Vet.Med.J.,Giza. Vol.49, No.2. (2001) :237-247.

SEROEPIDEMIOLOGICAL AND VIROLOGICAL STUDIES ON VIRUS-INDUCED TUMORS IN CHICKENS IN EGYPT*

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Received: 24.7.2000.
Accepted: 4.10.2000.

SUMMARY

Extensive epidemiological studies were conducted over four years to investigate the real cause and prevalence of virus-induced tumors among commercial meat-and egg-type chicken breeds, as well as native varieties of different age-groups and localities. Criteria used to establish an etiological diagnosis in problem flocks included, besides flock history, gross, histopathological and cytological examinations, virus isolation in chicken embryo fibroblast cultures (Line O) and identification by enzyme-linked-immunosorbent assay (ELISA) and/or indirect immunofluorescence (IFA), antigen detection in thin tissue sections by IFA, and reticuloendothelisosis virus (REV) proviral DNA detection in blood or tumor tissue by polymerase chain reaction (PCR). Moreover, chicken flocks were examined for viral antigen and/or antibody in plasma, egg albumen or serum by ELIA.

The results achieved indicated that Marek's disease virus (MDV), lymphoid leukosis virus (LLV), and REV were the common causes of neoplasms as single or mixed infections with variable incidence among the flocks. The sources of infection in the investigated flocks is discussed in the light of the obtained results.

INTRODUCTION

Chickens are subject to a variety of virus-induced transmissible tumors of distinct etiology. Three main disease complexes are so far Known:

* This work presents the results of a National Research Project on "Virus-Induced Tumors in Poultry", funded by the National Academy of Scientific Research and Technology.

Marek's disease (MD) caused by a herpes virus; leukosis sarcoma group caused by closely related retroviruses that induce different types of neoplasms including lymphoid leukosis (LL) which is the commonest naturally occurring form, and disease conditions associated with reticuloendotheliosis virus (REV) group.

These virus-induced tumors exist in poultry-producing countries throughout the world and are associated with varied economic losses to the poultry industry.

In Egypt, an increasing incidence of neoplasms in commercial meat-and egg-type chickens has been observed during the last two decades with the developing poultry industry, which was associated with serious losses. Diagnosis has been handcaped until a few years ago by lack of diagnostic facilities and expertness and depended mainly on gross lesions and histopathology (Agroudi et al., 1954; El-Sawy et al., 1992; El-Sawy, 1994; Fadel, 1994). This created discripancies and legal problems between flock owners and supplier companies relative to the etiological cause and source of infection.

In the present studies extensive pathological, virological and serological examinations were carried out over the period 1994-1998 to reveal the etiological and some epidemiological aspects of these tumors using current diagnostic techniques.

MATERIAL AND METHODS

Samples for examination:

Heparinized and non-heparinized blood samples, egg albumen, and tissue specimens were collected from suspect living and freshly dead birds as well as day-old chicks from commercial and native meat-and egg-type flocks raised in different localities, with history claiming variable tumor mortalities.

Laboratory host system:

Chicken embryo fibroblast (CEF) cell cultures (line O) prepared after Karel and Purchase (1989) from 9 to 11-day-old specific-pathogen-free (SPF) embryos (SPAFAS, Inc., Norwich, CT, USA) were used for isolation trials of LLV and REV.

Tissue culture media:

Dehydrated minimum essential medium (MEM; Sigma, USA) with Eagle's salts and L-glutamine without sodium bicarbonate was used after reconstitution and adjustment of pH to 7.2. Inactivated calf serum was added to the medium at concentration of 10% or 2% for growth and maintenance media, respectively.

Viruses and antisera:

* Reference LLV and REV and their chicken and rabbit antisera respectively, as well as monoclonal antibodies (Mabs) 11A25 and 11C237, specifically reactive against REV envelope

glycoprotein (gp62), were kindly provided by the Avian Disease and Oncology Laboratory, East Lansing, Michigan, USA.

* Fluorescein isothiocyanate (FITC) antimouse and antichicken IgG conjugates were supplied by KPL Inc., MD, USA, for detection of REV and LLV antigens by indirect immunofluorescence (IFA).
* Horse radish peroxidase (HRPO) antirabbit IgG conjugate was supplied b KPL, Inc., MD, USA, for detection of REV by enzyme-linked-immunosorbent assay (ELISA).

Commercial diagnostic ELISA:

LLV antigen and antibody detection kits as well as REV antibody detection kits were supplied by IDEXX laboratories, Inc., France.

Reagents and primers for REV polymerase chain reaction (PCR):

* Gene amplification PCR reagents kit with amplifier Tag DNA polymerase were supplied by Perkin-Elmer, Branchburg, NJ, USA.
* REV oligonucleotide primers and template DNA were kindly provided by the Avian Disease and Oncology Lab., East Lansing, Michigan, USA.
* Molecular size markers for electrophoresis were supplied by GIBCO BRL, England, and represented multiples of 123 bp.

Postmortem examination:

Sacrificed and/or freshly dead birds were autopsied for gross lesions suggestive of tumors, and samples were collected for the various assays.

Histopathological and cytological examinations:

Portions from suspected organs were fixed in 10% neutral formalin and processed in the usual way for paraffin sections which were stained with hematoxylin and eosin as well as methyl green pyronin for differentiation between LL and MD tumor cells (Payne and Fadly, 1997). Cryostat frozen tissue sections were also prepared for IFA examination.

Virus isolation and identification:

Buffycoat, palsma or 5-10% tissue homogenates in MEM were inoculated into CEF monolayers for 3 blind passages as described by Witter (1989). The presence of virus was determinaed by ELISA or IFA using cell culture lysates and culture cells, respectively.

Detection of REV and LLV antigens by ELISA:

The test for REV antigen detection in cell culture lysates was carried out after Cui et al. (1988) and Witter (1989), using a mixture (equal volumes) of Mabs 11A5 and 11C237 diluted 1:1000 in carbonate bicarbonate buffer (pH 9.5) for coating ELISA plates. Optical densities were read at 490 nm wave length by ELISA reader (SLT, Austria). Results were interpreted according to Smith et al. (1977) and Clark and Dougherty (1980), where

tested samples with optical density values of 0.2 above the value of known negative samples were considered as positive. Duplicate wells were used per sample and for positive and negative controls.

For LLV antigen detection in cell culture lysates, commercial LLV-antigen detection kits (IDEXX) were used and interpreted according to the instrucitons given by the manufacturer.

Detection of REV and LLV antigens by indirect immunofluorescence (IFA):

Detection of REV antigen in CEF cell cultures grown in microtitre wells was carried out as described by Witter (1989) and Aly et al. (1993), using Mab 11A25 (Cui et al., 1988) diluted 1:200 in PBS and FITC anti-mouse IgG conjugate diluted 1:100.

REV antigen detection in frozen tissue sections followed a simialr method described by Aly et al (1998).

Using LLV chicken antiserum and FITC anti-chicken IgG conjugate, LLV antigen detection in cell cultures or frozen tissue sections was carried out as described by Spencer (1987) and Fadly (1989).

Detection of REV proviral DNA by PCR:

DNA extraction from blood or tumor tissue was carried out according to Maniatis et al. (1982) and Aly et al. (1993), respectively. Amplification reactions were as described by Aly et al. (1993) and were conducted in COY tempcycler II model 110P.

Electrophoresis of PCR products (20ul) were added to gel loading buffer (2ul) as described by Aly et al. (1993), and bands were resolved in 1.5% agrose after electrophoresis in 1x Tris-Borate-EDTA buffer (TBE) for 2-3 hours at 80 volts (BIORAD,USA) and stained with ethidium bromide.

Detection of REV and LLV antibodies by ELISA:

Commercial ELISA kits (IDEXX) were used for antibody detection in sera from chicken flocks. The technique and interpretation of the test were according to the instructions given by the manufacturer.

RESULTS

The results are presented in tables (1-4).

Table (1): Results of examination of suspect chicken flocks for avian tumour virus infections as judged by histopatholoy and cytochemistry as well as virus and/or antigen detection tests.

Type and Source of Birds	No. of examined flocks	Age (wks)	Histopathol. & Cytochemical examination MDV	Results of examination (No.test + ve flocks/No. examined)					Interpretation													
				IFA (TS)		ELISA (CC) IFA (CCL)		PCR (BL/T)	- ve Flocks		+ ve flocks											
											MDV		LLV		REV		REV+ MDV		REV LLV		Total +ve flocks/Total No. examined	
				LLV	REV	LV	REV	REV	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Broiler Parent (Locally produced & imported as day old)	34	6-61	12/34	9/16	10/18	2/14	1/20	4/7	7	20.5	10	29.4	3	8.8	6	17.6	2	5.8	6	17.6	27/34	79.4
Commercial Layers (Locally produced)	9	19-46	3/9	2/2	1/4	0/3	0/6	2/3	1	11.1	3	33.3	2	22.2	3	33.3	0	0.0	0	0.0	8/9	88.8
Native Varieties (Local)	6	19-37	0/6	2/6	4/6	1/6	0/6	2/4	0	0.0	0	0.0	2	33.3	2	33.3	0	0.0	2	33.3	6/6	100.0
Total	49		15/49						8	16.3	13	26.5	7	14.3	11	22.4	2	4.0	8	16.3	41/49	83.6

LLV= Lymphoid leukosis virus; REV = Reticuloendotheliosis virus; MDV= Marek's disease virus;
IFA = Indirect immunofluorescence; ELISA = enzyme-linked immunosorbent assay; PCR = polymerase chain reaction;
TS = Tissue sections ; CCL = Cell culture laysate; CC = Culture cells; BL/T= Blood or tissue.

Table (2): Results of examination of sera and egg albumen from chicken flocks for lymphoid leukosis virus (LLV) antigen by enzyme-linked-immunosorbent assay (ELISA).

Type of Birds and Samples	Source	No. of examined flocks	No. of examined samples	ELISA + ve Samples		ELISA + ve Flocks	
				No.	%	No.	%
A- Day old chicken sera:							
• Broiler Parents	Imported	11	99	39	39.4	5	45.0
• Broiler Parents	Locally produced	25	364	93	25.5	20	80.0
• Layer Parents	Imported	1	4	3	75.0	1	100.0
Total		37	467	135	28.9	25	70.2
B- Growing & adult chicken sera:							
• Broiler Parents	Locally produced	13	370	270	73.0	13	100.0
• Native Varieties	Local	10	800	543	67.8	10	100.0
• Commercial Broilers	Locally produced	5	116	80	69.0	5	100.0
• Commercials Layers	Locally produced	15	261	41	19.0	11	73.3
Total		43	1547	934	60.4	39	90.6
C- Egg Albumen:							
• Broiler Grand Parents	Imported as day-old	1	61	6	9.8	1	100.0
• Broiler Parents	Loocally produced	20	333	47	14.1	15	75.0
• Commercial Layers	Locally produced	6	483	41	8.5	6	100.0
• Native Varieties	Local	12	898	387	43.0	11	91.6
Total		39	1775	481	27.0	33	84.6

Table (3): Results of examination of sera from chicken flocks for lymphoid leukosis virus (LLV) antibodies by enzyme-linked-immunosorbent assay (ELISA).

Type of Birds	Source	No. of examined flocks*	No. of examined samples	ELISA + ve Samples		ELISA + ve Flocks	
				No.	%	No.	%
A- Day old chicks :							
• Broiler Grand Parents	Imported	2	20	0	0.0	0	0.0
• Broiler Parents	Imported	21	185	23	12.4	6	28.6
• Broiler Parents	Locally produced	35	775	106	13.7	11	31.4
• Layer Parents	Imported	2	22	3	13.6	2	100.0
• Commercial Layers	Locally produced	1	4	0	0.0	0	0.0
Total		61	1006	132	13.1	19	31.1
B- Growing & adult chickens:							
• Broiler Parents	Imported & Locally produced	24	540	35	6.5	7	29.5
• Layer Parents	Imported	1	6	0	0.0	0	0.0
• Native Varieties	Local	11	139	13	9.3	7	63.6
Total		36	685	48	7.0	14	38.8

* Included flocks parallely examined for LLV antigen presented in table (2).

Table (4): Results of examination of sera from chicken flocks for reticuloendotheliosis virus (REV) antibodies by enzyme-linked-immunosorbent assay (ELISA).

Type of Birds	Source	No. of examined flocks*	No. of examined samples	ELISA + ve Samples		ELISA + ve Flocks	
				No.	%	No.	%
A- Day old chicks :							
• Broiler Grand Parents	Imported	2	20	0	0.0	0	0.0
• Broiler Parents	Imported	18	140	31	22.1	2	11.1
• Broiler Parents	Locally produced	28	423	26	6.1	6	21.4
• Layer Parents	Imported	2	7	0	0.0	0	0.0
Total		50	590	57	9.6	8	16.0
B- Growing & adult chickens:							
• Broiler Parents	Locally produced	24	494	326	6.5	20	83.3
• Layer Parents	Imported	3	38	1	2.6	1	33.3
• Layers	Locally produced	15	211	100	47.3	13	86.6
• Native Varieties	Local	6	99	57	57.5	4	66.6
Total		48	842	484	57.4	38	79.1

* Included flocks parallely examined for LLV antibodies presented in table (3).

DISCUSSION

MDV, LLV, and REV are the most common naturally occurring tumor viruses associated with lymphomas and considerable economic losses in chickens (Gavora et al., 1980; Payne and Fadly, 1997; Purchase, 1985). LLV induces B-cell lymphomas (Payne and Fadly, 1997), whereas MDV induces acute T-cell lymphomas with peripheral nerve lesions (Calnek and Witter, 1997).

On the other hand, REV can induce two types of lymphomas, bursal lymphomas resembling LL (Witter and Crittenden, 1979) and nonbursal lymphomas resembling MD (Witter et al., 1986), depending on the strain of virus and chicken.

In Egypt, an increasing incidence of tumor mortality has been observed among commercial layers, broiler and layer breeders, as well as nativechicken varieties since the early of the 1980's. Until a few years ago accurate, etiological diagnosis has been handcaped by lack of diagnostic facilities and expertness. Gross and histopathological examinations were the only tools that could be resorted to for tumor diagnosis. These methods although helpful, are now considered insufficient in many cases for accurate diagnosis and differential diagnosis (Shane, 1999).

The present work involves extensive studies over four years to reveal the viral cause and some epidemiological aspects of tumors among chicken flocks using current diagnostic methods.

In one study, 49 commercial meat-and egg-type flocks as well as native chicken flocks of different age-groups were investigated (Table 1). They were raised in different localities and had histories claiming losses due to tumors. Besides flock histories, two or more of the following criteria were used for diagnosis: gross, histopathological and cytological examinations, viral antigen detection in thin sections of tumor tissue by IFA, virus isoaltion in CEF cell culutres (Line O) and identification by ELISA and/or IFA, and detection of REV proviral DNA in blood or tumor tissue by PCR.

The results (Table 1) revealed that MDV, REV, and LLV as single and mixed infections were the common causes of neoplasms in 41 (83.6%) of 49 investigated flocks. They were diagnosed in 27 of 34 commercial broiler breeder flocks, 8 of 9 commercial layer flocks, and in 6 of 6 native flocks. MDV as single infection was the commonest cause in commercial broiler breeder flocks (10 of 34 flocks) and commercial layer flocks (3 of 9 flocks), thus involving 13 (13.7) of 41 flocks diagnosed as tumor virus infection. All these flocks had history of vaccination against the disease. Suboptimal management and mishandling of MDV vaccines likely contributed to MDV infections in vaccianted flocks.

Moreover, mixed infections with MDV and REV

were diagnosed in 2 additional commercial broiler breeder flocks, constituting 4,8% of 41flocks diagnosed as tumor virus infection. This might be due to the depressive effect on REV of vaccinal immunity to MD as has been reported by Witter et al. (1979).

On the other hand, LLV was diagnosed as single infection in 3 broiler breeder flocks and in 2 of each of commerecial layer and native chicken flocks, constituting 17% of the 41 flocks diagnosed as tumor virus infection, and as mixed infection with REV respectivley in 6 commercial broiler breeder and 2 native flocks constituting 19.5%. The tumorogenic role of REV in mixed infection with LLV is not clear, and indirect stimulation of the c-myc by REV for enhanced tumor formation by LLV seems possibel. Noori-Daloii et al. (1981) reported that in REV-induced lymphomas the dNA proviral genome was specifically integrated adiacent to c-myc, a cellular oncogen important in the induction of lymphoid leukosis by LLV.

REV was aslo found as single infection in 6 commercial broiler breeder flocks, 3 commercial layer flocks, and 2 native chicken flocks, constituting 26.8% of the 41 flocks diagnosed as tumor virus infection. Vertical transmission appeared to have contributed to the spread of LLV and REV infections and may by supported by the results of antigen and/or antibody detection in egg albumen or plasma and sera from day-old as well as growing and adult commercial meat-and egg-type breeder flocks, commercial broiler and layer flocks, as well as native chicken flocks (Tables 2-4).

In conclusion, the results of the present work indicate that MDV, LLV, and REV as single or mixed infections are common and widespread causes of tumors among chicken flocks in Egypt.

Recently, avian leukosis virus subtype J infection has been reported in imported broiler breeder flocks (Ahmed et al., 1999), which should be considered by tumor diagnosis. These results should draw the attention of the veterinary authorities and farm owners to the role of imported and locally produced retrovirus-infected breeding stocks and contaminated live vaccines in disseminating the infection.

In addition, breeders of native chickens should adopt a retrovirus-eradication program on their stocks at regular testing basis using current methods of virus and antibody detection.

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