



Molecular detection and phylogenetic analysis of avian reticuloendotheliosis virus from formalin fixed tissue by PCR in fancy chicken

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Received: 19-05-2018

Accepted: 14-07-2018

DOI: 10.18805/ijar.B-3640

ABSTRACT

The present study was carried out to identify avian neoplastic viruses in the formalin fixed tissues of fancy chicken. The tissue samples collected during necropsy were examined by histopathology. It showed lymphoid and reticular cell infiltration in kidney, liver and lungs. For identification and differentiation of avian neoplastic viruses, PCR was performed using primer sets specific for Marek's disease virus, avian leukosis complex and reticuloendotheliosis virus. It was found that tumors were REV originated. Further confirmation, purified PCR product was subjected to sequencing. It showed 99% homology with other REV isolates available in the NCBI database. The present communication describes infection of a fancy chicken with REV on the basis of histopathological findings as well as molecular methods.

Key words: Fancy chicken, PCR, Phylogenetic analysis, Reticuloendotheliosis virus.

INTRODUCTION

The most prevalent neoplastic viruses such as Marek's disease (MD), avian leukosis virus (ALV) and reticuloendotheliosis (REV) cause huge economic losses due to mortality and poor performance in poultry (Nair, 2013). Reticuloendotheliosis virus is an immunosuppressive and neoplastic condition caused by Gamma retrovirus belongs to Retroviridae family (Buchen-Osmond, 2004). The virus is morphologically and immunologically different from avian leukosis complex viruses. Various authors isolated REV from variety of avian species namely chickens, turkeys, ducks, pheasants, geese, Japanese quail, peafowl and prairie chickens (Nair *et al.*, 2013). The clinical disease associated with REV is acute reticular cell neoplasia, chronic lymphomas and an immunosuppressive runting disease (Crespo *et al.*, 2002). Various reports explained that REV as contaminant of Marek's disease and Fowl pox vaccines which resulted in delayed growth, feather abnormalities, anemia, and leg paralysis (Wei *et al.*, 2012).

It is evident from various studies that partial or full length REV sequences is integrated into the genome of Fowl pox viruses (Prukner-Radovic *et al.*, 2006). REV was also integrated into another DNA virus, Marek's disease as demonstrated by Davidson and Silva, ((2008). Many studies have reported that coinfection of REV with Marek's disease and Avian leukosis complex in poultry (Khordadmehr *et al.*, 2017).

REV was diagnosed by histopathology as well as various methods which include indirect immunofluorescence

test, enzyme linked immune sorbent assay (ELISA) and PCR. Recently, nucleic acid based diagnostic test have proved beneficial for detection and differentiation of viral neoplasms of poultry. The aim of the present study was to identify and differentiate the viral neoplasm of fancy chicken in the formalin fixed tissue by PCR.

MATERIALS AND METHODS

Sample collection: A total of 12 fancy chicken aged 24 weeks were maintained in hygienic manner for show purpose in Thanjavur district, Tamil Nadu. Around four birds showed dullness and depression in the flock and three birds died. During necropsy, liver, kidney and lung samples showing mild nodular lesions were collected in 10% buffered formalin for histopathology.

Histopathology: The samples collected during necropsy were fixed in 10% formalin, processed and embedded in paraffin wax. Hematoxylin and eosin stain were used for staining the tissue sections. Further, the histopathological lesions were analyzed using research microscope at various magnification.

DNA extraction, PCR and Sequencing: DNA was extracted from formalin fixed and paraffin embedded tissue by using HiPurA™ Paraffin-Embedded Tissue DNA Purification Kit as per manufacture's instruction. The obtained DNA was stored at -20°C until for further analysis. Then, Polymerase chain reaction was carried out by using previously reported primer sets for MDV, ALV and REV as shown in Table 1. The PCR reactions were carried out in 25

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Table 1: Primers used in this study

Target gene	Primer sequence	Size	Reference
REV LTR	LTRF2-GCGCTGGCTCGCTAACTG LTRR2- TTCGATCTCGTGTTCGTGATT	200 bp	Garcia <i>et al.</i> , 2003.
ALV Subgroup J	H5 - GGATGAGGTGACTAAGAAAG H7 - CGAACCAAAGGTAACACACG	545 bp	Smith <i>et al.</i> , 1998.
ALV Subgroup A-E	H5 - GGATGAGGTGACTAAGAAAG AD1 - GGGAGGTGGCTGACTGTGT	295–326 bp	Smith <i>et al.</i> , 1998.
MD Bam H1-H	TACTTCCTATATAGATTGAGACGT GAGATCCTCGTAAGGTGTAATATA	434 bp	Kalyani <i>et al.</i> , 2011.

μ L volume of 12.5 μ L of master mix (2x), 1 μ L of forward and reverse primer each (10 pmol/ μ L), 7.5 μ L of deionized water and 3 μ L of extracted DNA. PCR program was carried out as previously described for REV (Garcia *et al.*, 2003), MD (Kalyani *et al.*, 2011) and ALV (Smith *et al.*, 1998). The analysis of PCR product was carried out in 1.5 per cent agarose gel stained with ethidium bromide (0.5 μ g/ml) and documented under Gel documentation system. The amplicons were purified by using Hiyield plus PCR purification kit (cat#QPP100) following manufacturer's instruction.

The purified PCR amplicons were subjected to sequencing by Sanger dideoxy sequencing method in an automated sequencer. The multiple sequence alignment was carried out using the Mega 5.2 software to generate sequence analysis data. The sequences were aligned by multiple sequence alignment (Clustal W). The phylogenetic tree was constructed to estimate the relationship between sequences by using Neighbour Joining (NJ) algorithm using bootstrap values and distance in Mega 5.2 software. Homology searches were performed with the NCBI database and BLAST.

RESULTS AND DISCUSSION

On histopathological examination, tissue sections showed massive infiltration of small and medium lymphocytes, lymphoblasts, few macrophages and scattered

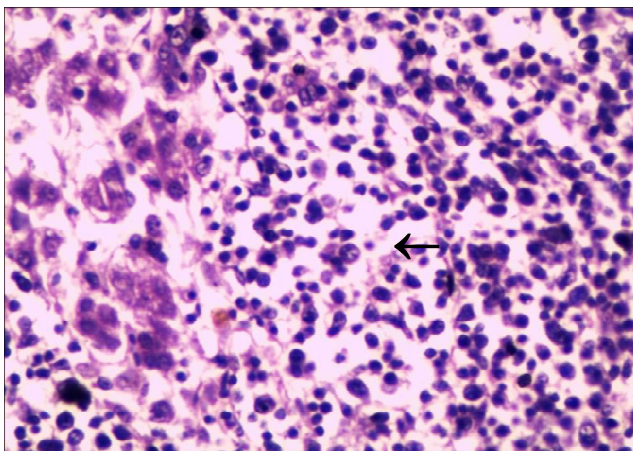


Fig 1: Liver: Infiltration of small (many), medium (few) lymphocytes and scattered reticular cells (arrow). H & E (400X).

reticular cells in liver, kidney and lung which results in loss of architecture of the organs. There were multifocal areas of lymphoreticular infiltration in the liver. Our histopathological findings were in accordance with Prezotto *et al.* (2016) stated in broiler chickens (Fig. 1 and 2). Further, DNA was extracted from formalin fixed tissue samples *viz.* liver, kidney and lung were subjected to PCR with specific primer sets for MD, ALV and REV. The samples were analyzed by PCR for the presence of ALV subgroups A-E and ALV subgroup J. It was found that no amplification was obtained from any of samples for ALV and MD. In the present study, neurological signs were not reported and also mortality was observed in the birds aged 24 weeks. It is considered that the tumors

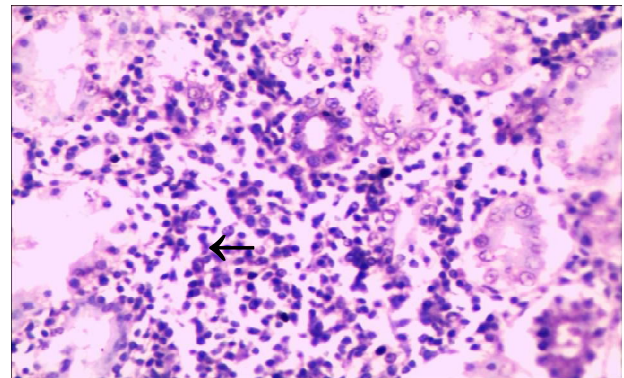


Fig 2: Kidney: Infiltration of small (many), medium (few) lymphocytes and scattered reticular cells (arrow). H & E (400X).

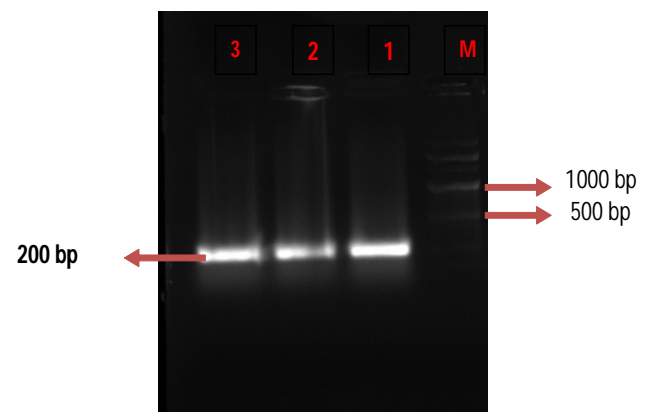


Fig 3: M-100bp ladder, 1 (liver), 2 (kidney) and 3 (lung) positive samples with a band at 200bp specific for REV LTR region

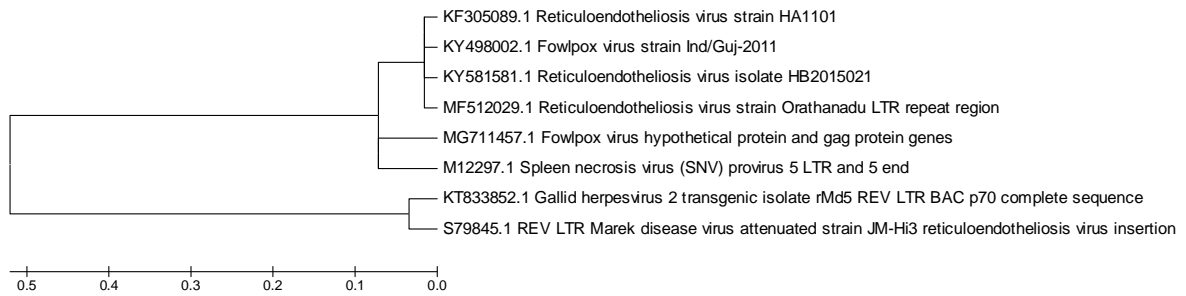


Fig 4: Phylogenetic tree (Neighbour - Joining) of REV of Orathanadu strain (MF 512029.1) based on nucleotide sequence

might be originated from retroviral infection. PCR using REV primers yielded approximately 200bp length of PCR product from all the samples of diseased birds. The PCR results for LTR region of REV were in agreement with previous reports for detection of REV in poultry (El-Sebelgy *et al.*, 2014). Due to the deficient of positive REV samples and also to support PCR results, the purified REV PCR amplicons were subjected to sequencing. The partial LTR sequence of REV was submitted to Gen Bank and the accession number MF512029 was obtained. The sequence analysis of the present study showed 99% sequence homology with other REV sequences available in NCBI database as demonstrated by blast. It is confirmed that the

present case is to be REV virus originated based on histopathology examination as well as molecular methods since adult chickens infected by REV is difficult to exhibit typical tumor or clinical symptoms. The partial sequences of LTR of REV was closely clustered with integrated REV genome insert in cutaneous fowl pox virus strain (MG711457.1), REV strain HB2015021(KY581581.1), Gallid herpesvirus 2 transgenic isolate rMd5 REV LTR BAC p70 (KT833852.1), REV strain HA1101, REV strain HA9901(KF305089.1) with nucleotide identity 99%. In the present study, REV infection in fancy chicken was demonstrated by the examination of gross and histopathological lesions as well as confirmatory molecular methods.

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