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Rapid detection of fowl adenovirus field samples using loop-mediated isothermal amplification assay

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Fowl adenoviruses (FAV) are causative agents of hydropericardium syndrome (Ganesh *et al*. 2002), inclusion body hepatitis (Winterfield *et al.*1973) resulting in heavy mortality and economic loss in poultry industry. The major structural proteins of FAV are hexon and fibre gene, the hexon is the major capsid protein of the non-enveloped icosahedral virion on which type, group and sub-group specific determinants are located (Norrby 1969). Routine diagnostic methods widely used for screening of FAV are virus isolation in cell culture, immunofluorescence, ELISA, Polymerase chain reaction combined with RFLP analysis. These methods are often time-consuming and require sophisticated equipment. Hence, an alternative technique called Loop-mediated isothermal amplification (LAMP) was employed for the rapid detection of fowl adenoviruses in this study.

LAMP assay can be performed in isothermal conditions 60–65ºC without sophisticated instruments and reaction time takes only 60 mins. These advantages have enabled the use of LAMP for the rapid detection of various viral pathogens (Yasuhiko *et al*. 2008, Das *et al*. 2011, Yongchang *et al*. 2009, Shanthi *et al*. 2007, Yi *et al.* 2011). The attractive feature of LAMP is that the results can be determined by naked eye as a white precipitate at the end of the reaction incubation (Parida *et al*. 2004). On addition of SYBR green DNA binding dye, the positive samples showed fluorescence under uv-light (Ashok *et al.* 2010). In this study, the result of LAMP assay was compared with conventional PCR method in terms of sensitivity. The specificity of LAMP products were ascertained by sequencing of LAMP products and Real time PCR.

Sample Collection and LAMP assay: One hundred and thirty four tracheal and cloacal (pooled) swabs were collected from birds suspected for fowl adenoviruses. DNA isolation was done using a commercial kit (Macherey-Nagel, Gmbh and Co, Germany) according to the manufacturer's instructions. The DNA extracted from chicken liver cells infected with fowl adenoviruses serotype 4 and uninfected chicken liver cells were used as positive and negative controls respectively. The conventional PCR was done using already published hexon gene (H3/H4) specific primers (Raue and Hess 1998). Primers were designed for LAMP assay based on the published sequences available in Genbank data using Primer explorer V3 software (http://primerexplorer.jp/e/intro/index.html) and presented in Table 1.The isolated DNA was also used as a template for LAMP assay as described by Parthiban *et.al*. 2012. For initial optimization, the reaction mixture was incubated from 60ºC to 63ºC for 60 mins and the reaction was stopped by heating at 80ºC for 5 minutes. Five µl of PCR and LAMP products of field samples along with positive and negative controls were analysed in 1.5% agarose gel electrophoresis and viewed under UV trans-illuminator after ethidium bromide staining.

The specificity of LAMP primers were checked by using DNA of other avian DNA viruses viz Marek's disease virus

Table 1. Primers designed for FAV LAMP assay

S.no	Hexon gene	Sequences (5'-3')
1.	Forward primer (F3)	GACCCCAACATGATTCTGCA
2.	Reverse primer (B3)	AATAGTAAGTGCCGTGGAGC
3.	Forward inner primer (FIP)	TCCATGGGCATGAAGTTAGC CA-GCAATGATCTGAGAGCC GAC
4.	Backward inner primer (BIP)	ACAAGCAATCAGCTCG AGCTCA-ACGCATTCTTGGCA CCTAG
5.	Loop forward (LF)	AGAGTACACGATCGAAG CTCC
6.	Loop backward (BF)	TGCTCAGAAACGCCACT AACG

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and Avian Leucosis virus. The high molecular weight band of LAMP product was excised and purified using a commercial gel purification kit. The purified DNA band was sequenced using 3 pairs of primers F3-B3, FIP-BIP and LF-LB as mentioned in Table 1. The sequences were subjected to BLAST analysis and homology percentage was determined with other FAV sequences in Genbank database. All the 28 LAMP positive samples and 10 random LAMP negative samples were further confirmed by real time PCR using already published primers (Penelope *et al*. 2009) to amplify the hexon gene to check the specificity of LAMP assay. The concentration of FAV positive DNA sample was determined using nanodrop and it was 10 fold serially diluted and subjected to PCR and LAMP assay to assess its sensitivity. The SYBR green DNA binding dye (1×1000) was added to amplified product and incubated for 15 min in dark at room temperature. The colour changes in positive and negative samples were compared.

The LAMP assay successfully amplified the target hexon gene sequence of fowl adenovirus when incubated at 63ºC for 60 min. A ladder-like pattern was seen in agarose gel electrophoresis (Fig. 1) as reported by Yongchang *et al.* 2009. Out of 134 field samples screened, 28 samples were positive by both PCR and LAMP assay. Hence, LAMP assay was found to be alternative diagnostic assay in field condition. The LAMP positive samples exhibited a green fluorescence when viewed under UV-light conditions on addition of SYBR green dye (Fig. 2) as reported by Ashok *et al.* 2010. The LAMP primers did not react with Marek's disease virus and avian leucosis viruses which clearly indicated the specificity of the assay. The specificity of LAMP assay was further confirmed by sequencing of the LAMP amplified products. The sequencing data exhibited 96% homology with other FAV sequences available in Genbank (GenBank Acc. No. JQ034221, JQ034222). In real time PCR, qPCR products in agarose revealed only one amplicon size of 191bp (Fig. 3) and similarly in the melt- curve analysis revealed only one peak at 88°C for all the LAMP positive samples and no peak was obtained at this temperature for the LAMP negative samples (Fig.5) and it coincide with the results of Penelope *et al.* 2009. The sensitivity of conventional PCR was 100ng of DNA whereas on the other hand, LAMP assay detected upto 10pg level of DNA (Fig. 4). This indicated the 100–fold sensitivity of LAMP assay compared to conventional PCR. Similarly Hatem *et al.* (2010) reported that as little as 10 fg level of cyprinid herpes virus-3 DNA could be detected using LAMP assay. In order to avoid false positive reactions, we have used all the reagents in small aliquots, filtered tips, PCR work station and positive and negative controls in each experiment as described by Yi *et al.* (2011).

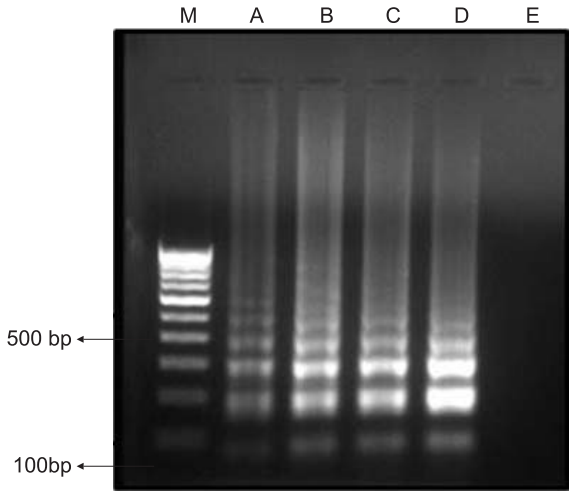

Fig. 1. Screening of Field Samples of FAV by LAMP assay M, 100bp ladder; A-D, FAV positive samples; E, negative control.

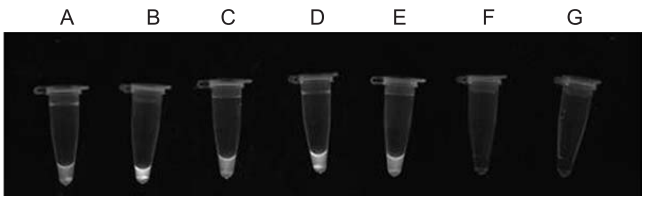

Fig. 2. Visual detection of LAMP products using SYBR Green I dye; A - E, Positive samples; F and G, negative samples.

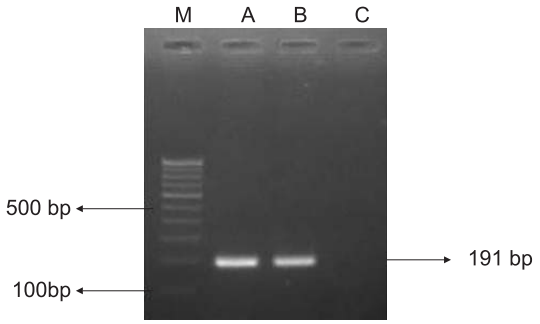

Fig. 3. Agarose gel analysis of the Q PCR products. M, 100bp DNA ladder; A and B, fowl adenovirus positive samples; C, negative control.

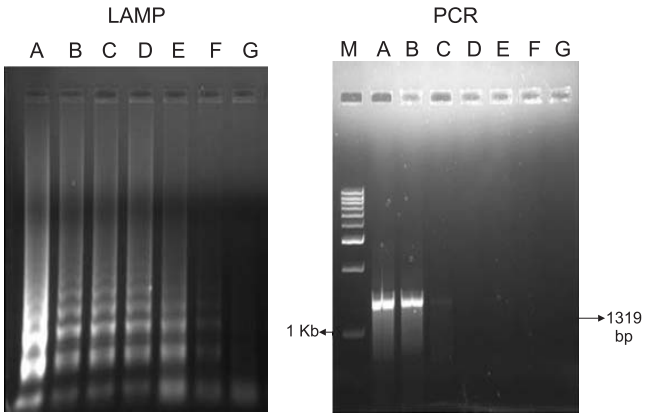

Fig. 4. Sensitivity of LAMP and PCR assay of FAV. A–G, 10^{-1} to 10^{-7} dilutions of positive FAV DNA. A, 1µg, B, 100ng, C, 10ng, D, 1ng, E, 10pg, F, 1pg.

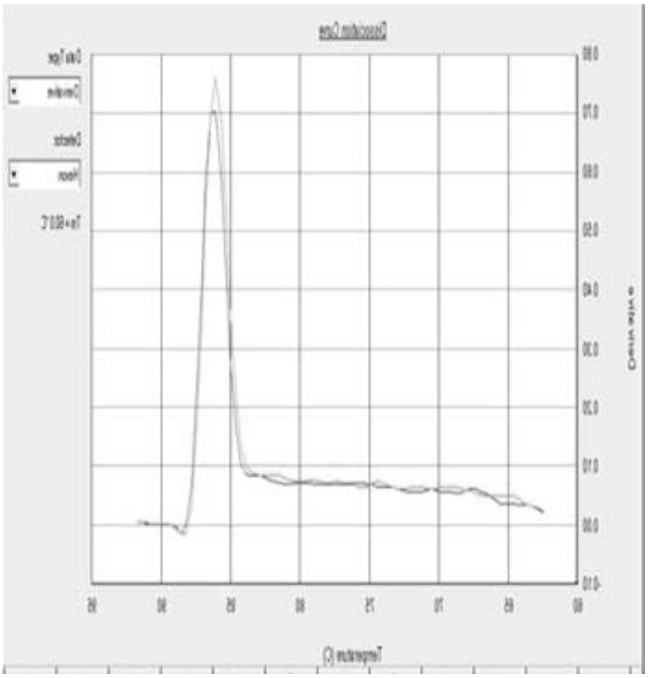

Fig. 5. Melt curve analysis of LAMP positive and negative sample using SYBR Green Real Time assay.

SUMMARY

Loop-mediated isothermal amplification was employed for the detection of fowl adenoviruses as a simple, rapid and field based diagnostic assay. Out of 134 field samples screened, 28 samples were positive by both PCR and LAMP assay. The LAMP assay primers were found to be highly specific and it detected only fowl adenovirus samples and it did not react with other avian viruses like Marek's disease virus and avian leucosis virus. Conventional PCR detected 100ug level of DNA whereas LAMP assay detected up to 10ng level of DNA. It clearly indicated that LAMP assay was 100-times more sensitive than conventional PCR. The specificity of LAMP assay was confirmed by sequencing of the LAMP amplified products and real time PCR. The LAMP positive reaction can be easily visualized under UV trans-illuminator by the addition of SYBR green dye. Thus, LAM Passay proves to be a sensitive, rapid, less-laborious and cost-effective technique for the diagnosis of fowl adenoviruses in field conditions.

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