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Research Article

Molecular identification of Reticuloendotheliosis virus in turkey and poultry layer farms of Tamil Nadu, India

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Keywords

Reticuloendotheliosis,
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Abstract

Reticuloendotheliosis virus is an avian type-C retrovirus which transforms pre-B and pre-T lymphocytes, causing bursal and T-cell lymphomas in chickens and turkeys, classified within the family *Retroviridae*, subfamily *Orthoretrovirinae*, genus *Gammaretrovirus*. Suspected tissue samples viz liver, spleen were collected from chicken and turkey at Namakkal, Tamil Nadu and initially screened for histopathological examination by using paraffin fixed sections. Histopatholoy suspected tissue sections were further confirmed by immunohistochemistry using REV polyclonal serum (US biological). Due to absence of positive control for polymerase chain reaction (PCR), Deoxy Ribonucleic acid (DNA) isolated from immunohistochemistry positive tissue section and tissue were utilized. By this the tissue samples were screened by 5'LTR-PCR. The PCR products were further confirmed by sequencing and BLAST analysis. The field strain sequences were aligned using GENE TOOL software and analyzed with similar sequences available in Genbank using phylogeny construction (MEGA 5.0). The virus is isolated in CEF cell culture system and the presence of virus was confirmed by ELISA using REV polyclonal serum. The positive DNA samples were further confirmed with REV- envelope specific PCR to confirm the provirus rather than LTR sequences.

Introduction

Reticuloendotheliosis virus is an avian type-C retrovirus which transforms pre-B and pre-T lymphocytes, causing bursal and T-cell lymphomas in chickens and turkeys. The International Committee on Taxonomy of Viruses has classified REVs within the family *Retroviridae*, subfamily *Orthoretrovirinae*, genus *Gammaretrovirus* [1]. Infection of newly hatched chickens and turkeys by replication defective strain T virus results in high mortality from neoplastic disease, acute reticulum cell neoplasia 1-3 weeks later. It is uncertain whether this form of disease occurs in the field. Non defective strains of REV induce two types of chronic lymphoid neoplasia. First, bursal dependent B cell lymphomas of visceral organs indistinguishable from lymphoid leukosis occur in chickens after a long latent period (4-10 months). Second, bursal independent T cell lymphomas have been induced experimentally that are comprised of large uniform lymphoreticular cells that arise in various visceral organs and peripheral nerves. Birds infected with nondefective REV may develop a runting syndrome, with abnormal feathering and sometimes infiltration of peripheral nerves [2].

In India, the marek's disease was observed in 1970, the time when poultry industry experienced a sudden spurt, both because of import of foreign breeds and also due to intensive rearing [3]. Poultry oncogenic viruses MDV and ALV, from different parts of India were reported namely Punjab, Haryana, Delhi, Tamil Nadu [4], Assam, Andhra pradhesh, Karnataka and Gujarat. However, there is not much information about the prevalence of REV in India.

Materials and Methods

Sample Collection

A total of 202 suspected tissue samples viz liver, spleen were collected from chicken at Namakkal, Tamil Nadu, India and the same tissue pieces were stored in formalin for histopathological studies.

Histopathological examination

All the samples were initially screened for histopathological examination by using paraffin fixed sections.

Immunohistochemistry

Based on histopathology results, the samples were further analyzed using immunohistochemistry using polyclonal antibodies against REV (US biologicals).

DNA isolation

All the suspected tissue samples were subjected to DNA extraction using previously described method [5]. Due to absence of positive control, immunohistochemistry positive tissue section was utilized as positive and DNA extraction from this section was done as per the method described by [6].

Polymerase chain reaction

The 5'LTR primers were used for initial screening of samples for REV. The primers were reported previously [7], [8]. The reaction conditions used for amplification were as follows: Initial denaturation 94°C – 1 min followed by 35 cycles of Denaturation 94°C – 1 min, Annealing 46°C – 1 min, Extension 72°C – 1 min and final extension 72°C – 5 min.

Sequencing

The purified PCR products were subjected to sequencing and the sequencing datas were analyzed using NCBI Blast analysis.

Phylogenetic analysis

The forward and reverse sequences obtained from cycle sequencing were aligned as single PCR product sequence using GENE TOOL 1.0 software. The similar sequences of different strains were obtained from NCBI database and used for phylogenetic tree construction using MEGA 5.0 software.

Isolation and identification of virus

Chicken embryo fibroblast culture (CEF) was used for isolation of REV. The PCR positive samples were used as inoculum. The virus in cell culture was identified by ELISA using virus specific polyclonal antibodies (US biologicals).

Reticuloendotheliosis virus *envelope* gene specific PCR

PCR primers reported earlier by Kim and Tripathy (2001) [9] were used.

Primer	Target sequence	Size
Fp: 5' CGAGAGTGGCTCGCGAGATGG 3' Rp: 5' ACACTACATTTCCCCCTCCCTAT 3'	*env* gene of REV (FJ496333.1)	642 bp

The 20.0µl volume of reaction mixture consisted of Red dye master mix(2x)- 10.0µl, forward primer- 1.0µl (20 pmoles), reverse primer- 1.0µl (20 pmoles), DNA template- 2.0µl and nuclease free water- 6.0µl. The reaction conditions used for amplification were as follows: Initial denaturation 94°C – 1 min followed by 35 cycles of Denaturation 94°C – 1 min, Annealing 55°C – 1 min, Extension 72°C – 1 min and final extension 72°C – 5 min.

Results

Gross pathology

In cases positive for reticuloendotheliosis virus, nodular lesions were noticed in the visceral organs especially in the liver and spleen. Intestinal tumors were noticed in some positive cases (Fig. 1).

Histopathology

Reticuloendotheliosis virus affected tissue section showed homogenous monomorphic lymphoid infiltration.

Immunohistochemistry

Reticuloendotheliosis virus positive signals were seen in cytoplasm and nuclei of affected liver tissue section (Fig. 2).

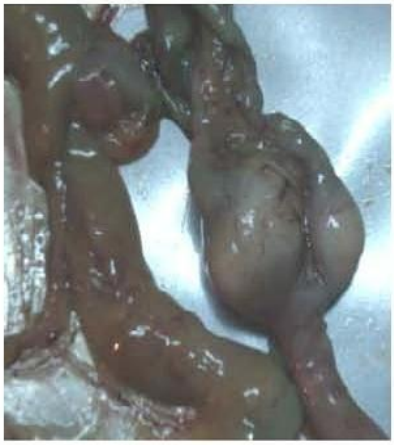

Fig. : Turkey - Intestinal tumour showing REV positive by PCR

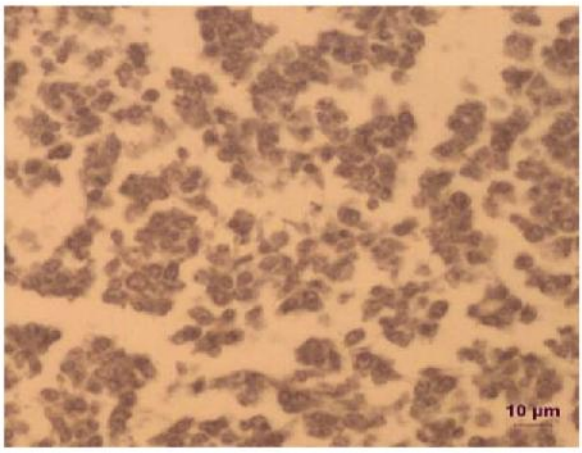

Fig. : Liver-Reticuloendotheliosis virus-positive signals were seen in cytoplasm and nuclei (DAB Bar=10 µm)

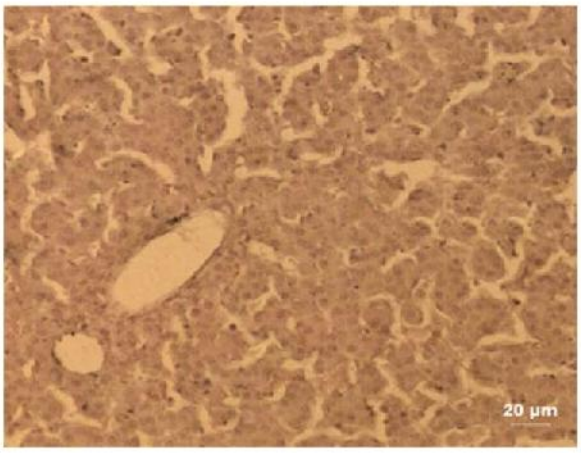

Fig. : Liver-normal-no virus signals were seen in cytoplasm and nuclei (DAB Bar=20 µm)

Detection of REV genome from the biological samples

Reticuloendotheliosis viruse (5' LTR – 291bp) were checked by using the template DNA extracted from immunohistochemistry positive tissue sections and tissues. Sensitivity of these primers was checked by using different dilutions of positive tissue DNA samples (Fig. 3). A total of 202 suspected samples were screened for REV and by PCR. The immunohistochemistry positive tissue DNA sample and uninfected tissue DNA sample were used as positive and negative controls respectively. The results were listed in Table.1.

Table.1: Total number and type of samples screened by PCR for avian oncogenic viruses

Species	Sample type	Total number of samples screened by PCR	Number of positive samples
			REV
Chicken	Liver	70	8
	Spleen	48	8
	Bursa	7	0
	Kidney	5	0
Turkey	Liver	5	2
	Spleen	5	0
	Intestine	3	2
Total		202	20

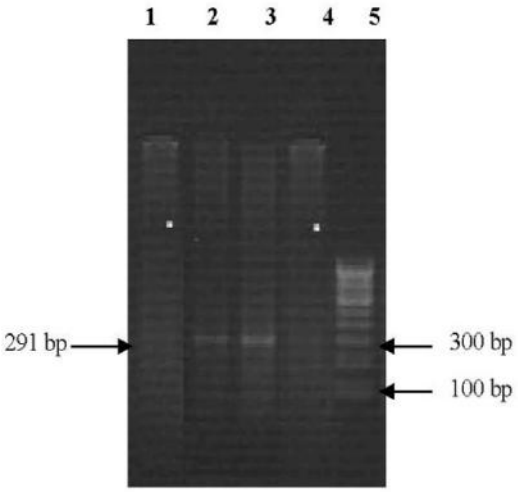

Fig : Specificity of LTR primers

Lane 1: IHC-ALV positive tissue section DNA
Lane 2: IHC-REV positive tissue sectionDNA
Lane 3: IHC-REV positive tissue DNA
Lane 4: IHC-ALV positive tissue DNA
Lane 5: DNA molecular weight marker (100 bp)

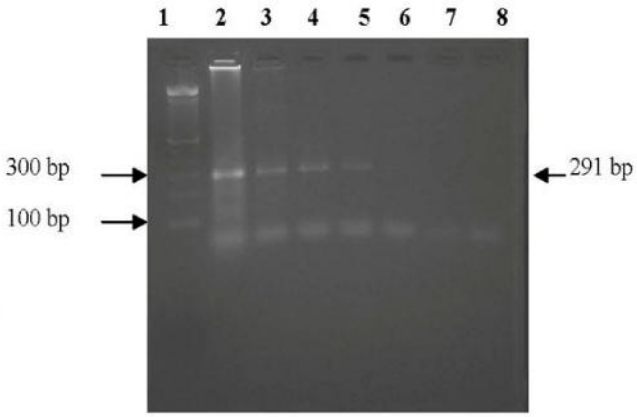

Fig : Sensitivity of LTR primers using positive tissue DNA

Lane 1: DNA molecular weight marker (100 bp)
Lane 2: Positive tissue DNA (1237.8 ng/µl)
Lane 3-8: 10^1 to 10^6 dilutions of positive tissue DNA

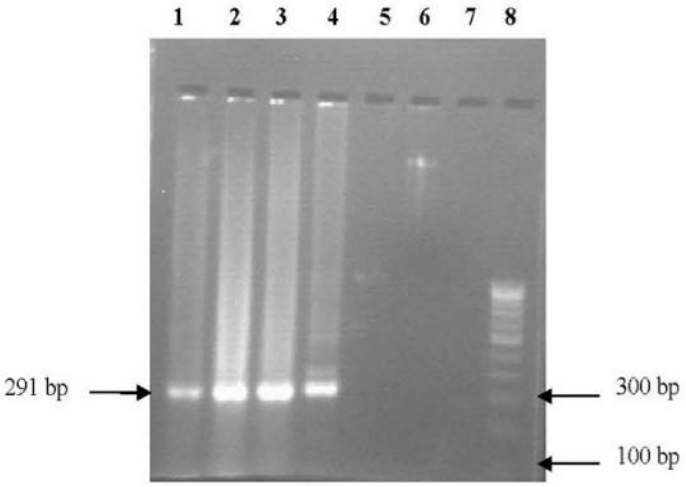

Fig : Screening of samples by LTR primers
Lane 1- 4 : Positive tissue samples
Lane 5, 6 : Negative tissue samples
Lane 7 : Nuclease free water (without DNA)
Lane 8 : DNA molecular weight marker (100 bp)

Fig. : Reticuloendotheliosis virus- PCR amplification- LTR primers- 291bp

Sequence analysis

The purified PCR products of REV were sequenced and the sequences obtained were analyzed using BLASTn to check for the homology (www.ncbi.nlm.nig.gov/blastn). The percentage homology of our field sample sequences with other GenBank sequences were listed in table (Table. 2).

Table.2: Comparison of nucleotide sequence homology for *LTR* sequence of REV (field strain) with *LTR sequence* of other REV virus strains available in GenBank

S. No.	Accession No.	Organism and strain	Homology expressed in percentage
1.	FJ496333.1	Reticuloendotheliosis virus strain ZD0708 from China, complete genome	97%
2.	FJ439120.1	Reticuloendotheliosis virus strain chicken/3337/05, complete genome	97%
3.	FJ439119.1	Reticuloendotheliosis virus strain goose/3410/06, complete genome	97%
4.	DQ387450.1	Reticuloendotheliosis virus strain APC-566, complete genome	97%
5.	GQ870290.1	Reticuloendotheliosis virus LTR, partial sequence	97%
6.	AY842951.1	Reticuloendotheliosis virus strain HA9901 from China, complete genome	95%
7.	DQ003591.1	Reticuloendotheliosis virus strain SNV from USA, complete genome	92%

Phylogenetic analysis

In REV, the phylogenetic tree was constructed using the *LTR* (227 bp) sequence of Tamil Nadu field strain and reference strains present in the NCBI database. When rooted by the mid-point method, the Tamil Nadu field strain formed a group with the strains ZD0708 (China), 3337/05 (Taiwan), 3410/06 (Taiwan) and APC-566 (USA) (fig.4).

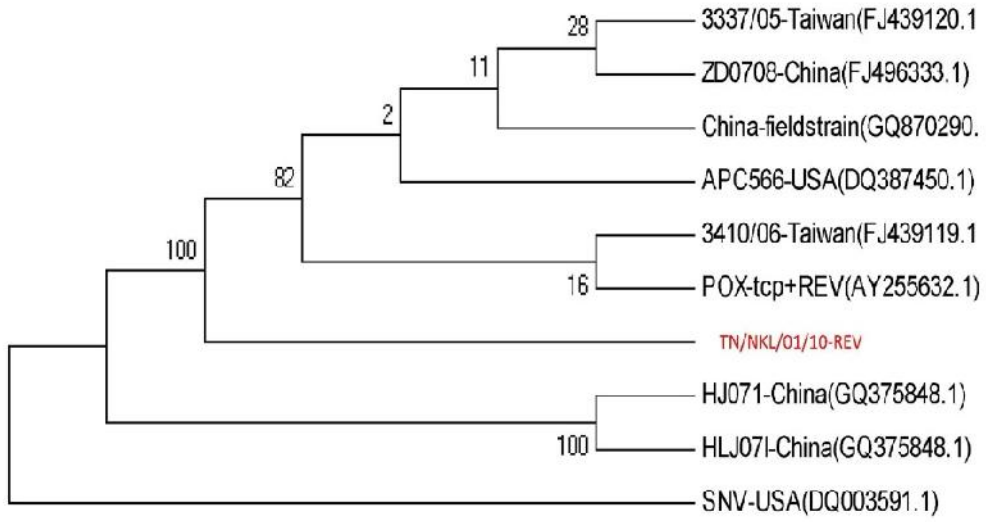

Fig. : Phylogenetic analysis of Tamil Nadu REV field strain - LTR sequence (227 bp)

Isolation and identification of virus

CEF infected with 5th passage REV produced no cytopathic effect after 5th day of infection. Hence, presence of virus in the infected culture was confirmed by ELISA titre values using virus specific serum (polyclonal serum against REV, US biologicals) (Table.3) of infected culture fluid in comparison with uninfected culture fluid clearly indicated the multiplication of virus in cell culture system.

Table.3: ELISA OD values (at 492nm) for identification of REV in cell culture

Antigen	Primary antibody	ELISA OD value MEAN ± S.D
CEF infected with REV	REV specific polyclonal antibody	0.868 ± 0.110
uninfected CEF	REV specific polyclonal antibody	0.148 ± 0.039
CEF infected with REV	ALV specific polyclonal antibody	0.082 ± 0.014

REV *envelope* gene specific PCR

The REV positive DNA samples showed the expected amplicon size of 642bp by using REV *envelope* specific primers (Fig. 5).

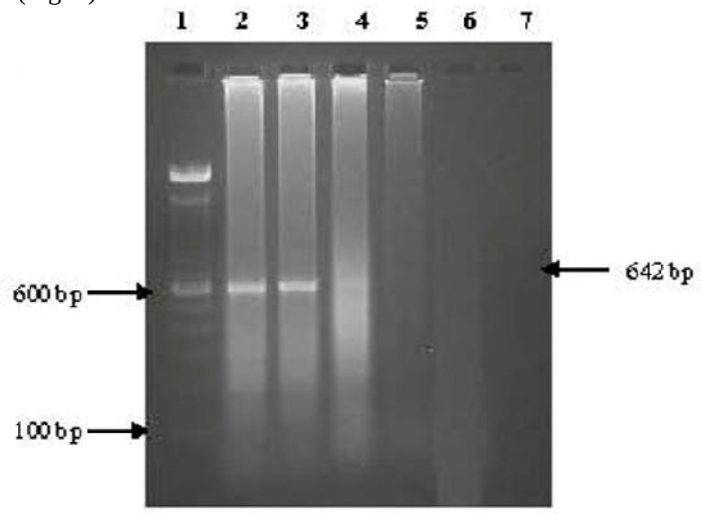

Fig : REV envelope specific PCR

Lane 1 : DNA molecular weight marker (100 bp)
Lane 2-6 : Suspected tissue samples
Lane 2, 3 : Positive tissue samples
Lane 4-6 : Negative tissue samples
Lane 7 : Nuclease free water (without DNA)

Discussion

The gross lesions caused by infection with avian oncogenic viruses overlap and are of a low degree of pathognomy, and diagnosis based on gross lesions is often obscure, veiled and specific laboratory diagnosis are needed [10]. So, samples suspected for Reticuloendotheliosis virus were collected after post-mortem findings and analysed by further molecular diagnosis in this study. In histopathology, REV infection shows uniform population of lymphoid cells but there is no possibility to distinguish between ALV and REV [11]. Hence the microscopic findings should be confirmed with immunological techniques. Here in this study, immunohistochemistry was used for identification of virus in suspected tissue sections using virus specific antibodies. In immunohistochemistry, heat induced antigen retrieval (HIER) in acidic buffer (Citrate buffer pH 6.0) was used for antigen retrieval. The positive labeling of reticuloendotheliosis virus particles as inclusion bodies was present both in the cytoplasm and nuclei of cells [12].

The PCR appears to be a method of choice for the diagnosis of avian oncogenic viruses as it overcomes the veiled aspects of differential diagnosis [13]. So screening of multiple numbers of samples for Reticuloendotheliosis virus was done using PCR in this study. Due to non-availability of reference strains of REV using the immunohistochemistry positive tissue DNA samples as positive controls, the large numbers of DNA samples were screened for REV. The detection of REV in the DNA from tissue samples by PCR amplification using 5'LTR primers yielded an amplicon of 291 bp which implies that the genome of the REV in the sample as reported by [7][8].

The results were further confirmed by sequencing of amplicon and BLAST analysis. The REV 5'LTR sequence of field sample has homology of 97% with the strains ZD0708 (China), 3337/05 (Taiwan), 3410/06 (Taiwan), APC-566 (USA) and grouped with these strains on phylogenetic analysis. Hence phylogenetic analysis clearly indicated that our field strains were closely related with other reference strains present in the NCBI database.

Cytopathic effects may not be seen on primary isolation of REV. Hence, the presence of virus is confirmed routinely by the demonstration of viral antigen in infected cell culture using polyclonal or monoclonal antibodies by immunofluorescence, immunoperoxidase staining, complement fixation or enzyme immunoassay. In comparative studies, enzyme immunoassays were more sensitive than complement fixation tests and indirect immunofluorescence was more sensitive than indirect immunoperoxidase or immunoelectron microscopy [14]. So, ELISA for REV was done for identification of virus and the presence of virus was further confirmed using PCR by amplifying the LTR region.

Detection of proviral DNA by polymerase chain reaction (PCR) assays that amplifies the 291bp product of REV 5' LTR has been shown to be a sensitive and specific method for detection of various strains of REV [7], [8]. Garcia *et al.* (2003) found that assays amplifying REV envelope and REV 3' LTR sequences provided a more accurate diagnosis than PCR amplifying the REV 5' LTR region alone. The authors reported that to differentiate virus strains that carried intact REV provirus form those that carried solo 5' LTR sequences, positive PCR results with primers that amplified the 5' LTR should be confirmed with more specific PCR assays such as the envelope or the REV 3' LTR PCR. Hence in this study, 5' LTR and envelope primers were used to get accurate results.

Because of the usually sporadic and subclinical nature of REV infections, no control procedures have been necessary commercially. However, it is probable that eradication could be achieved by prevention of vertical transmission through testing egg albumen samples for REV gs antigen, testing males, and rearing progeny in isolation. An intriguing recent finding has been evidence for the presence of REV genetic sequences integrated on occasions into MDV genome [15]. This finding raises the possibility that the pathogenicity of MDV could be modified, and also that REV (and perhaps other retrovirus genomes) could be transmitted within the MDV genome [16].

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