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RESEARCH ARTICLE

Standardization and Comparison of Loop mediated isothermal amplification with conventional PCR on MD positive archived samples

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Abstract

Marek's disease is a Lymphoprliferative disease of poultry caused by alphaherpesvirus. Despite the ubiquitous use of vaccination to control losses, MD still affects a poultry industry with an estimated annual loss up to US $2 billion. Therefore, it is essential to better the ability of detection of MD virus during the early stages of an outbreak. The aim of the study was to develop a loop-mediated isothermal amplification (LAMP) method for the simple, rapid and inexpensive detection of MDV from the tissues of MD positive birds and to compare with diagnostic efficacy of conventional PCR. The LAMP was established using a set of three pair of primers specific to MEQ gene for detecting serotype 1 MDV. The amplification was able to finish in 60 min under isothermal condition at 65°C by addition of *Bst* polymerase. The results were visualized directly under daylight or ultraviolet light by addition of SYBR Green I dye. The Assay demonstrate a significant advantage of loop-mediated isothermal amplification in diagnosis of Marek's disease compared to conventional PCR as it can be carried out in field condition where rapid diagnosis is required.

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Introduction

Marek's Disease (MD), recently classified as OIE list B disease, is a lymphoproliferative disease of chickens that virtually exists in all commercial chicken flocks (Calnek, 2001). MD is caused by Marek's disease virus (MDV), a cell-associated virus belonging to the family Herpesviridae, in the genus Mardivirus. . The disease is characterized by number of conditions such as lymphomas of visceral organs (ovaries, liver, spleen and kidneys), unilateral or bilateral enlargement of nerves particularly sciatic nerve and brachial nerve due to infiltration of lymphoblasts; graying of iris due to lymphoblastoid cell infiltration (ocular form) and cutaneous form characterized by nodular lesions at the base of feather follicles. Generally, young birds at any age are susceptible but in most cases susceptibility is seen at four weeks of age. Clinical signs appear at about 2-5 months of age in sexually immature birds. But the disease has also been reported in adult birds Ahmed (1982).

MDV strains (MDVs) have been classified into three serotypes that have major differences not only in the genome but also in the biological features. Serotype 1 MDVs includes all the oncogenic strains and their attenuated forms; serotype 2 are non-oncogenic viruses isolated in chickens; and serotype 3 are non-oncogenic virus isolated in turkeys, generally known as herpesvirus of turkeys or HVT (Bulow et al., 1975a), (Bulow et al., 1975b). Among those three serotypes, only serotype 1 are oncogenic, which can be further classified into four pathotypes including mild (mMDV), virulent (vMDV), very virulent (vvMDV), and very virulent plus (vv+MDV) (Witter, 1997). The MD virus initially replicates in a cell-associated form in the lymphoid tissues. Then, from 10 days post infection

onwards, high levels of MDV antigens are expressed, and the cell-free virus is shed with the skin and feather debris throughout the life of an infected bird which is the source of infection for other chickens, via a respiratory route (Baigent and Davison, 2004). Several genes unique for MDVs have been identified. Among these genes, the MEQ gene is specific to serotype 1 MDVs. The vaccination can prevent formation of tumor but generation of infectious virus is not prevented. However, MD is still a problem throughout the world. Several PCR and real-time-based techniques have been developed for detection, as well as quantification, of the MDV genome of field and vaccine strains (Becker et al., 1992), (Handberg et al., 2001), (Silva, 1992), Zhu et al., 1992). These techniques use blood, organ samples, and feather tips for detection and quantification. However, PCR requires an expensive thermal cycler and operator skill, which are limited in the field. Additionally, virus isolation using chicken embryo fibroblast (CEF) cell culture takes more than 7 days for a definite diagnosis. To overcome the difficulties of PCR-based techniques, (Notomi et al., 2000) developed termed LAMP that amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions. This technique requires a Bst polymerase to displace DNA strands in isothermal condition. The reaction uses forward inner and backward primer (FIP and BIP), outer forward and backward primer (F3 and B3) and forward and backward loop primers (LF and LB) which enable faster formation of loop and hairpin-like structures allowing faster detection of the specific DNA. As LAMP recognizes the target by six distinct sequences initially and by four distinct sequences afterwards, it is expected to amplify the target sequence with high specificity.

Since the technique was first described, it has been applied for the detection of several poultry pathogens (Chen, 2008, 2010), (Deng et al., 2010), (Xie et al., 2010), (Xu et al., 2009).

As LAMP recognizes the target by six distinct sequences initially and by four distinct sequences afterwards, it is expected to amplify the target sequence with high specificity.

The advantages of this technique are that it requires only 30 to 60 min and can be performed at a single temperature ranging from 60°C to 65°C. The reaction can be carried out in a water bath and the results can be read with the naked eye. Hence, the present study was carried out to develop a diagnostic method based on the LAMP reaction for rapid detection of the MD viral genome from archived tissue samples positive for Marek’s disease. The sensitivity and specificity of LAMP were compared with those of conventional PCR.

Material and Methods

Standardization of LAMP

Archived DNA Samples (N=19) positive for Marek’s disease stored at -20ºC were used for standardization of Loop-mediated isothermal amplification (LAMP) Kaur (2010). (Table 1) provides the details of poultry birds from which, LAMP was standardized using archived DNA samples.

Preparation of tissue inoculum

The samples were washed with PBS twice and then triturated with the help of sterilized beads using PBS as a diluent to make 10% (w/v) suspension. The suspension was then centrifuged at 2500 rpm for 10 minutes. The supernatant was collected and stored at -20ºC until further processing for extraction of nucleic acid.

DNA extraction

The tissue samples which were stored at -20ºC were taken out and thawed to make a uniform solution. To 50 µl of 500 µl tissue inoculums, 500 µl lysis buffer (1M Tris HCL Ph 8.0, 5M NaCl, 0.5M EDTA, 10% SDS) and 25 µl Proteinase ‘K’ (20 mg/ml) in sterile eppendorf tubes. The samples were incubated in water bath at 56ºC overnight and DNA was extracted by phenol/chloroform extraction method as described by (Wozniakowski, 2011). Concentration and purity of DNA was assessed by UV spectrophotometry using Nanodrop system (Nanodrop 2000C, Thermo Scientific, USA).

LAMP for meq gene of MDV

As the meq gene is present only in the serotype 1 (oncogenic MDV) of the MDV thus it was targeted as the main gene for LAMP reaction. The six primers (Table 2) consisted of one pair of each outer primers (F3 and B3), inner primers (FIP and BIP), and loop primers (LF and RF) were designed [R Angamuthu,] and synthesized by Integrated DNA Technologies, India. The LAMP reaction was carried out in 25 µl reaction containing 10x Buffer, 25 mM $MgSO_4$, 5 M Betaine, 10 mM dNTP Mix , 10 pmol each of F3 and B3, 40 pmol each of FIP and BIP, 20 pmol each of LFP and LRP, and 4 µl DNA template (50ng). The mixture was heated at 95ºC for 5 min, then chilled on ice, 8 U Bst DNA polymerase (New England Biolabs) were added, incubated at 65ºC for 60 minute in a water bath and heating at 80ºC for 5 min to terminate the reaction. The LAMP products were analyzed by 2% agarose gel electrophoresis, under UV transillumination and naked-eye observation of a colour change after the addition of 0.5 µl of SYBR green I dye (1000X) (Invitrogen).

PCR amplification of the MEQ gene using LAMP F3 and B3 primers

PCR amplification of the MDV-1 was standardized using DNA extracted from archived tissues from the Marek's disease positive cases. This amplification of MEQ gene of MDV-1 was performed by using LAMP F3 and B3 primers (Table 3).

Results

Standardization of LAMP on MD positive archived samples

Archived tissue samples, positive for Marek's disease stored at -20ºC (Kaur 2010), were used for standardization of Loop-mediated isothermal amplification (LAMP). DNA was extracted from these tissue samples (N=19) for standardization of LAMP.

The LAMP reaction was standardized using varying ratio of primers, $MgSO_4$ and dNTPs. The results were observed at the amplification temperature of 60^oC to 65^oC at the reaction time for 60 min. The expected results and optimization of LAMP was obtained at reaction temperature of 65^OC at reaction time of 60 min.

The LAMP products were analyzed under UV light transillumination which appeared fluoresced apple green for positive cases by addition of SYBR green I dye (Fig.1). Positive cases showed apple green colour whereas negative appeared brown when visualized by the naked eye (Fig. 2).

Out of 19 tissue DNA samples processed, a ladder like DNA bands of different sizes were amplified from 18 MD positive samples indicating the presence of MEQ gene in 18 samples (Fig.3).

PCR amplification using LAMP F3 and B3 primers

PCR amplification of the MEQ gene of MDV-1 was carried out from the archived tissue DNA samples using outer primers F3 and B3 designed for LAMP. Out of 19 archived tissues DNA samples, amplification of 180bp was obtained in 18 samples which were also found positive with LAMP (Fig. 4).

Table 1: Details of poultry birds from which LAMP was standardized using archived DNA samples

S. No.	Type of bird	Age	Number of samples
1	RIR	18-24wks	2
2	Broiler	Adult*	4
3	RIR	Adult*	13
Total			19

*more than 24 wks of age

Table 2: Primers used for LAMP for MDV-1 Virus

Primer	Sequence	Primer size
F3-MDV	5' CGT CCC TGC GTG TAC AGT 3'	18
B3-MDV	5' GGA ACC GGA GCA ATG TGG 3'	18
FIP-MDV	5' AGC AGT CCA AGG GTC ACC GTT TTT GGC TG TCA TGA GCC AGT T 3'	43
BIP-MDV	5' CGC AGC ATC CCG TTC CTG AAT TTT GTT AGG TTC ATC CGG TGA GG 3'	44
LFP-MDV	5' GGT ACC GCC ATA GGG CA 3'	17
LRP-MDV	5' CCT CCC ATT TGC ACT CCT CC 3'	20

Table 3: Primers used for PCR amplification of MDV-1

Primer	Sequence	Primer size	Gene	Product size
F3-MDV	5’ CGT CCC TGC GTG TAC AGT 3’	18	MEQ gene	180bp
B3-MDV	5’ GGA ACC GGA GCA ATG TGG 3’	18		

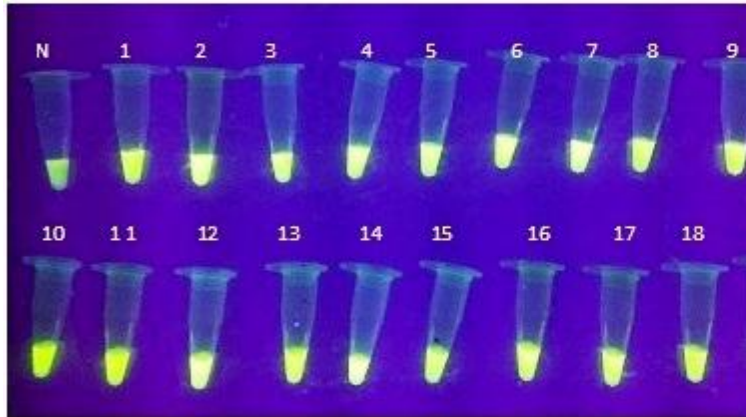

Figure 1: LAMP products of archived samples under UV transillumination
(N: Negative sample, 1-18: Positive samples)

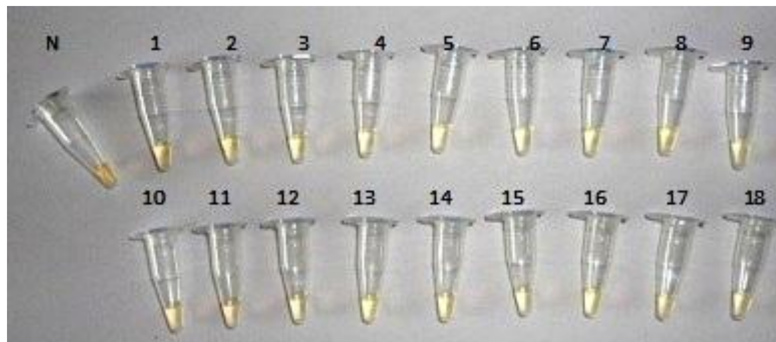

Figure 2: Visual detection of LAMP products of archived samples
(N: Negative sample, 1-18: Positive samples)

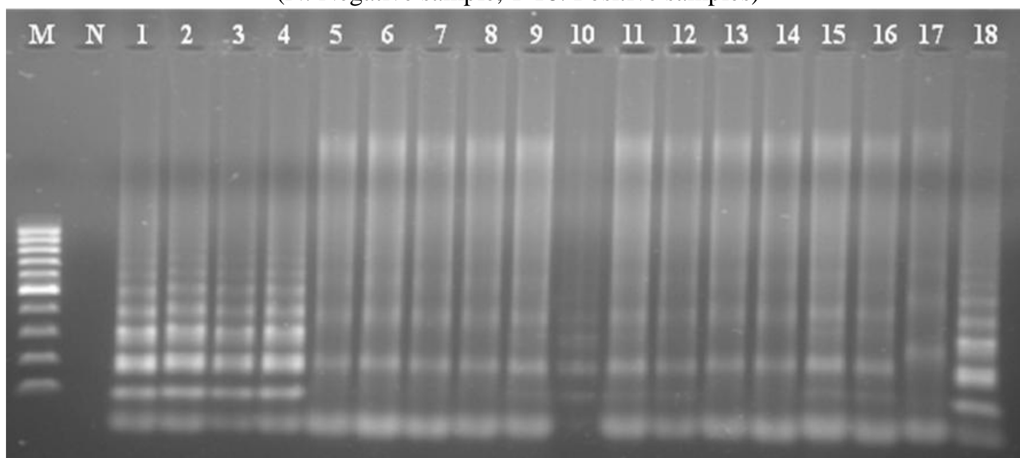

Figure 3: LAMP amplified product of DNA of archived tissue samples

Lane M : 100bp plus Molecular weight marker
Lane N : Negative sample
Lane 1 to Lane 18 : Positive samples

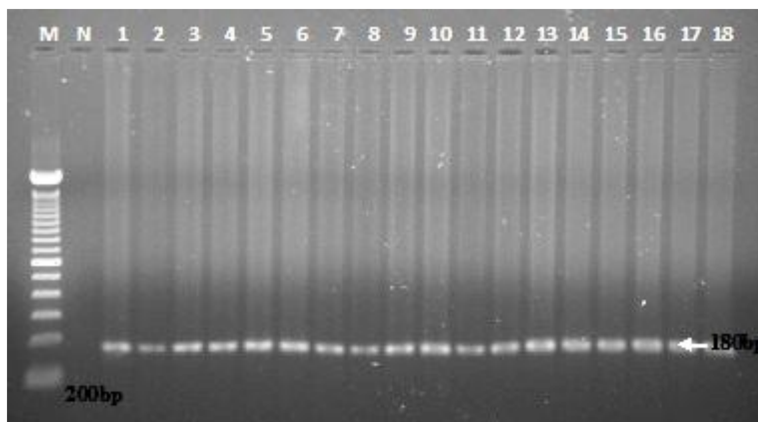

Figure 4: PCR amplicons (~180bp) from archived tissue DNA samples

Lane M : 100bp plus Molecular weight marker
Lane N : Negative sample
Lane 1 to Lane 18 : Positive samples

Discussion

Several PCR and real-time-based techniques have been developed, for detection, as well as quantification, of the MDV genome of field and vaccine strains. PCR testing methods have been developed in order to quantify viral loads (Baigent et al., 2004), (Bumstead et al., 1997), (Yunis et al., 2004) which have enabled the viral loads in shed dust to be directly measured (Islam et al., 2007, 2008). While MDV replicates in semi-productive, latent and transformative modes in the various organs of the birds, the feather follicle epithelium (FFE) cells are a productive site MDV replication and horizontal dissemination (Calnek and Witter, 1997). This feature was utilized to determine the presence of MDV by polymerase chain reaction (PCR) (Handberg et al., 2001). MDV specific primers employed in the present study could differentiate between pathogenic and non-pathogenic serotype-1 MDV with their ability to amplify BamH1-H 132 bp tandem repeat from the genomic sequences of pathogenic MDV-1 only (Becker et al 1992). Furthermore, PCR assays described in this study, take several hours to complete including electrophoresis time making it time consuming diagnostic method, a technique that requires a well-established laboratory, a thermal cycler and a gel system to visualize respective amplified products. Additionally, virus isolation using chicken embryo fibroblast (CEF) cell culture takes more than 7 days for a definite diagnosis.

The most important aspect of the present study was to diagnose and differentiate the Marek’s disease in a simple manner and in a more rapid way because of certain deficiencies of the standard diagnostic methods based on necropsy, histology, immunohistochemistry and conventional PCR. Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method that synthesizes large amounts of DNA in a species specific-manner with high efficiency, speed and a low cost (Notomi et al., 2000). Bst polymerase which has strand displacement activity, mediates auto-cyclic DNA synthesis with loop-forming primers to yield long-stem loop products under isothermal conditions (60-65°C for about 60 min) and can be inactivated at 80°C after the LAMP reaction is complete (Notomi et al., 2000), (Nagamine et al., 2002). This technique uses four to six primers that recognize six to eight defined regions of the target DNA.

There is an early cell-associated cytolytic infection of lymphocytes, however acute infections revert into latency 7 days later. Latently infected T lymphocytes harbor the MDV genome and express limited viral antigens. The virus is transported through the body via the bloodstream to visceral organs, peripheral nerves and FFE (Baigent et al., 2005). MDV replication in the FFE is fully productive and results in the release of enveloped, cell-free virus particles that are shed with skin and feather debris (Carrozza et al., 1973), therefore feather tips have long been used for the detection of MDV (Calnek et al., 1970), (Rangga and Cho, 1982). Feathers can be sampled readily from live birds and feather tip extracts are useful sources of MDV DNA used for PCR amplification (Davidson and Borenshtain, 2003). Therefore, LAMP was optimized to detect MDV from feather follicles.

The MEQ gene was selected as a target gene for LAMP analysis, as it is present only in the stereotype 1 of the MDV. LAMP primers were designed as per (Angamuthu et al., 2012) and synthesized by Integrated DNA Technologies, India. The six primers consisted of one pair each of the outer primers (F3 and B3), inner primers (FIP and BIP), and loop primers (LF and RF). The expected results in the present study and optimization of LAMP were obtained at reaction temperature of 65°C at reaction time of 60 min with the loop primers. Without loop primers the LAMP reaction usually takes 90 minutes to amplify the target template (Angamuthu et al., 2012). Loop primers could accelerate the reaction because they hybridize to the stem-loops, except for those loops that are hybridized by the inner primers and prime strand displacement DNA synthesis (Nagamine et al., 2002). The loop primers could also provide higher specificity, as the six primers could recognize the eight distinct regions of the target DNA (Parida et al., 2004).

As describe by (Wei et al., 2012) MDV LAMP is approximately 100-fold more sensitive than the conventional PCR when using the same template. Similarly, (Angamuthu et al., 2012) has observed that the LAMP assay had a detection limit of 10 copies of the MEQ gene of serotype 1 MDV, whereas the PCR assay was able to detect 100 copies of the MEQ gene of MDV. The detection limit of the LAMP assay was 10-fold higher than that of conventional PCR. This sensitivity translated to the successful analysis of MDV from clinical samples that identified MDV in 20 out of 21 samples screened compared with 14 out of 21 samples identified by conventional PCR in the present study. Similarly, LAMP was able to detect MDV-1 from feather follicle of 29 live birds out of 30 as compared to 17 out of 30 by MDV-specific PCR.

LAMP analysis of DNA samples from clinical cases and feather follicles of live birds was also compared with other diagnostic tests such as MDV-specific PCR, histopathology and immunohistochemistry using the kappa statistic as suggested by Everitt (1989). The calculated kappa values (Kappa value at 95% CI) showed slight degree of agreement between LAMP and MDV-specific PCR, histopathology and immunohistochemistry. Similarly, a slight agreement was observed between LAMP and MDV-specific PCR (K=0.086) in detection of MEQ gene of MDV-1 from feather follicles of the live birds. This clearly indicated that LAMP assay optimized during the present study has edge over other diagnostic techniques like histopathology, immunohistopathology and conventional diagnostic PCR.

These findings demonstrate a significant advantage of LAMP in diagnosis of MDV compared either with PCR or virus isolation procedures as it can be carried out in most situations where rapid diagnosis is required, like in field conditions. A water bath or block heater is sufficient for DNA amplification because the method requires isothermal conditions. In addition, amplified LAMP products could be visualized by the addition of SYBR Green I dye without the need for gel electrophoresis; this makes it a very simple and cheap diagnostic tool. LAMP assays, therefore can generate results quickly in less well equipped laboratories or in field conditions. These observations suggested that the new MDV LAMP assay described here would be more practical for routine MDV screening procedures compared with conventional PCR or virus culture procedures.

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