Currently, MD has been successfully controlled by vaccination and all three serotypes of MDV including an attenuated strain of serotype 1 (MDV-1), CVI988 (Rispens *et al*., 1972), have been used as vaccines. MDV belongs to a group of strongly cell-associated avian herpesviruses that have been subdivided into three serotypes. Serotype 1 viruses are pathogenic chicken viruses and their cell culture attenuated variants. Serotype 2 viruses are the naturally occurring non-pathogenic chicken viruses. The non-pathogenic herpesvirus of turkeys or HVTs are also designated as serotype 3 viruses (Bulow and Biggs, 1975a, b). It is reported that the direct detection of MDV in the peripheral blood or tissue samples is not simple (Silva, 1992). Normally peripheral blood lymphocytes are seeded on susceptible cells and the cultures are incubated until viral plaques appear. At this point, an immunofluorescent assay (Lee *et al*., 1983) or enzyme linked immunosorbent assay (Davidson *et al*., 1988) can be used to identify and differentiate between the three MDV serotypes. Once viral plaques appeared, it is also possible to extract the viral DNA and differentiate the MDV serotypes on the basis of their restriction endonuclease patterns (Ross *et al*., 1983; Silva and Barnett, 1991). Unfortunately, these procedures require the inoculation of susceptible cells in cell culture in order to amplify the DNA sequences. The present study describes a simple PCR procedure to detect MDV from cell culture or directly from clinical samples and differentiation of MDV from HVT.

Materials and Methods

Viruses

Vaccine strains of CVI988 (serotype 1-MDV-1) and non-pathogenic turkeys HVT FC126 (serotype 3-MDV-3) were obtained from commercial vaccine vials (Lohman Animal Health GmbH, Germany). Field isolates of MDV-1 were obtained from Razi Vaccine and Serum Research Institute. These viruses were propagated in chicken embryo fibroblasts (CEF), harvested when cytopathic effects were confluent and used for the extraction of total cellular DNA. Clinical samples (nerve tissue) from suspected diseased broiler chickens were also used for DNA extraction.

Extraction of total DNA

Inoculated CEF cell culture with MDV field isolates, homogenates of nerve tissue samples and Marek vaccine strains were used separately for DNA extraction using a DNA extraction solution. Briefly, 900 μl of extraction solution was added to 200 μl of each sample and vortexed. 400 μl chloroform was added and the sample was gently inverted five to six times and incubated at room temperature for 3 min. After 10 min centrifugation (10,000 Xg) the upper phase was transferred to a new tube and equal volume of cold propanolol was added then incubated on ice for 15 min. Total DNA was precipitated (12,000 Xg) and washed with 70% ethanol. The resultant DNA was semi-dried, dissolved in dH_2O and stored at -20ºC until used.

Polymerase chain reaction (PCR)

Total extracted DNAs were used for PCR to amplify a part of glycoprotein A (gA) gene. The specific oligonucleotide primers were previously published (Zhu *et al*., 1992). The sequence and location of primers are showed in Table 1.

PCR mixture (50 μl) contained 1.5 mM $MgCl_2$, 10X PCR buffer (5 μl), 35 pmol of each primer, 2 mM of dNTPs and 0.5 U of Taq DNA polymerase (Roche, Germany). Template DNA was added to the reaction mixture at a concentration of 0.5-2 μg. The amplification reaction was performed in a DNA thermal cycler (TECHNE, UK). Temperature cycling for PCR consisted of 94ºC for 3 min (one cycle), 94ºC for 30 sec, 55ºC for 30 sec and 72ºC for 30 sec, repeated for 30 cycles, followed by 72ºC for 10 min. 7 μl of the amplified product was electrophoresed in 1% agarose gel at 80 volts for 45 min using tris-borate-EDTA buffer. The DNA bands were visualized by UV transluminator after ethidium bromide staining.

Sequencing of PCR products

Amplified products were purified using a High Pure PCR Product Purification Kit (Roche, Germany) and each PCR product was sequenced using its specific primers.

Results

DNA extraction

Presence of a single DNA with high molecular weight of all tested samples was observed in the agarose gel (Fig. 1). Amplification of the MDV-1 gA gene sequence of non-pathogenic serotype MDV-1 vaccine virus (CVI988-Rispens vaccine) gave a positive result of DNA amplification with the expected size of 200 bp, using MDV-gA-1 primers. Similar results were obtained when DNA from the cell cultures inoculated with field isolates and clinical samples were used in PCR using the same set of primer. No amplified product was observed when DNA of HVT or negative

Table 1. The sequence of oligonucleotide primers

Primer	Sequence	Location of primer
MDV-gA-1 primer-F	CATGCAAGTCATTATGCGTGAC	696-717
MDV-gA-1 primer-R	TGTTTCCATTCTGTCTCCAAGA	895-874
HVT-gA-3 primer-F	CGCGTACTGCGCCTGACG	231-248
HVT-gA-3 primer-R	CAACTTCGCTCTTGACG	618-602

Fig. 1: Total DNA extracted from different samples in 1% agarose gel. Lane 1: molecular weight DNA marker (Ladder 100); Lane 2: MDV-1 vaccine strain (CVI988); Lane 3: CEF cell culture inoculated with MDV field isolate; Lane 4: nerve tissue sample from diseased bird and Lane 5: HVT vaccine strain (FC126)

Fig. 2: PCR test with primers from gA gene specific for MDV-1 virus DNA which allow the specific detection of MDV-1. The PCR products (200 bp) were electrophoresed in a 1% agarose gel and stained with ethidium bromide. Lane 1 and 7: molecular weight DNA marker (Ladder 100); Lane 2: MDV-1 vaccine strain (CVI988); Lane 3: CEF cell culture inoculated with MDV field isolate; Lane 4: nerve tissue sample from diseased bird; Lane 5: HVT vaccine strain (FC126) and Lane 6: uninoculated CEF cell culture (negative control)

Fig. 3: PCR test with primers from gA gene specific for HVT virus DNA which allow the specific detection of HVT. The PCR products (388 bp) were electrophoresed in a 1% agarose gel and stained with ethidium bromide. Lane 1 and 7: molecular weight DNA marker (Ladder 100); Lane 2: HVT vaccine strain (FC126); Lane 3: MDV-1 vaccine strain (CVI988); Lane 4: CEF cell culture inoculated with MDV field isolate; Lane 5: nerve tissue sample from diseased bird and Lane 6: uninoculated CEF cell culture (negative control)

control (uninoculated CEF cell culture) were examined in PCR (Fig. 2). However, when HVT samples and HVT-gA-3 primers were used in PCR, a 388 bp PCR product was amplified while no amplified product was observed from MDV-1 viruses (CVI988 vaccine strain), CEF cultured field isolates and tissue samples. In addition, the negative control (uninoculated CEF cell culture) was remained negative (Fig. 3).

Sequencing of PCR products

The sequence of 200 bp PCR product is showed in Fig. 4. Specificity of the PCR products were confirmed when high homology (99%) of sequenced data with gA gene of MDV type 1 (Accession number AY129979) was observed.

Discussion

MDV has been subdivided into three serotypes: serotype 1 (MDV-1), containing oncogenic strains of MDV and attenuated or

TGTTTCCATTCTGTCTCCAAGATACATAGACAGATCCAGGGGGATAAAAGTGTCTACC

GATACAAGATGCCTTGTAATTTTCTCCGCTGAGGACTGGAGGGGCCAGTACATCCACT
GATGCAGGTCGTTGACACACACGTATGTAAATATGTTTATCTATTAAAGGCCGATTAA
AATGGTCACGCATAATGACTTGCATG

Fig. 4: The nucleotide sequence of PCR product

non-oncogenic variants derived from them, serotype 2 (MDV-2), naturally occurring non-pathogenic strains and serotype 3 (MDV-3), HVT (Zhu *et al*., 1992). The gA or A antigen of MDV-related viruses is immunologically cross reactive among three serotypes of MDV (Rispens *et al*., 1972; Hirai *et al*., 1986). The genes encoding the gAs of MDV-1 and HVT are located at the same positions on these genomes with strong homology between these viruses (Fukuchi *et al*., 1985; Isfort *et al*., 1987; Binns and Ross, 1989; Ihara *et al*., 1989; Kato *et al*., 1989). The nucleotide sequence homology between the gA genes of MDV-1 and HVT was showed to be 73% (Kato *et al*., 1989). Differentiation of oncogenic and non-oncogenic MDV-1 viruses based on genetic differences has been attempted due to its importance in controlling the disease. Structural changes in the MDV genome during attenuation of MDV have been reported including amplification of the 132 bp direct repeat in the BamHI-H (Maotani *et al*., 1986), 200 bp deletion in the BamHI-L (Wilson and Coussens, 1991), 400 bp deletion in the BamHI-A regions (Hooft *et al*., 1999) and insertion of 178 bp sequence in the meq gene (Lee *et al*., 1999). However, the causative relationship between those changes and loss of oncogenicity of MDV-1 is still unknown. One of the early reports on differentiation of pathogenic and non-pathogenic MDVs was based on amplification of tandem direct repeats of 132 bp sequence within MDV genome (Silva, 1992). However, experimental results were not consistent with known MDV-1 samples when examined in our hand. The published primers used in this study (Zhu *et al*., 1992) were specific for MDV and could be used for detection of MD infection in suspected birds. In order to differentiate between MDV-1 and HVT, specific primers were used (Table 1). The first primer pair (MDV-gA-1) allowed amplification of the 200 bp DNA sequence that codes for a portion of MDV-1-gA (Ihara *et al*., 1989). The second primer pair (HVT-gA-3) allowed amplification of the 388 bp sequence that codes for a portion of HVT-gA (Kato *et al*., 1989). Using the first set of primers revealed that all cell cultured field isolates and tissue samples which contained MDV, belonged to MDV-1 serotype. Since very virulent MDVs could be isolated from vaccinated chickens (Powell and Lombardini, 1986; McKimm-Breschkin *et al*., 1990; De Laney *et al*., 1995), this assay can be used for confirmation of very virulent MDV in diseased birds based on clinical signs. The PCR amplification of MDV sequence is a direct detection assay and is therefore similar to isolating virus in CEF cell culture (Silva, 1992). However, PCR is extremely simple to perform and results can be obtained in less than one day. Unlike virus isolation that depend on obtaining viable viruses, samples for PCR analysis do not have to be frozen or protected from inactivation. In addition, PCR is the only rapid and sensitive test to detect the presence of MDV in the sample. In the present study, a diagnostic PCR technique that allows detection of MDV-1 in cell culture, clinical specimens from infected commercial chickens and differentiation from HVT is described. Consistent results were obtained when repeated experiments with different samples were conducted. The PCR allows the detection of MDVs from clinical materials including tissue samples of diseased chickens and does not require in vitro isolation of the virus in CEF cultures.

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