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Research Article

MOLECULAR CHARACTERIZATION AND HISTOPATHOLOGICAL ANALYSIS OF AVIAN RETICULOENDOTHELIOSIS VIRUS FROM GUINEA FOWL IN TAMIL NADU

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ABSTRACT: The present study was carried out to detect the causative agent of sudden death in guinea fowl flock maintained at organized poultry farm, Orathanadu. On post mortem examination, liver was enlarged and revealed multiple grayish areas. The spleen was enlarged and kidneys were pale and swollen. Breast muscle was very thin and the keel bone was prominent. Representative tissue samples from liver, kidney spleen were collected for histopathological examination and revealed lymphoid and reticuloendothelial cells in kidney, liver and spleen. PCR was used for detection as well as differentiation of avian neoplastic viruses such as Marek's disease virus, avian leukosis complex and reticuloendotheliosis virus using specific primer sets. The sample was found to be positive for avian reticuloendotheliosis virus and negative for Marek's disease virus and avian leukosis complex. The positive PCR amplicons were subjected to nucleotide sequencing for further confirmation of avian reticuloendotheliosis virus. The sequence analysis showed 99% homology with other published REV isolates available in the NCBI database. The present study reported the first incidence of avian reticuloendotheliosis virus in guinea fowl flock based on gross as well as histopathological lesions and molecular methods in India.

Keywords: Avian reticuloendotheliosis virus, Guinea Fowl, PCR and Phylogenetic analysis.

INTRODUCTION

Neoplastic diseases in poultry have been classified into two categories as virus associated neoplasms and neoplasms without known etiological agent. Marek's disease (MD), avian leucosis complex (ALC) and reticuloendotheliosis (RE) are virus associated neoplastic diseases in poultry [1].

MD is caused by a double stranded DNA virus belongs to the family Herpesviridae, genus *Mardivirus* and species *Gallid alphaherpesvirus* 2. MD is a lymphoproliferative neoplastic disease characterized by paralysis, cellular infiltrations in parenchymal organs and tumors in visceral organs including skin, muscle and nerves [2]. ALC includes avian retroviruses which cause lymphoid leucosis and has been classified under the family Retroviridae, genus Alpha retrovirus and consists of subgroups A, B, C, D and J based on antigenic nature of viral envelop glycoproteins [3].

RE is caused by avian reticuloendotheliosis virus belongs to the family Retroviridae, subfamily gamma retrovirinae [4]. RE virus affects chickens and turkeys and rarely ducks, geese, Japanese quail, pheasants and peafowl and prairie chickens [5]. There were many reports of avian reticuloendotheliosis virus in chickens, Turkey, backyard and fancy chickens in India [6, 7, 8, 9]. The disease is characterized by high mortality, abnormal feathering, runting syndrome, immunosuppression [10], tumors in liver, spleen, intestine and heart with diffuse infiltration of neoplastic reticular cell [11].

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Above neoplastic diseases can occur as co-infection due to integration of part or whole of the avian reticuloendotheliosis viral genome into field and vaccine strains of MD and fowl pox viruses [12, 13, 14]. The oncogenic avian viruses are detected and differentiated by conventional virus isolation and histopathology. Serological methods such as ELISA, agar gel precipitation and fluorescent antibody tests are used for detection of antibodies against oncogenic avian viruses. Among the serological techniques, ELISA is found to be more reliable [15, 16].

Now-a-days, molecular methods such as PCR, qPCR followed by sequencing are being routinely used to detect and characterize the diseases. These molecular diagnostic methods are more sensitive, less time consuming than conventional methods [17]. The present paper describes the detection and differentiation of the avian neoplastic diseases in Guinea fowl using molecular methods.

MATERIALS AND METHODS

Collection of samples

An organized poultry farm at Orathanadu taluk in Thanjavur district reported sudden mortality of guinea fowl flock aged 24 weeks without any clinical signs. The flock is having 28 birds with weekly mortality of 3 birds. Systematic postmortem was carried out in randomly selected dead birds from the flock at Department of Veterinary Pathology, Veterinary College and Research Institute, Orathanadu. Representative tissue samples from liver, kidney and spleen were collected for histopathological examination and for molecular diagnosis.

Cytology

Impression smears were collected from liver samples in clean grease free glass slide for cytological examination. The prepared smears were fixed in 95% methanol and dried. Smears were stained with Giemsa's stain.

Histopathology

The respective tissue samples collected during necropsy were fixed in 10% neutral buffered formalin and processed as per standard paraffin embedding technique [18]. Tissue sections of 4-5 µm thickness were prepared and stained by Hematoxylin and eosin stain as per standard protocol [18]. Further, the histopathological lesions were analysed using research microscope at various magnifications.

DNA extraction

Tissues in sterile container were subjected for genome extraction. DNA was extracted from tissue samples such as liver, kidney and spleen by using DNeasy blood and tissue extraction kit (Qiagen) as per manufacturer's instructions. Extracted DNA samples were stored at -20oC until further analysis.

Polymerase Chain Reaction (PCR)

PCR was carried out for detection of MD virus, ALC virus and RE virus by using already published primers as depicted in Table 1. A total of 20 µL thermal reaction was carried with 10µL of master mix (2x), 10 pmol/ µL concentration of forward and reverse primers each 1 µL, 6 µL of molecular grade water and 2µL of DNA sample. The thermal conditions were set in the thermal cycler (Mastercycler® nexus gradient) as previously described for REV [12], MDV [19] and ALV [20]. Agarose gel electrophoresis was employed to view the amplified product using 1.5 % agarose gel and ethidium bromide (0.5µg/ml). The image was analysed using the Gel documentation system. The positive PCR amplicons of LTR gene of REV were purified by PCR purification kit (Hiyield plus cat#QPP100) as per the manufacturer'sinstructions.

Sequence analysis

Nucleotide sequencing was done using the purified LTR gene of REV amplicon by Sanger dideoxy sequencing method in an automated sequencer (M/s. Eurofins, Bangalore). The published sequences were retrieved from NCBI database and multiple sequence alignment (Clustal W) was carried out using the Mega XI software. The phylogenetic analysis was performed to find out the evolutionary relationship of the sequences by using Maximum likelihood algorithm with 1000 bootstrap values and distance in Mega XI software.

RESULTS AND DISCUSSION

Reticuloendotheliosis viruses consist of numerous strongly associated amphotropic retroviruses under the reverse transcribing RNA virus family. The prototype REV strain named as REV-T was first detected in 1957 from leukosis like outbreak among turkeys in USA [21]. REV-T is considered as a defective strain which causes acute oncogenicity because of carrying oncogene [22]. After the detection of REV-T, several avian species including ducks, chickens, geese, turkeys, peafowls, prairie chickens, pigeons, pheasants, and Japanese quails were affected by other non defective REV strains [23].

Table. 1. Primers used for detection and differentiation of avian oncogenic viruses.

Target genes	Primer sequences	Product Size
REV LTR	5'-GCGCTGGCTCGCTAACTG-3'	200 bp
	5'-TTCGATCTCGTGTTTGTTCGTGATT-3'	
MDV	5'-TACTTCCTATATAGATTGAGACGT-3'	434 bp
Bam H1-H	5'-GAGATCCTCGTAAGGTGTAATATA-3'	
ALV Subgroup J	5'-GGATGAGGTGACTAAGAAAG-3'	545 bp
	5'-CGAACCAAAGGTAACACACG-3'	
ALV	5'-GGATGAGGTGACTAAGAAAG-3'	326 bp
Subgroup A-E	5'-GGGAGGTGGCTGACTGTGT-3'	

Avian reticuloendotheliosis is an infectious disease causing neoplasm primarily chickens and turkeys and rarely in other birds such as ducks, geese, pheasant and peafowl. In the present study, REV was detected in Guinea fowl flock aged 24 weeks old. Clinically the disease is characterized by abnormal feathering, reduced body weight, anaemia, runting and immunosuppression but apparently no clinical signs were observed in the present study.

On gross examination, liver lobes were diffusely appeared enlarged with multiple, scattered, grayish white, elevated, firm foci. On incision, it extended deep into the parenchyma. Spleen was appeared paler and enlarged. Kidneys were pale and swollen with multiple greyish white firm foci. Breast muscles were pale, thin with prominent keel bone.

Gross lesions were in accordance with previous documented evidences. It has been reported that the enlargement and nodules of liver, spleen, kidney and lymphoid tissues of intestinal tract in poultry [24]. The nodular lesions in visceral organs such as liver, spleen and intestine in REV infection were reported in chickens and turkeys [8]. Else, in contrary to the previous reports, there were no evident of tumor nodules in visceral organs rather than diffuse enlargement in many birds. Cytological examination of liver impression smears revealed sheets of individual small to medium sized pleomorphic lymphocytes. Cytology was used previously for detection and differentiation of MDV and ALV. In MD, the tumors composed of mixture of small, large lymphocytes, plasma cells and macrophages whereas ALC composed of primarily large lymphoblasts with more or less uniform size**.** In the present study, cytological examination of liver tissues was appeared to be similar to MD which furthermore have-to-have additional diagnostic tests to differentiate the MDV and REV.

The histopathological findings of liver, kidney, spleen and intestine revealed massive proliferation and infiltration of pleomorphic lymphocytes and scattered reticular endothelial cells which resulted in loss of architecture. Liver revealed multifocal areas of proliferative neoplastic cells in the sinusoids which resulted in atrophy of hepatic cords. Pleomorphic lymphocytes and reticuloendothelial cells were found to be diffuse in the interstitum of kidney causing widening of interstitum, atrophy of tubules and glomerulus. Spleen revealed pleomorphic lymphocytes with complete disappearance of red pulp. Intestine revealed pleomorphic lymphocytes and reticuloendothelial cells in the mucosa and found penetrating deep into the tunica muscularis. The above findings were similar to that of previous reports in chicken [9] and quail [25].

The diagnosis and differentiation of avian oncogenic viruses based on cytology and histopathological lesions in tissues is found to be difficult and inaccurate. Tissue changes induced by oncogenic viruses most particularly the lymphoid cell proliferations shows alikeness between the viruses. Hence, molecular diagnosis is warranted. Although avian neoplastic viruses can be differentiated by using immunohistochemistry, usage of specific antibodies to oncogenic viruses is found to be challenging [26]. To overcome the limitations, PCR found to be the best technique for detection as well as differentiation of avian oncogenic viruses.

PCR was performed for MDV, REV and ALV subgroups including A-E and J using specific primer setsto confirm and to differentiate poultry oncogenic viruses in the present study. The samples were found to be positive for REV by PCR using LTR gene with product size of 200bp length (Figure 7). The tissue samples were negative for MDV and ALV subgroups. The PCR results confirmed that the source of neoplastic condition in guinea fowl was from REV infection.

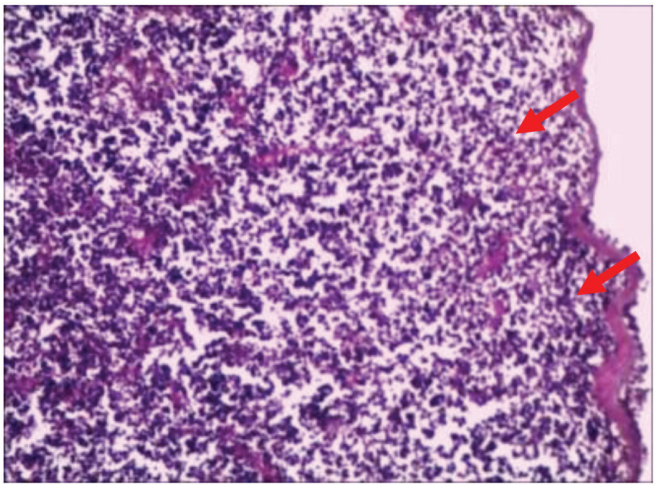

Fig. 1. Infiltration of pleomorphic lymphocytes and reticular endothelial cells in the spleen (Arrow) - H&E x100.

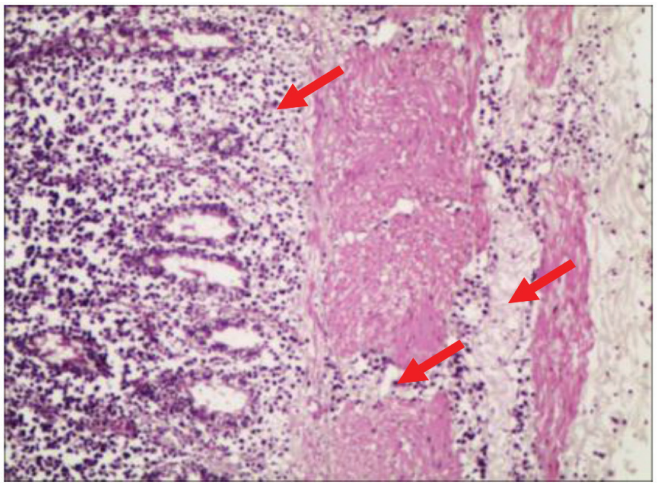

Fig. 2. Infiltration of pleomorphic lymphocytes and reticular endothelial cells in all tunics of intestine (Arrow) - H&E x100.

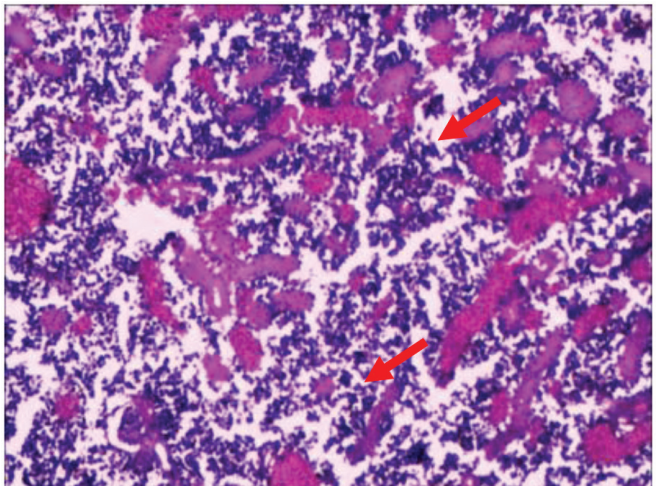

Fig. 3. Infiltration of pleomorphic lymphocytes and reticular endothelial cells in the interstitium of kidney (Arrow) - H&E x100.

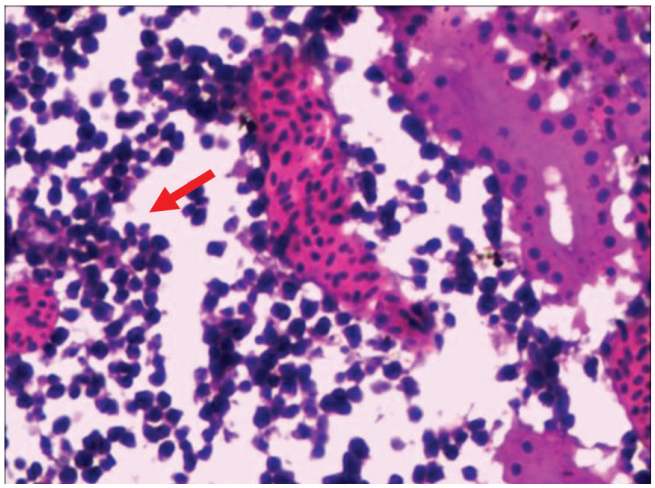

Fig. 4. Infiltration of pleomorphic lymphocytes and reticular endothelial cells in the interstitium of kidney (Arrow) - H&E x400.

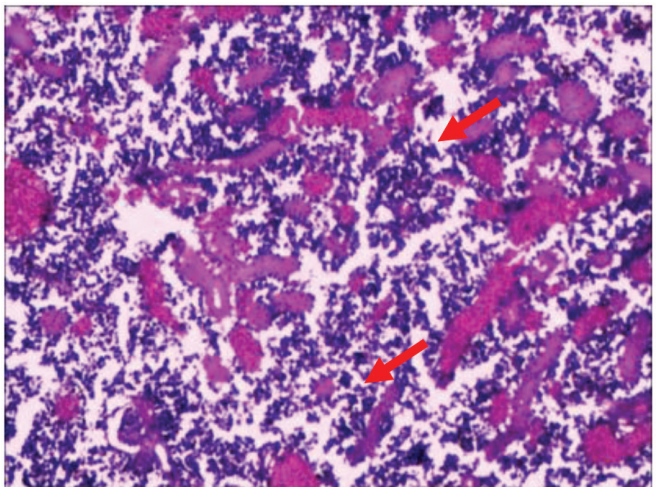

Fig. 5. Pleomorphic lymphocytes and reticular endothelial cells replace the hepatic cords of liver (Arrow) - H&E x100.

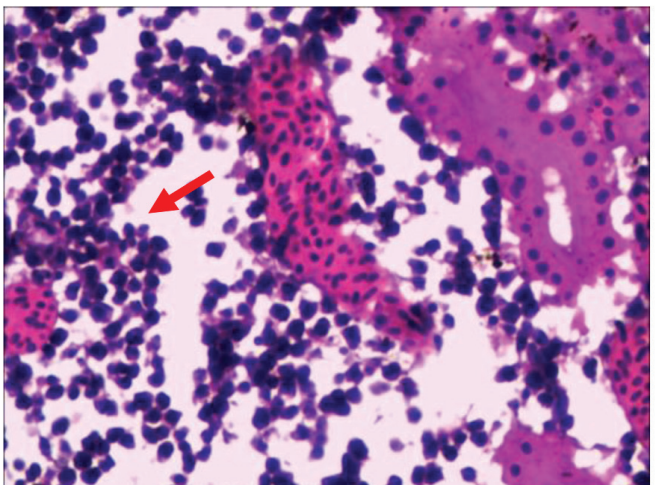

Fig. 6. Pleomorphic lymphocytes and reticular endothelial cells replace the hepatic cords of liver (Arrow) - H&E x400.

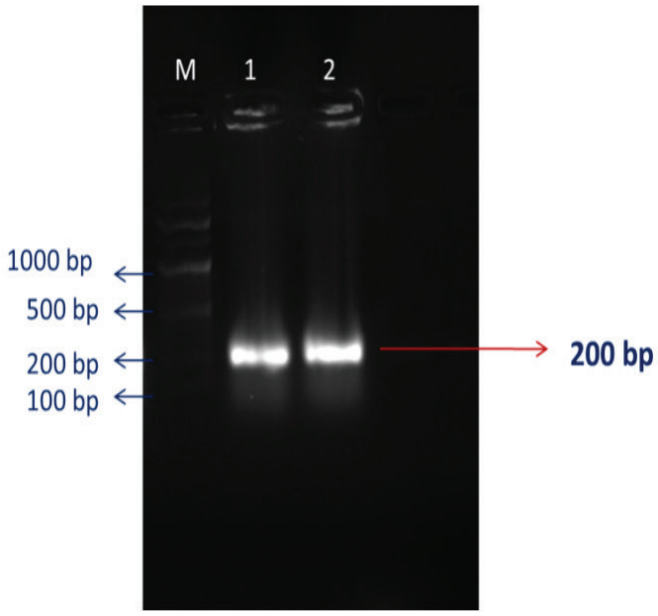

Fig. 7. Agarose gel electrophoresis showing amplicons specific to LTR gene of REV. (Lane M: DNA Marker, Lane 1 & 2: Samples).

The PCR results were found to be consistent with previous reports for detection of REV in tumor samples using LTR gene. Multiplex PCR was used for differential detection of MDV, ALV and REV in poultry laying hens and turkeys using LTR as one of the primer for detection of REV [8]. REV was detected and characterizedby amplifying the partial gene of LTR with a product size of 200bp in length from visceral tumors in broiler breeder chickens [27].

The sequences were closely related with other reference sequences of REV available in NCBI database. The nucleotide sequence of REV in the present study was found to have 100% identity with REV strains of chickens reported in Tamil Nadu (Accession numbers MH365472 and MF512029) and more than 99% identity with other REV strains of pigeon (Accession number PP526161), Muscovy duck (Accession number MN812764) and chicken (Accession number MF631845) in the countries *viz*. Thailand, China and Brazil respectively.

Fig. 8. Phylogenetic tree of avian reticuloendotheliosis virus of Orathanadu strain in guinea fowl (MH 536198.1) based on nucleotide sequence.

CONCLUSION

The sequence obtained in this study was clustered in the group comprising of REV sequences of other countries which clearly indicated that the tumors were originated from REV. Hence, the present study concluded that avian reticuloendotheliosis virus is circulating in the flock which is responsible for mortality in Guinea fowl.

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