



Original article

doi: 10.12980/jclm.4.2016j5-105

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Molecular detection of Marek's disease virus antigen A in fowls infected with Marek's disease

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ARTICLE INFO

Article history:

Received 2 Jul 2015

Received in revised form 9 Oct 2015

Accepted 12 Nov 2015

Available online 23 Dec 2015

Keywords:

Marek's disease

Antigen A

Tandem repeat

Tumorigenic

Tissue distribution

ABSTRACT

Objective: To investigate the presence of Marek's disease virus in broiler flocks in Iran using PCR method.

Methods: A total of fifty broilers with suspected cases of Marek's diseases were selected purposively. Gross and histopathological examinations were done. Tumoral and non-tumoral tissues were collected. Viral DNA in tissue was extracted following standard procedure and, a 314 bp fragment of antigen A gene was amplified. Differentiation of detected viral DNA as belonging to pathogenic or non-pathogenic strain was performed by amplification of 434 bp fragment of tandem repeat.

Results: Antigen A was detected in tumoral and non-tumoral tissues obtained from all the birds. DNA detected in all tumoral tissues from all the birds belonged to pathogenic Marek's disease virus strain. Histopathologic examinations showed different degrees of lymphocytic infiltration in sampled tissues.

Conclusions: In tumorigenic stage of Marek's disease, antigen A could be distributed to almost every tissue.

1. Introduction

Marek's disease (MD) is an important cause of lymphoproliferative lesions in chickens and is caused by a virus belonging to herpesvirus[1,2]. This virus consists of three serotypes. Serotype 1 is oncogenic MD virus (MDV), serotype 2 is nononcogenic of MDV while serotype 3 is herpesvirus of turkeys (HVT) virus[3].

All poultry races being farmed are sensitive to this disease[4]. The disease is spread via inhalation of aerosol droplets in poultry farm. Infection starts by the phagocytes in respiratory system and then the virus spreads to lymphatic tissues[5]. Within some weeks, B lymphocytes appear as tumor in the body[2]. MD can occur in 4 different forms: neural (nerve), cutaneous (skin), visceral (internal-organ) and ocular (eye) forms[6].

Even after vaccination, infection in the flock results in considerable damages. Diagnosis of MD disease is based on clinical signs and pathological changes (gross and microscopic observation). Early detection of Marek's infection is crucial for

devising strategies for control of possible outbreaks. In most of the studies, disease lesions are reported at older age. Under field conditions, determination of the exact incubation period under farm conditions is difficult. Chronic symptoms have also been reported in 3-4 week-old pullets, and in acute disease, the disease symptoms is observed after 8-9 weeks. In these cases, determining the time and infection conditions is very difficult[1].

In 1991, some researchers associated the factor of the disease with infectious multiple sclerosis or neural sclerosis in human beings[7]. The pathogen is a cell-associated herpesvirus and there is evidence regarding the isolation of this virus from feather follicle epithelium of infected chicks and this herpesvirus is spread via feather follicle epithelium[4].

The global vaccination of commercial bird flocks in the past 30 years reduced MD disease considerably all over the world however, dispersed epidemics have been reported in commercial flocks[2]. Therefore, rapid and exact diagnosis methods of MD and detection of pathogenic MDV strains are of great importance. Diagnosis of MD disease has been based on viral isolation, serological and molecular techniques. PCR is showed to be a valuable and rapid tool for diagnosis of animal and human diseases[8-14]. The present study aimed to detect MDV in

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suspected cases of MD in broiler birds in Iran using PCR method.

2. Materials and methods

2.1. Sampling

Suspected birds from broiler flocks slaughtered at Golpayegan slaughter houses located in Isfahan, Iran and referred to veterinary clinic at Islamic Azad University of Shahrekord Branch were used in the study. Postmortem examination of the carcasses was performed following standard procedure. Gross lesions that enabled suspicion of MD included tumor in the visceral organs and enlargement of or loss of cross striations and/or discolouration of nerves such as peripheral, celiac, anterior mesenteric, brachial plexus, sciatic, splenic and vagus nerves. Tumoral and non-tumoral tissues were collected from each bird and stored in 10% buffered formal saline for histopathological examination and at -20 °C in refrigerator for molecular studies. Study of tissue distribution of MDV in infected fowls was done on 110 histopathologic lesions.

Degenerative lesions in bone marrow and other visceral organs and microscopic visible atheromatous lesions in coronary arteries, aortas, and major aortic branches were investigated, and these parts of MD microscopic lesions were observed.

These samples were taken from lymphoid tumors of skin, liver and spleen and they consisted of healthy and pathologic samples. After being in formalin solution for 4 to 5 days (after being transferred to lab, formalin solution is changed at the first day), the samples were evaluated for tissue fixation.

The samples were sliced with microtome and stained in histopathology laboratory by hematoxylin & eosin (H&E) and then they were observed under optical microscope.

Detection of MDV in suspected cases of MD in broiler birds in Iran was carried out using PCR test. All MD positive carcasses in terms of visceral organs like liver, skin, nerves, thymus, spleen, bursa, proventriculus and kidney were investigated. After identification of MD infected cases, tissue distribution of antigen A of MD in other organs was investigated.

2.2. DNA extraction

Samples for PCR analysis were transported to the Biotechnology Research Center of Islamic Azad University of Shahrekord

Branch in cool box with ice packs and were stored under -20 °C for further use. DNA was extracted from the collected samples by phenol-chloroform as described by Sambrook and Russel[15]. One positive control containing HVT-Rispens vaccine strain and one negative control containing water were included in each experiment.

2.3. Gene amplification

Two pairs of specific primers for the “antigene A” gene and 132 bp tandem repeat primers were used (Table 1). The amplification was done using thermal cycler (Mastercycler gradient, Eppendorf, Germany) in a final reaction volume of 25 µL. The PCR mixture consisted of 1 µg of DNA sample, 1 µmol/L of each primer, 2 mmol/L MgCl₂, 200 µmol/L diethyl-nitrophenyl thiophosphate, 2.5 µL of 10× PCR buffer and 1 unit of *Taq* DNA polymerase (Fermentas, Germany). The following conditions were applied: initial denaturation at 95 °C for 5 min followed by 30 cycles, denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min, elongation at 72 °C for 1 min. The program was followed by final elongation at 72 °C for 5 min.

2.4. Analysis of PCR products

The amplified products were detected in 1% agarose gel electrophoresis. The electrode buffer was TBE (Tris base, 10.8 g; boric acid, 5.5 g; 4 mL of 0.5 mol/L ethylene diamine tetraacetic acid, pH 8.0). Aliquots of 10 µL of PCR products were applied to the gel. Constant voltage of 80 V for 30 min was used for products separation. After electrophoresis the gel was stained with ethidium bromide and photographed under ultraviolet light. After extraction of DNA from tumor tissues amplification was done targeting antigen A 314 bp.

2.5. Histopathology test

Samples of different organs were kept in formalin buffer 10% and were transferred to pathology laboratory for histopathologic evaluation. After rinsing of fixed tissue samples (in formalin 10%) and embedding in paraffin, section diameter 4-5 µm stained by H&E was studied by optical microscope in terms of MD tissue lesions. In H&E method, cells cores became blue. The cells cytoplasm and creatine and elastic fibers and fibrin

Table 1

PCR primers used for detection of MDV.

Target gene	Primers sequence	Amplicon size (bp)	Annealing temperatures (°C)	Reference
Antigen A	F: 3'-GAG GTA CCT CAT GGA CGT TCC ACA-5' R: 3'-ACA TTC TTT TCG TTG GCG TGG TAT-5'	314	57	[8]
Tandem repeat 132 bp	F: 5'- TAC TTC CTA TAT ATA GAT TGA GAC GT-3' R: 5'- GAG ATC CTC GTA AGG TGT AAT ATA-3'	434	57	[8]

became light red. Collagen, reticulin, nerves and amyloid were pink. Red globules were orange. After staining the slides, they were interpreted and read to evaluate the results. Histopathology sections were investigated qualitatively. Only the lesions in each section were mentioned in qualitative description.

3. Results

3.1. Molecular detection of MDV

The results showed that in all MD infected cases in tumor creation stage, antigen A is detected in all tissues. In addition, to separate pathogenic strains from non-pathogenic tissues, 434 bp of repeat fragment was amplified. The results showed that all positive samples and MD infected cases were pathogenic. It seems that in tumor creation stage antigen A of MD is detected in all tissues.

In all of the infected birds, antigen A was found in tumoral and non-tumoral tissues. Based on amplification of tandem repeat fragment, all of tumoral birds were infected with pathogenic MDV. PCR amplified products from various organs buffy coat for antigen A gene (314 bp length) in all samples from confirmed cases was detected as positive control (HVT + Rispens vaccine) and for 132 bp tandem repeat (434 bp length) (Figure 1). In positive control (HVT + Rispens vaccine), 132 bp repeat fragment was not amplified. In all positive samples, repeat fragment with length of 434 bp were amplified. The evaluation of positive samples with repeat fragment specific primer 132 bp showed that in all samples, 434 bp was amplified but in positive control (HVT + Rispens vaccine), 434 bp of repeat fragment of 132 bp was not observed.

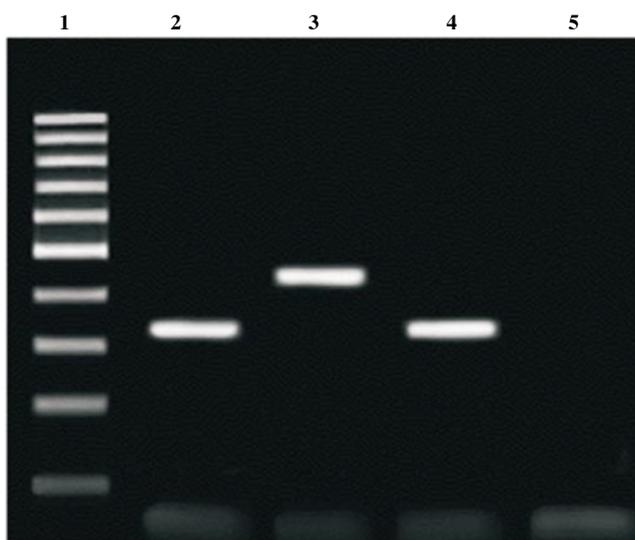


Figure 1. Gel electrophoresis for antigen A gene and tandem repeat of MDV.

Line 1: 100 bp DNA ladder (Fermentas, Germany); Line 2: Positive samples for antigen A gene (314 bp); Line 3: Positive samples for tandem repeat (434 bp); Line 4: Positive control for antigen A gene (HVT- Rispens); Line 5: Negative control (without DNA).

3.2. Histopathologic diagnosis

Localized enlargement caused the affected portion to be 2-3 times normal size even larger in some cases. Lymphomas occurred in one or more of a variety of organs and tissues. Lymphomatous lesions was found in the gonad (especially the ovary), lung, heart, mesentery, kidney, liver, spleen, bursa of Fabricius, thymus, adrenal gland, pancreas, proventriculus, intestine, iris, skeletal muscle and skin, visceral tumors. Skin lesions (scablike brownish crust formation) in external and internal crural and dorsal cervical tracts were observed. Lesions in the pectoral muscle varied from tiny whitish streaks to nodular tumors. Gross changes in eyes due to pigmentation loss in the iris (gray eye), irregularity of the pupil and severe atrophy of the bursa of Fabricius and thymus were also observed.

Histopathologic examinations showed different degrees of lymphocytic infiltration in sampled tissues. The analysis of microscopic sections of the birds, in which MD virus is confirmed, showed that besides tumor tissues, other tissues had microscopic lesions. Macroscopic lesions in muscle and nerve and liver and heart of bird infected with MD are shown in Figure 2 and Figure 3. Microscopic lesions in liver, skeletal muscle, heart muscle, skin, proventriculus and lung of bird infected with MD are shown from Figure 4 to Figure 9.

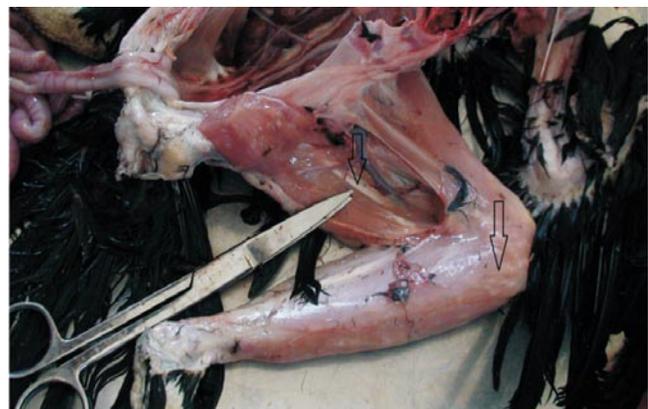


Figure 2. Macroscopic lesions in muscle and nerve of bird infected with MD. Muscle: White areas as diffused on muscle with sciatic nerve enlargement.

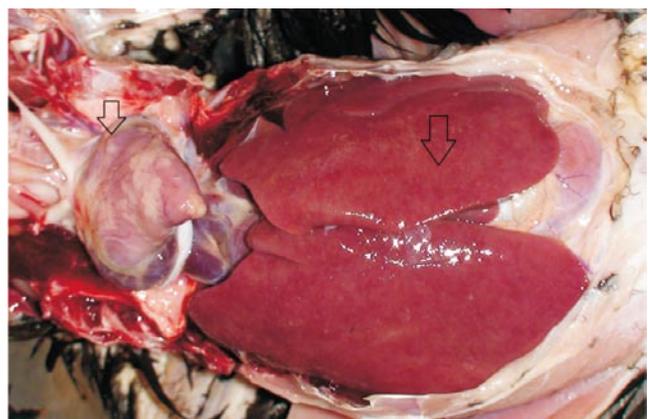


Figure 3. Macroscopic lesions in liver and heart. Liver: White areas as diffused in liver, liver is pale and enlarged. Heart: White and large areas of outer level of heart in the chicks infected with MD.

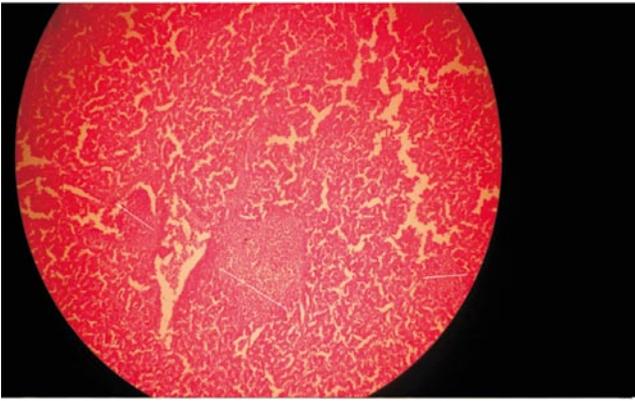


Figure 4. Microscopic lesions of the liver of birds with MD. Microscopic sections of liver have neoplastic foci with pleomorphic cells. Neoplastic foci include lymphoblast and lymphocyte cells with various small or average sizes. Lesion site is shown by lines (H&E, 40×).

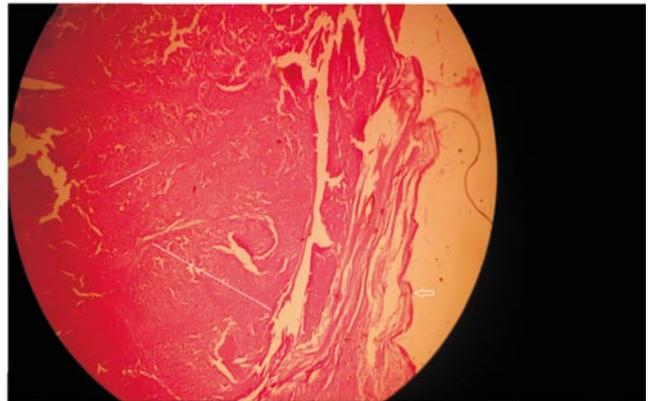


Figure 7. Microscopic lesions of skin of birds with MD. Diffused proliferation of lymphoid cells is seen in dermis of skin. The lesion site is shown by line and epidermum and coverage tissue with arrow (H&E, 40×).

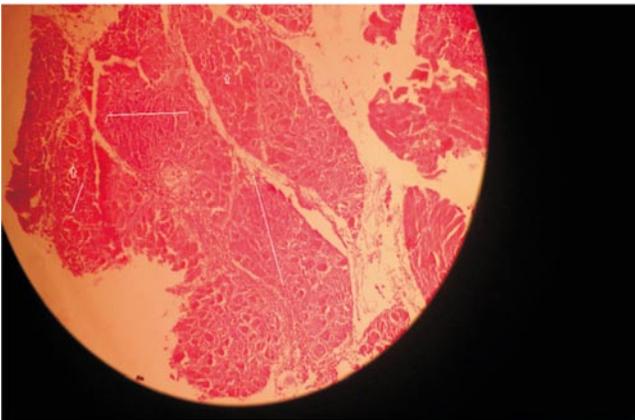


Figure 5. Microscopic lesions of skeletal muscle of birds with MD. Microscopic sections of skeleton muscle have neoplastic foci as lymphocytic pleomorphic as focal and diffused between the muscles. The lesion site is shown by line and section of muscle with arrow (H&E, 40×).

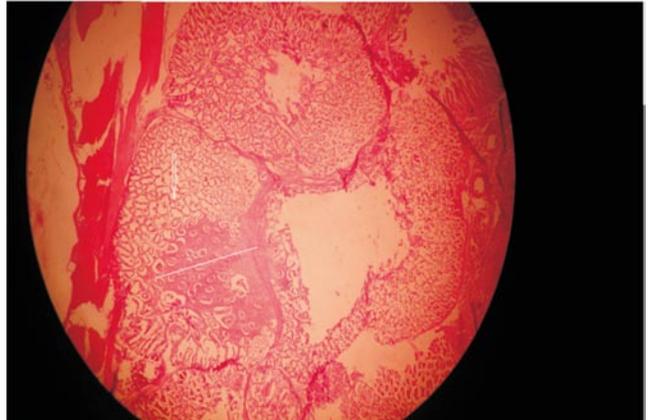


Figure 8. Microscopic lesions of proventriculus of birds with MD. Proliferation of lymphoid cells, diffused pleomorphic tumor around glands and lobules of proventriculus can be observed. The lesion site is shown with line and atrophy of proventriculus glands with arrow (H&E, 40×).



Figure 6. Microscopic lesions of heart muscle of birds with MD. Proliferation of pleomorphic cells is focal under heart endocardium and atrophy of myofibroblasts is under pleomorphic cells. The lesion site is shown with arrow and proliferation of pleomorphic cells with line (H&E, 40×).

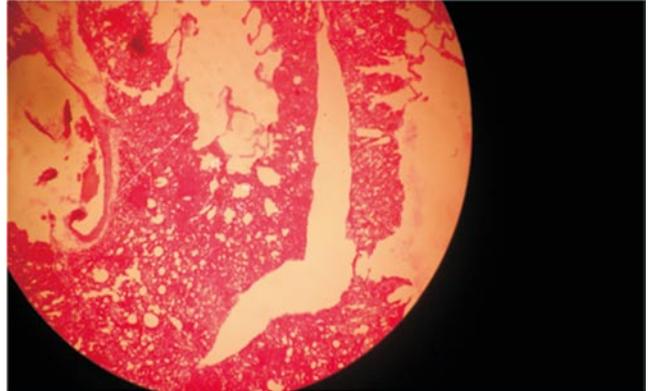


Figure 9. Microscopic lesions of lung of birds with MD. Proliferation of polymorphic cells in topology tissue and parenchyma wall and increase in the thickness of atrium level or alveolus with proliferation of pleomorphic cells can be observed. The line indicates topology tissue around parenchyma and arrow indicates increase of thickness of atrium wall and alveolus in the figure (H&E, 40×).

4. Discussion

MD is a viral lymphoproliferative disease that causes tumor lesion among laying and broiler breeding flocks, thus this disease

causes low production and huge economic loss. This disease is common in poultry above 6 weeks[16]. There is no vaccination against this disease among broiler flocks in Iran and vaccination is carried out on laying and broiler breeding flocks. In many

countries, control of MD is by vaccination[16].

Zhu *et al.*[17], Becker *et al.*[18] and Kozdrun *et al.*[6] described the best way to distinguish pathogenic strains from non-pathogenic strains of MD using this primer. In the current study, a primer based on antigen A was used to detect MD virus and also this primer was used to separate strains from the primer based on repeat row of 132 bp.

Initial results of antigen A in the collected blood samples from Golpayegan slaughter site showed that only 2.95% of samples were infected with MD disease and based on production of 132 bp repeat fragment, all of them are pathogenic viruses. The lack of formation of 434 bp fragment in extracted DNA from MD disease showed the pathogenic nature of detected cases. The present study evaluated the tissue distribution of antigen A of MD virus in various organs. Thus, various organs of birds with MD were sampled and they were also analyzed besides PCR results evaluation. The results showed that in all samples of infected birds, proliferative microscopic lesions were observed and most of the lesions were not macroscopic and only microscopic infiltration of lymphoblastic cells were observed.

PCR results of sampling organs showed that in all tissue samples, antigen A was detected and in PCR of all tissue samples from the infected birds, 132 bp tandem repeat (434 bp length) was amplified and the pathogenic nature of all pathogenic strains was confirmed from molecular aspect. Fodor *et al.* showed that microscopic lesions in visceral organs with contagious MD disease can have negative impact on growth and performance and production[19].

Four phases of infection *in vivo* were recognized: 1) early productive-restrictive virus infection causing primarily degenerative changes; 2) latent infection; 3) a second phase of cytolytic, productive-restrictive infection coincident with permanent immunosuppression; and 4) a proliferative phase involving nonproductively infected lymphoid cells that may or may not progress to the point of lymphoma formation[16]. And based on the results of PCR and pathology, it seems that sampling stage in all cases was about tumor creation in this stage, besides microscopic lesions of antigen of virus is detected in the tissues with lesions.

The observation of histopathology sections showed that most microscopic lesions of inflammation and infiltration of mononucleosis cells and the inflammatory reactions were mostly due to the effect of focal accumulated in the surrounding tissues.

Generally, the results of the study show that rearing broiler chickens to 56 days can increase the risks of the disease and based on the previous reports the growth and production period should be reduced to 42 days to 56 days. There are many health problems in broiler chick flocks at high breeding age and it is

recommended to match poultry breeding age in Iran to European standards.

Regarding MD virus tissue distribution in various organs, the results of the present study showed that in the birds infected with MD in tumor creation stage the presence of lesions is associated with genome detection in other organs.

It is recommended to monitor the broiler birds' condition in all breeding period regarding MD virus. Also, by isolation of MD virus from broiler birds, we can investigate its pathogenic nature under empirical conditions and quarantine. Finally, under quarantine conditions, based on the challenge of MD virus to sensitive birds, virus tissue distribution can be evaluated in various disease stages.

Many studies showed that herpes virus infection can endanger the health of human being[16,20]. According to the studies, DNA of MD disease has some similarities with DNA of herpes virus of human lymphotropic[16,20]. Lymphocytes type B are susceptible to this virus (3%-25% of the lymphocytes are infected), and this makes them sensitive to Epstein-Barr virus. This virus causes cancer and in non-human primates creates lymphoma tumors. This virus is also found in Burkitt lymphoma, nasopharyngeal cancer and cancer of the salivary gland[20].

Herpesvirus of MD disease is similar to alpha-herpesviruses and one of them is herpes simplex 2 alpha herpesviridae which is associated with cervical cancer among women[21]. According to the studies, the cervical cancer was highly observed in birds farming areas[1]. It is observed that this virus can proliferate under laboratory conditions and mammals culture media. According to the studies, lymphoma is mostly observed among farmers compared to other groups of society[1].

Evidences showed that MDV, human herpes simplex virus, and cytomegalovirus, all belonging to herpesvirus, can cause atherosclerosis among humans[16,20,22]. Atherosclerosis of birds is used as a model for atherosclerosis of cytomegalovirus in human being[21].

Diagnosis of MD disease is mainly based on clinical symptoms and tests on microscopic and macroscopic lesions of tissues. Diagnosis methods to confirm the disease include virus isolation, detection of virus antigens and serology methods as ELISA, Agar gel immunodiffusion[8]. Due to the crossing reactions among serotypes, we cannot detect MD disease definitely. Even after virus isolation in cell culture, due to neutralization of viruses in serotypes 2 and 3 with anti-serotype antibodies, a definite diagnosis is challenging[6]. Thus, definite diagnosis of infection by MDV is only possible using molecular tests. In addition, PCR test that enables differentiation of oncogenic and non-oncogenic strains of MDV serotype 1, and the vaccine strains of MDV (*i.e.* serotypes 2 and 3) have been described[3,23].

Becker *et al.* designed two pairs of primers which were able to detect MDV with 314 bp fragment amplification from antigen genome A and to confirm the pathogenic potential of the viruses following amplification of 434 bp of 132 bp repeat fragment[18]. The 132 bp repeat fragment is repeated in various strains of MDV serotype 1 and it is repetitive depending upon its repetition times. All the samples with clinical symptoms for MD were infected for this virus. According to these findings it seems that other factors, except the MDV, have an important role in tumor lesions.

Conflict of interest statement

I declare that I have no conflict of interest.

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