

# Serological Investigation and Molecular Characterization of Reticuloendotheliosis Virus from Breeder Flocks in Pakistan by Polymerase Chain Reaction

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## ABSTRACT

**OBJECTIVE:** The goal of this study was to provide the authentic information about the incidence of reticuloendotheliosis virus in Pakistan because several epidemiological studies suggested a link between avian oncogenic viruses and different types of cancers in people working in poultry industry.

**METHODOLOGY:** Cross sectional study to determine the REV infection status. Presence of antibodies in serum were observed using ELISA and confirmation of REV genome was made by polymerase chain reaction using long terminal repeat specific primers to amplify a 291bp fragment of REV viral genome from tumor samples of liver collected from breeder flocks with average age of 30 weeks. In all 480 serum and liver samples were taken from 24 poultry breeder across Punjab and Khyber Pakhtunkhwa.

**RESULTS:** Out of 480 serum samples 42 serum samples which were found positive for REV antibodies by ELISA. When subjected to PCR revealed n=37 to bear REV genome.

**CONCLUSION:** It is concluded that reticuloendotheliosis virus is present in breeder flocks across a wide range of area across Pakistan which can be hazardous to public health.

**KEY WORDS:** Reticuloendotheliosis virus, Polymerase chain reaction, Pakistan

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## INTRODUCTION

Reticuloendotheliosis virus is a retrovirus. Retroviruses are enveloped RNA viruses. They integrate themselves into the host's cell genome which aids in their detection from the genome of host's cell DNA. They replicate by RNA dependent reverse transcription. REV group is divided into two groups. REV replication defective strain T and REV non defective strain A. REV T strain infects avian species and has the most oncogenicity, causes tumors of spleen and kidney<sup>1</sup>.

Indigenous and spontaneous infection of REV is seen in many avian species such as chicken, Japanese quail, turkeys, pheasants, geese and ducks. Cases of reticuloendotheliosis in avian species were observed throughout the world. Its frequency is increasing day by day as there is no such vaccine available<sup>2</sup>.

Reticuloendotheliosis virus is transmitted vertically, horizontally, by vector and through contaminated vaccines. The most important source of spread of REV which has been reported, is by the unintended and incidental contamination of vaccines especially Marek's disease and fowl pox disease vaccines by

reticuloendotheliosis virus<sup>3</sup>. Many economic losses of poultry flocks were noted and proclaimed due to REV contaminated vaccines.

Reticuloendotheliosis is a neoplastic condition which is of great commercial and economic importance. Reticuloendotheliosis is responsible for inducing various disease syndromes which includes most importantly runting disease syndrome, immunosuppression and lymphoproliferative disorders and lymphomas. Runting syndrome is a group of diseases in which a majority of chickens in the poultry flocks are of smaller size than normal. It is due to inappropriate growth. Thus the flock's average weight is markedly low. It is also characterized by slow and abnormal feathering, broken feathers also referred to as helicopter feathers<sup>4</sup>. Diarrhea is also seen. It is mostly due to the REV non defective T strain. When REV infection occurs at an early age, it is responsible for producing T cell and B lymphomas especially with non-defective strain T. Lymphomas of B cell origin are reported to be produced by REV non defective strain (REV-T) Due to conflicting and confounding pathogenesis and pathology associated with

neoplastic diseases of birds e.g. Marek's disease, avian leucosis and reticuloendotheliosis, polymerase chain reaction (PCR) gives the most definitive affirmation in the diagnosis of REV<sup>5</sup>.

Common antigenicity has been discovered between the glycoprotein antigens obtained from neoplasms of chickens and antigens obtained from variety of human cancers. This suggests that retroviruses are possible etiological agents in human cancers<sup>6</sup>.

#### **Goals of Study**

This research will give the evidence about the infectious disease under study, provide the authentic information about incidence and will guide the veterinary experts and risk management authorities to diagnose this emerging virus in the flocks so that prevention and control measures should be practiced.

#### **METHODOLOGY**

This cross-sectional study was conducted at Institute of Molecular Biology and Biotechnology (IMBB department), The University of Lahore. It was an approved project of IMBB department, UOL. Study was carried out during September 2016 – April 2017.

#### **Inclusion Criteria**

Samples were collected from those breeder flocks that had mortality rates greater than 5% and showed depressed growth, abnormal feathering, decreased hatchability and neoplastic changes in viscera. There was no epidemic at the time of sample collection.

#### **Exclusion Criteria**

Samples were not collected by those flocks whose mortality rates were less than 5% and did not show the characteristic symptoms of reticuloendotheliosis virus.

#### **Sample Collection**

Simple Random Sampling technique was used in two steps. In first step breeder farms were randomly selected from Punjab and KPK and in second step the diseased chickens were randomly selected from those breeder farms. Serum and liver samples n=480 each were collected during the months of September 2016 – December 2016 randomly from chickens of poultry breeder farms which showed depressed performance and neoplastic lesions in viscera. There was no epidemic at that time. Total 300 samples were collected from Punjab and 180 from KPK. The average age of those flocks was 30 weeks. Diseased chickens n=20 were separated from each flock and their serum and liver samples were taken for the detection of REV antibodies for ELISA and molecular characterization by PCR respectively<sup>6</sup>.

#### **Detection of Antibodies to Rev By Elisa**

The serum samples were tested for REV antibodies

by ELISA using REV antibody Test kit (IDEXX laboratories). ELISA was performed according to the instructions provided in the manual. If the S/P value (sample to positive ratio) was greater than 0.50 (SP >0.50) the result was considered positive. For precision of results samples were run in duplicates<sup>7</sup>.

#### **MOLECULAR CHARACTERIZATION OF REV**

##### **Inclusion Criteria for Molecular Characterization**

Only those samples were processed for molecular characterization whose serum counterparts were positive for REV antibodies by ELISA<sup>4</sup>

##### **DNA Extraction from Liver Samples**

DNA was extracted from liver samples for the molecular characterization by PCR. DNA was extracted by using QIAamp DNA mini kit (QIAGEN). This kit is designed for quick and effective purification of genomic DNA from frozen tissue samples. The extracted DNA was then stored at -20 °C<sup>2</sup>.

##### **Polymerase Chain Reaction**

The primers were used against the long terminal repeat regions (LTR) of REV genome. Primers were used to amplify a 291bp amplicon of LTR of REV genome. The primers used for this purpose were (5'-CATACCTGCAGCCAATGGTT-3') and (3' AATGTTGTACCGAAGTACT-5'). The Genbank accession no followed was KX278301<sup>8</sup>.

PCR reaction was performed in a 50µl reaction mixture, containing 5µL PCR buffer, 3µL MgCl<sub>2</sub>, 1µL dNTPs, each primer 1µL, Taq DNA polymerase (Fermentas) 1µL and 1µL template DNA PCR conditions in the thermal cycler (Applied Biosystems 2720) were: initial denaturation at 95°C for 5 minutes; 32 cycles of denaturation at 95°C for 30 seconds; annealing at 55°C for 1 minute; primary extension at 72°C for 1 minute; final extension at 72°C for 6 minutes and final hold at 4°C. After completion of polymerase chain reaction the samples were loaded on the gel. Each well was loaded with 7.5 µL of sample alongside 3-5 µL of 100bp DNA ladder or marker. Gel was run at 120 volts for 40 minutes in gel electrophoresis instrument and then visualized by UV transilluminator<sup>8</sup>.

#### **RESULTS**

Out of the Four Hundred and Eighty serum samples Forty two were found positive for REV antibodies on ELISA and genomic DNA was identified in 37 samples using polymerase chain reaction. Percentages were calculated using SPSS v 21 according to area wise positive isolates.

##### **TOTAL NO OF POSITIVE SAMPLES FOR REV ANTIBODIES ON ELISA**

Of all Forty two were found positive for REV

**TABLE I: DETAILS OF FLOCKS, TOTAL NO OF SAMPLES AND NUMBER OF POSITIVE SAMPLES**

Farm no	Area	District	Province	Age of flock	Total no of samples	Positive samples on ELISA	Percentage of positive isolates	Mortality rates
1	Pattoki	Kasur	Punjab	27 weeks	20	8	40 %	7%
2	Behali	Mansehra	KPK	34 weeks	20	15	75 %	21%
3	Sherwan	Abottabad	KPK	38 weeks	20	19	95 %	30%

**TABLE II: ELISA RESULTS OF THE POSITIVE SAMPLES**

Sample No	SP Ratio	Sample No	SP Ratio
1.	8.917	22.	1.328
2.	6.321	23.	2.543
3.	5.216	24.	7.961
4.	1.397	25.	5.552
5.	2.544	26.	3.606
6.	3.890	27.	2.371
7.	4.578	28.	9.846
8.	6.552	29.	1.254
9.	4.310	30.	1.987
10.	7.358	31.	5.364
11.	4.695	32.	4.212
12.	2.361	33.	7.457
13.	12.115	34.	2.981
14.	5.961	35.	2.397
15.	2.367	36.	5.006
16.	4.211	37.	4.070
17.	8.312	38.	1.798
18.	4.555	39.	3.432
19.	5.639	40.	3.257
20.	3.746	41.	4.147
21.	1.264	42.	2.305

antibodies on ELISA. Eight samples were positive out of 20 samples from a poultry breeder flock from Pattoki, Kasur (40% positive isolates), 15 serum samples were positive out of 20 from a poultry breeder flock from Behali, Mansehra (75 % positive isolates) and 19 serum samples were positive on ELISA from a breeder flock from Abottabad, KPK (95% positive isolates). Details of flocks, total no of samples and no

of positive samples are listed in Table I. Values of sample to positive ratios (SP values) determined as mean of optical absorbances are given in Table II. The value if was greater than 0.50 declared a positive result on ELISA.

#### **TOTAL NO OF SAMPLES POSITIVE ON PCR**

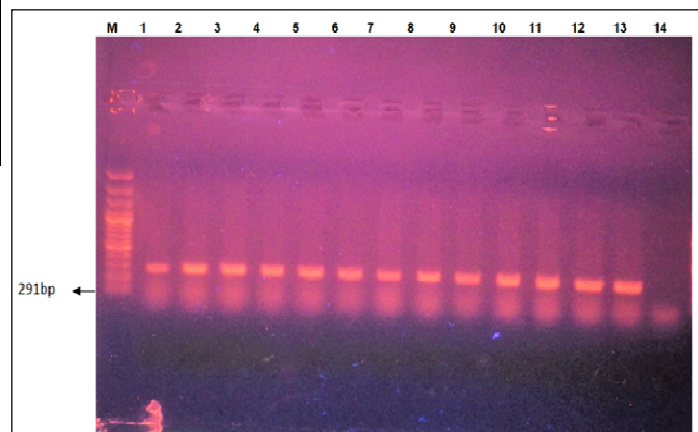
DNA was extracted from liver samples and molecular characterization was done by polymerase chain reaction. Out of Forty two samples found positive by ELISA the genomic DNA was identified in 37 samples. Results received upon gel electrophoresis are shown in the figures 1, 2 and 3. The figures show the bands of LTR fragments of REV genome (291bp) as discrete bands.

#### **PROVINCE WISE COMPARISON OF REV**

Out of Four Hundred and Eighty samples Three hundred samples were taken from Punjab and One Hundred eighty from KPK. Out of Three hundred samples from Punjab 8 samples were found to possess REV antibodies (2.7% positive isolates). Out of One hundred eighty samples from KPK 34 samples were found positive for REV antibodies (18.9% positive isolates). The data shows the incidence of REV to be higher in KPK than in Punjab.

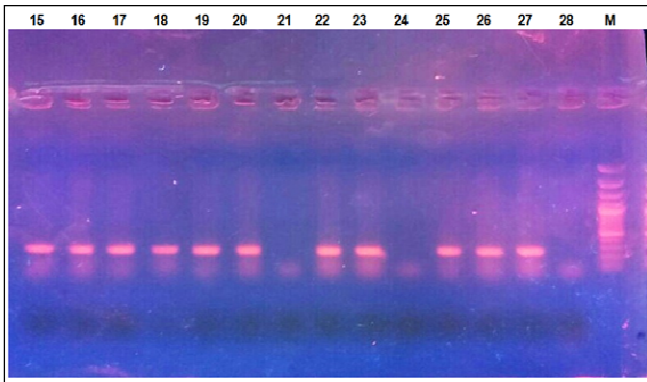
#### **Figure 1: Appearance of bands of LTR fragment of REV genome (291bp) on gel electrophoresis.**

**Lane M : Molecular marker (100 bp DNA ladder); Lane 1-14 : Samples**

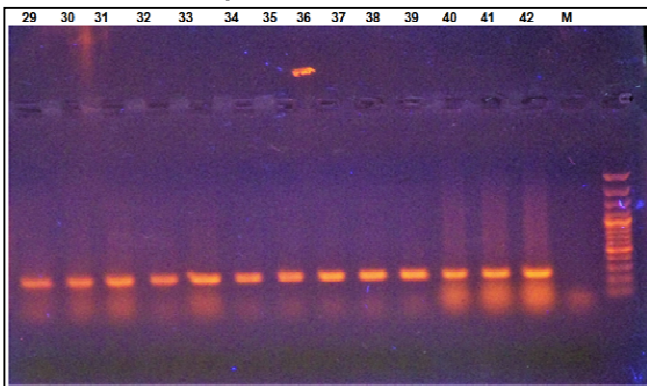




**Figure II: Appearance of bands of LTR fragment of REV genome (291bp) on gel electrophoresis. Lane M : Molecular marker (100 bp DNA ladder); Lane 15-28 : Samples**



**Figure III: Appearance of bands of LTR fragment of REV genome (291bp) on gel electrophoresis. Lane M : Molecular marker (100 bp DNA ladder); Lane 29-42 : Samples**



## DISCUSSION

In this study the signs and symptoms that were typical to REV were detected and were noted in chickens in breeder flocks at an average age of 32 weeks. In general this study shows the presence of REV in Pakistan. Although the incidence is higher in KPK than Punjab but the significant presence of reticuloendotheliosis virus in samples from Punjab cannot be ruled out.

Liver samples taken from diseased chickens were investigated by PCR for the presence of REV genome. Amplification product at 291bp was indicative of the REV genome. These assays are the diagnostic tools in active REV infection that causes runting disease syndrome and immunosuppression with high mortality and morbidity. There are other techniques e.g. immunohistochemistry and viral isolation which are diagnostic for detection of REV but PCR is the most sensitive and specific<sup>9</sup>. This study provides the specific results about the prevalence of REV virus in chickens in breeder farms in Pakistan.

This study reveals that the age at which REV virus infects birds is between 25-35 weeks and older the flock age the more is the infection and the mortality rate as shown by the results. In Farm 1 (age 27 weeks) 40% positive isolates were detected and mortality rate was 7%, Farm 2 (age 34 weeks) showed 75% positive isolates and the mortality rate was 21% whereas the highest infection rate was found from Farm 3 (age 38 weeks), 95% positive isolates and 30% mortality rate. This shows that REV infection is more severe in older flocks. These results are in harmony with the studies done by Yang *et al.* which showed that the sero-prevalence of REV increased with increasing age of the flock but not immediately after the administration of Marek's disease and fowl pox disease vaccines, whereas the study done by Wei *et al.* illustrated the outbreak of REV infection in young flocks which revealed that the infection of REV in young chickens was most probably due to administration of REV contaminated vaccines or by congenital transmission. Flocks that were found positive for REV in our study were of older age, thus the exposure of REV was assumed to come from environment because it was impossible to have such high maternal antibodies level for all of them as they were more than 25 weeks old and the vaccines used in these flocks had no storage and can't be verified one by one and the infection caused by contaminated vaccines was detected in young flocks<sup>2</sup>.

In this study we also observed that although 3 breeder flocks were found positive for REV antibodies, but their positive rates were quite different from each other as the lowest positive rate was 40% for Farm 1 and the highest positive rate was 95% for Farm 3 was observed. This may be related to the susceptibility to REV of different native chickens or the situation in different regions of Punjab and KPK. These results correlate with the results of study done in native chicken flocks in China.

Due to financial crisis faced by poultry industry, this study aimed to provide information about this newly emerging virus in Pakistan which will help in the management authorities to not only diagnose it timely but it will also save the foreign exchange which is being spent on the definitive diagnosis of the infections in poultry. These viruses have been shown to be capable of infecting human cells invitro and induce neoplastic changes. Studies also showed that poultry workers and subjects in general population are commonly infected with these viruses and these viruses are responsible of the development of cancers of major sites i.e cancers of buccal and nasal cavities, rectum and esophagus, pancreas, trachea, lungs, brain, ovary and tumors of hemopoietic system<sup>11</sup>. These risks were not limited to cancers only but

neurological disorders and diseases of cardiovascular and urinary systems were also detected.

## CONCLUSION

From this study it is concluded that reticuloendotheliosis virus is present in poultry breeder farms in some of the regions of Pakistan and is one of the cause of mortality and morbidity. The study shows some numbers of confirmed cases of REV, which though are significant, are still lower than the suspected ones. The findings of this study gives the sufficient information that will guide the poultry industry officials about the presence of this newly emerging virus so efforts may be made for its prevention and control and direct steps should be taken to look after the poultry workers during carrying out tasks that put them on risk of exposure to the infectious agents. Another important implication of this study is that the general population may be at risk of developing neoplasms from exposure to live poultry or their products or by ingesting raw meat or by receiving contaminated vaccines with poultry oncogenic viruses.

## RECOMMENDATIONS

The poultry industry is a backbone of farming in Pakistan with a substantial impact on state's GDP which is 1.3 %<sup>12</sup>. It has a major influence in the economy of Pakistan but nowadays it has been facing serious challenges due to outbreaks of infectious diseases which confer economic losses are mostly caused by viruses including certain uncommon and unknown ones such as REV. Hence, future studies may be directed to check the prevalence and clinical outcomes of REV on a larger sample size and may be at other or different time of the year. Molecular characterization through genome sequence analysis of the virus strain shall provide information about the strains prevalent in the region (Pakistan) and preparation of vaccines against these indigenous strains for effective control as they infect a wide avian host range<sup>13</sup>. Its potential to exist as a provirus with other viruses is yet another point to consider the REV seriously<sup>14</sup>. Future studies are immediately required to investigate that possibility of avian oncogenic viruses to present as a public health problem

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