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M.M. El-Sebelgy, B.M. Ahmed, N.S. Ata & H.A. Hussein

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Full Length Article

Molecular detection and characterization of reticuloendotheliosis virus in broiler breeder chickens with visceral tumors in Egypt

M.M. El-Sebelgy [a], B.M. Ahmed [b], N.S. Ata [a], H.A. Hussein [b,*]

[a] Department of Microbiology and Immunology, National Research Centre, Dokki, Egypt
[b] Department of Virology, Faculty of Veterinary Medicine, Cairo University, Giza 11221, Egypt

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Reticular cell infiltration;
REV-LTR

Abstract In the present study, reticuloendotheliosis (REV) provirus DNA was detected by PCR using LTR (long terminal repeat)-specific primers to amplify 200 bp fragment of the REV viral genome in tumor samples collected from broiler breeder flocks with 30–40 weeks of age demonstrating neoplastic lesions. Histopathology examination of the liver tumor tissue showed reticular cells infiltration and proliferation replacing hepatic parenchyma. Sequence analysis of the amplified PCR products revealed genetic similarity to REV-LTR in MDV (Marek Disease Virus) JM-Hi3. SPF (specific-pathogen free) chicks (1-day old) were experimentally inoculated with liver homogenate of the REV-positive sample and the chicks were housed for 8 weeks. Visceral organs and sera were collected from inoculated chicks at 3 and 6 weeks post inoculation. REV was detected by PCR in the organs of the inoculated chicks at 6 weeks post inoculation.REV antibodies were detected in sera of the inoculated chicks at 3 (3 out of 10 samples) and 6 (one of 2 samples) weeks post inoculation. Histopathology examination of liver and spleen collected from inoculated chicks showed the characteristic reticular cells infiltration in both organs. The study reports the existence of REV associated with visceral tumors in broiler breeder flocks in Egypt. The sequence of the detected virus was submitted in NCBI GenBank with access number KC018475.

* Corresponding author. Address: Professor and chairman of the department of virology, Faculty of veterinary medicine, Cairo University, Egypt. Tel.: +202 35710309; fax: +202 35729659.
E-mail addresses: husvirol@link.net (H.A. Hussein), best_vet007@yahoo.co.uk, bestvet007@gmail.com (M.M. El-Sebelgy), vet_factor@yahoo.com (B.M. Ahmed), nagwaata@yahoo.com (N.S. Ata).

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1. Introduction

Reticuloendotheliosis virus (REV) belongs to the retrovirus family, genus Gammaretrovirus. The genome of retroviruses consists of single-stranded RNA with positive polarity and present in two copies [1]. The genomic structure of REV consists of a group-specific antigen (gag), protease (pro), polymerase (pol), and envelope (env) regions flanked by long-terminal repeats (LTRs) [2]. The gag gene encodes five structural

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proteins, p10, p12, pp18, pp20 and p30. The p30 (30 kDa) protein is the major REV group-specific antigen [3]. The env gene encodes two envelope glycoproteins, gp90 and gp120 [4]. The pol gene encodes a reverse transcriptase similar to those of mammalian type retroviruses and differs from the Avian Leucosis–Sarcoma Virus [5,6].

REV is immunologically, morphologically and structurally distinct from the leukemia/sarcoma group. REV can cause acute reticular cell neoplasia, chronic neoplasia of lymphoid tissues and other organs, immunosuppression and runting disease syndrome in domestic poultry as well as other avian species. However, natural outbreaks of REV in commercial poultry are rare [7].

It was suggested that REV is a potential contamination hazard in the use of chicken embryos and cells for preparation of vaccines [8], and REV infection can persist at the same production site over a period of several years [9].

Reticuloendotheliosis virus has been isolated from Marek's disease vaccines [10]. Also, high mortality rate, neurological symptoms, and feathering abnormalities (nakanuke) were reported in chickens vaccinated with a contaminated Marek's disease (HVT) vaccine. These cases were attributed to REV, which was detected as a vaccine contaminant in one commercial vaccine batch [11]. REV was isolated from a commercial Marek's disease vaccine (herpesvirus of turkeys) by serial passage of the REV-contaminated vaccine on chicken embryo fibroblast (CEF) and detected REV antigen using a fluorescence antibody test [12]. In the Middle East and Africa, REV-associated lymphoma, not related to a contaminated vaccine, has been reported [13,14].

Few studies have reported REV in Egypt [15–17]. In the present study, we investigate the association of REV with tumor collected in year 2005 from broiler breeder flocks in Giza Governorate, Egypt.

2. Materials and methods

2.1. Sampling

A total of 50 tumor tissue samples were obtained in the production period from 5 broiler breeder flocks (10 samples each), where the farms were located in Giza Governorate. Tumors from liver, spleen, ovaries and testicles were removed aseptically and divided into 2 parts, the first one was kept in buffered formalin 10% for histopathology and other part was kept frozen at −70 °C and used for DNA extraction. Additional flock information provided in Table 1.

2.2. PCR oligos and PCR condition

Primers LTRF2 (5′-GCGCTGGCTCGCTAACTG-3′) and LTRR2 (5′-TTCGATCTCGTGTTTGTTCGTGATT-3′) [18] were used to amplify a 200 bp amplicon from REV LTR. DNA was extracted from frozen tissue samples using Biospin Tissue Genomic DNA Extraction Kit according to manufacturer's recommendation (BioFlux-Japan).

PCR was carried out using Taq PCR Master Mix (Promega-US) with total volume of 50 µl as follows; 25 µl Master Mix, 1 µl forward primer, 1 µl reverse primer followed by 22 µl nuclease-free water and finally 1 µl of extracted DNA. PCR program condition was carried out according to [18] with some modification using Applied Biosystems GeneAmp PCR System 9700 applying the following program; initial denaturation cycle at 94 °C for 5 min, 40 cycles consisted of denaturation at 94 °C for 30 s, annealing at 58.5 °C for 30 s, extension at 72 °C for 45 s followed by a final extension cycle at 72 °C for 5 min. The PCR product was visualized by electrophoresis of 5 µl product in 1.5% agarose in 1X TAE, ethidium bromide was added to a concentration of 0.5 µg/ml for nucleic acid visualization. The remaining 20 µl were submitted to "VACSERA Gene Analysis Unit, Egypt" for sequencing and result received as hard copy.

2.3. Sequence analysis and GenBank submission

The results of sequencing were analyzed by BLAST web tool of the GenBank (NCBI) http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome then sequences were downloaded and imported into BIOEDIT version 7.0.1.4 for multiple alignments.

MEGA version 5.05 was used to construct phylogenetic trees for the amplified sequence with the highly similar sequences in regard to BLAST result with the exclusion of 100% similar sequences. One thousand bootstrap replicates were conducted to assess the statistical support for the tree topology. Sequence submission followed the instructions of the BankIt tool of the GenBank http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank. The obtained accession number for the submitted sequence is KC018475.

2.4. SPF chick inoculation

A total of 30 one-day-old SPF chicks were kept in incubator, inoculated group $n = 20$, control group $n = 10$, both groups were fed and watered as recommended. The inoculated group received 0.5 ml positive sample homogenate each subcutaneously. First euthanasia applied 3 weeks after inoculation and second one after 6 weeks. Sera were separated and stored at −20 °C for testing and organs were taken for histopathology examination. Chicks were euthanized at 3 and 6 weeks PI to follow up the serological response of the inoculated positive sample homogenate and also to check for histopathology at that age.

2.5. Histopathology

Autopsy samples were taken initially from (a) liver of clinically affected broiler breeder bird and (b) liver and spleen of experimentally-inoculated SPF chicks and both were fixed according to Bancroft [19]. The obtained tissue sections were stained by hematoxylin and eosin stain for histopathological examination using light microscope at magnification forces 40×, 64×, 80× and 160×.

2.6. ELISA

Sera of experimentally inoculated one-day-old SPF chicks after 3 and 6 weeks post inoculation were collected and tested by ELISA kits to detect antibodies of REV (IDEXX Laboratories, USA). Sera were not collected from breeder flocks because the suspicion at that time was not REV, no available

Table 1 History of flocks from which the samples were collected.

No. of flocks	1	2	3	4	5
Breeds	Tumor samples were submitted to the lab without breed information due to the commercial facet of such information that may harm the chick provider(s)				
Average age	32–40 weeks				
Vaccination history against MDV	HVT and CVI988-Rispens				
Symptoms	Visceral tumors upon post-mortem examination				
Average mortality rate	2–5%				
Egg production	Decreased hatchability and fertility				

kit for REV and the flocks were screened for ALV serologically and found negative by ELISA.

3. Results

3.1. Polymerase chain reaction

The results of amplification of 200 bp fragment of REV proviral DNA were positive for the original tumor samples and also from the spleen collected from the inoculated SPF chicks with original spleen homogenate (Fig. 1).

3.2. Phylogenetic analysis results

The phylogram results are presented in Fig. 2 where Neighbour-joining phylogenetic tree based on nucleotide sequence showing the clustering of the detected REV sequence (KC018475) with the highly similar REV LTR sequences (Table 2). One-thousand bootstrap replicates were conducted to assess the statistical support for the tree topology. The numbers at the forks indicated the bootstrap values (1000 replicates). The figure shows the phylogenetic tree of the obtained sequence compared to other REV different strains available in GenBank. The obtained sequence was clustered with REV LTR strains and constitutes different group from those used in the analysis.

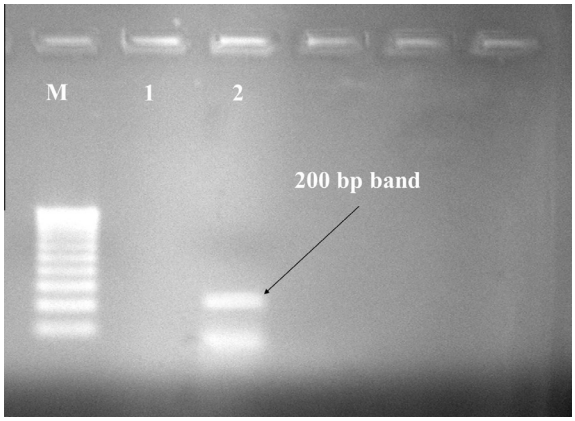

Figure 1 Ethidium bromide stained agarose gel containing the PCR amplification products of the 200 bp fragment of REV proviral DNA in original tumor sample. Lane M: represents the 100 bp molecular weight marker (Promega, USA). Lane 1: represents the negative control. Lane 2: represents the positive sample.

3.3. Histopathological findings

The collected tissue samples of original tumor sample and those of the inoculated SPF chicks were subjected to histopathological examination using H&E. Sections showed the characteristic REV reticular cells proliferation and infiltration, blood vessel dilation and congestion. Detailed description of the histopathological findings is shown in Fig. 3. Regarding gross lesions for organs collected from SPF chicks, there were no lesions observed.

3.4. ELISA

REV antibodies were detected in sera of sacrificed chicks at 3 and 6 weeks interval post-inoculation of SPF chicks. After inoculation with viral homogenate, 4 chicks were able to develop detectable antibody response using ELISA, 3 at 3 weeks of age and 1 at 6 weeks of age.

4. Discussion

REV can integrate into the genome of large DNA viruses including Marek's Disease and fowlpox [20]. REV genetic recombination and high rate of association was revealed, during fowlpox virus (FPV) vaccines examination [21]. REV can co-infect with other viruses [22] and cause contamination of a variety of poultry biologics [10,11,18,23,24]. The first genomic recombination between retroviruses and herpesviruses in chickens involved a cell culture MDV with a REV-LTR insertion [20] where as GX0101 strain of MDV was the first isolated field case with an LTR insert of REV origin [25]. The phenomena of natural genetic recombination between REV and MDV or FPV warned that the co-infection and recombination of REV with other viruses could speed up evolution of some viruses. Therefore, REV infection not only caused tumors and immunosuppression in chickens, but also had other negative potentials of accelerating other viral mutations [26]. REV infection in Egypt was detected in ducks [16], in commercial chicken and turkey flocks [15] and as fowl pox vaccine contaminant [17].

In the present study, conventional PCR has been used for detection of REV in the form of REV-LTR. Such PCR has been previously employed by others as a means of differential detection of avian oncogenic viruses including REV in multiplex PCR [27], detection of replication-defective and replication-competent REV provirus [28], both in field and vaccine strains [23,29,40]. REV-LTR fragment of 200 bp was amplified using specific primers [18]. REV was detected in original tumor collected from broiler breeder farm in Egypt and in the organ

Table 2 BLAST result of the obtained sequence shows maximum identity and the accession numbers of the REV reference strains viruses used in constructing the phylogenetic tree.

Accession number	Description	Country of origin	Max. identity (%)
AY842951.1	Reticuloendotheliosis virus strain HA9901 from China, complete genome	China	91
S79845.1	{REV LTR} [Marek disease virus, attenuated strain JM-Hi3, reticuloendotheliosis virus insertion, Insertion, 583 nt]	USA	89
S70398.1	{LTR, U3, R and U5 regions, long terminal repeats, provirus} [reticuloendotheliosis virus A REV-A, A, Genomic RNA, 545 nt]	USA	89
GQ415646.2	Reticuloendotheliosis virus strain HLJR0901 from China, complete genome	China	94
HQ111429.1	Fowlpox virus strain DCEP25 hypothetical protein, partial cds; Reticuloendotheliosis virus proviral remnant LTR, complete sequence; and protein kinase-like protein gene, partial cds	China	94
GQ375848.1	Reticuloendotheliosis virus strain HLJ07I, complete genome	China	94
FJ496333.1	Reticuloendotheliosis virus strain ZD0708 from China, complete genome	China	94
FJ439120.1	Reticuloendotheliosis virus strain chicken/3337/05, complete genome	China	94
FJ439119.1	Reticuloendotheliosis virus strain goose/3410/06, complete genome	China	94
DQ387450.1	Reticuloendotheliosis virus strain APC-566, complete genome	USA	94

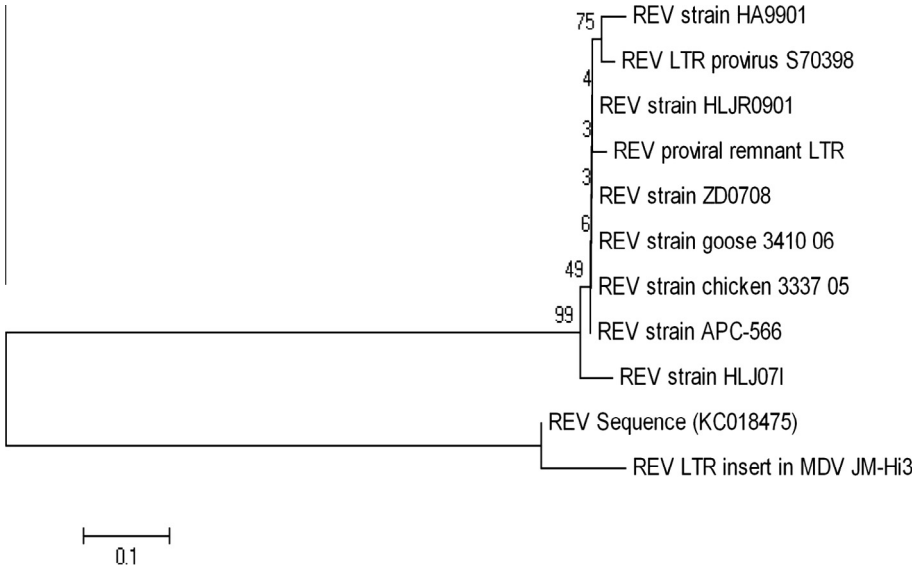

Figure 2 Neighbour-joining phylogenetic tree based on nucleotide sequence showing the clustering of the detected REV sequence (KC018475) with the highly similar REV LTR sequences.

collected from SPF chick 6 weeks post-inoculation with the homogenate obtained from the original tumor. This confirms the presence of proviral load of REV-LTR and also the reproducible infection of the virus in SPF chicks.

In addition, confirmation of REV infection by serological procedures usually involves the detection of antibodies in sera from chickens inoculated with suspect virus or from affected chickens. Antibodies are induced with various frequencies and persist for varied period. Using ELISA assay has been shown to be a sensitive and reliable method for detection of REV antibodies [30–32]. ELISA kits for antibody detection (FlockChek) are commercially available and were used in this study. The sera of SPF chicks experimentally inoculated with positive sample homogenate obtained from original breeder flock, yielded positive for the presence of REV-specific antibodies at 3 and 6 weeks interval using commercial ELISA kit (IDEXX) [17,33], which supports the assumption of the presence of replication-competent virus [23] and thus the infection of the original breeder broiler flock in Egypt by REV virus.

Nucleotide sequence analysis of the amplified PCR product revealed that the detected REV strain was closely related to the REV LTR insert in MDV strain JM-Hi3 and from a separate clade distinct from the other REV different strains (REV strain HA9901, REV LTR provirus S70398, REV strain HLJR0901, REV proviral remnant LTR, REV strain ZD0708, REV strain goose 3410 06, REV strain chicken 3337 05, REV strain APC-566 and REV strain HLJ071) with nucleotide homology ranging from 89–94% (Table 2).

Histopathology examination as a confirmatory diagnostic method in tumor induced by viruses in chicks has been considered one of the main procedures for differentiation of the causative agents for tumors in chickens [2]. Gross examination of the liver with tumor collected revealed massive enlargement

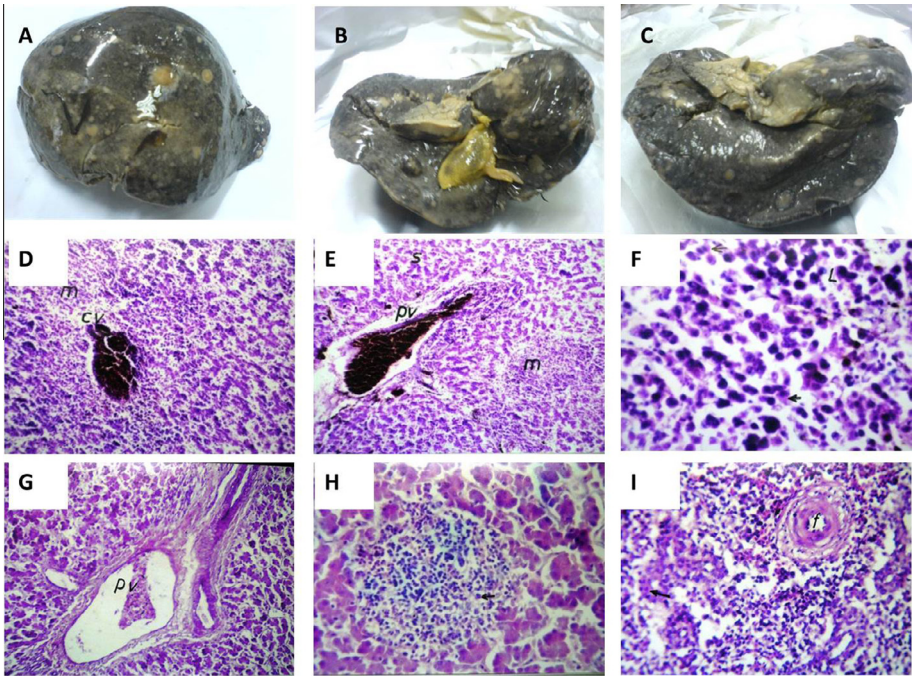

Figure 3 (A, B and C) Gross examination of liver designate massive enlargement with irregular surface, multi-focal pale areas of necrosis with neoplastic lumps consistent with REV clinical picture. (D, E and F) Represent histopathological picture of liver original sample, (D) liver of bird showing severe congestion in the central vein (CV) with massive number of proliferated and infiltrated lymphoid cells and primitive reticular cells in focal manner (m) H and E ×40, (E) liver of bird showing severe congestion in the portal vein (PV) and sinusoids (S) with focal lymphoid cells and reticular cells proliferations replacing the hepatic parenchyma (m) H and E ×40, and (F) liver of bird showing the magnification of (E) identify the focal lymphoid cells (mature and immature) (L) as well as large primitive reticular cells proliferation (arrow) and infiltration replacing the hepatic parenchyma H and E ×160. (G, H and I) Represent histopathological picture of liver and spleen 6 weeks post-inoculation, (G) liver of chicken showing severe congestion and dilatation of the portal vein (PV) H and E ×40, (H) liver of chicken showing reticular cells infiltration (arrow) in between the hepatocytes H and E ×80 and (I) spleen of chicken showing hypertrophy with edema in the wall of follicular blood vessels (f) with diffuse reticular cells infiltration (arrow) in splenic tissue H and E ×64.

of the liver, which appeared hemorrhagic. There were multifocal irregular pale areas on cut and uncut surfaces. The hepatic parenchyma contains large neoplastic lumps or multicentric and expansive nodular lesions, which might be explained as degeneration, cell lysis and massive infiltration of neoplastic lymphoid cells. These findings were previously reported by others [27,34–37]. Moreover, histopathology of liver of experimentally infected SPF chicks revealed typical pathognomonic lesions of REV, where severe congestion in the central and portal veins as well as hepatic sinusoids associated with degeneration and necrosis in the hepatocytes with massive number of pleomorphic lymphoid cells and reticular cell infiltration replacing the hepatic parenchyma which is considered REV-characteristic lesion [16,17,27,38].

Indeed, this present study is the first report entailing the presence and reproducibility of REV detected in visceral tumors of broiler breeder flocks in Egypt 2005. Further investigation is still needed for a complete detailed report on the source of infection.

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