Cytological and immunocytological detection and differentiation of Marek's disease and lymphoid leucosis in poultry

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Abstract Marek's disease (MD) and lymphoid leucosis (LL) are the major diseases causing lymphoid tumors in chickens accounting for high economical losses. Gross examination could not yield definite diagnosis owing to their similar presentation of lesions. Thus present work was aimed for diagnosis and differentiation of MD and LL by utilizing simple cytology and novel immunocytology techniques. Cytological examination was carried out on slides with tumor touch imprints stained by simple Giemsa staining. The diagnosis was mainly achieved based on morphology of cell population. In the present study, out of a total of 595 cases examined, 502 cases had pleomorphic lymphocytic cell population suggestive of MD and 53 cases had uniform lymphocytic/lymphoblast cell population suggestive of LL, while the rest 40 cases remained inconclusive. A definitive diagnosis was achieved after performing immunocytology using specific antibodies that revealed 518 cases had reactivity for Meq oncoprotein specific for MD and 77 cases showed immunoreactivity for IgM in transformed B-cells confirming LL. The technique of immunocytology which has been useful for detecting human viral pathogens and MD in poultry has been applied for the first time as a novel, simple, rapid and inexpensive

technique that could be used as an alternate test to effectively detect and differentiate MD and LL in poultry.

Keywords Cytology · Immunocytology · Lymphoma · Lymphoid leucosis · Marek's disease · Meq oncoprotein · Detection and differentiation · Poultry

Introduction

Marek's disease virus (MDV) and lymphoid leucosis virus (LLV) are oncogenic viruses that affects poultry health worldwide [12, 13, 25]. Marek's disease causes morbidity up to 50% and mortality up to 100% in an affected flock depending on various factors like pathotype and dose of virus, age of the host, maternal antibody, genetic susceptibility, other immunosuppressive diseases and other factors including stress. It was estimated that in the USA, loss due to Marek's disease (MD) was at least 1 billion US dollars [20]. Economic losses caused by avian leukosis is due to tumour mortality and reduced production performance in chickens [25].

Rapid and more sensitive laboratory techniques are required to diagnose and differentially diagnose viral-induced lymphoid tumours in poultry viz. Marek's disease (MD) and lymphoid leucosis (LL) [8, 18, 19, 24]. A variety of criteria are commonly used in the diagnosis of poultry lymphomas. Epidemiological criteria, mainly the age of affected chickens, can aid in the diagnosis since MD lymphomas tend to appear earlier than lymphomas induced by retroviruses. Although true in some cases, the age criterion is not absolute because MD lymphomas can also appear in older chickens [22].

In most cases, the diagnosis relies on pathological criteria, especially distribution of the lesions and histological

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characteristics of the tumour cells [17]. However, pathological criteria are not always adequate. Tumour cell type and molecular characterization of integrated retrovirus are useful criteria for diagnosis, although not widely used [11]. These two diseases are difficult to be differentiated grossly and sometime microscopical picture also raise questions. When one of the diseases is suspected it becomes mandatory to 'rule out' or 'rule in' the presence of other disease. Any of these diseases can occur in combination, and also genome of one virus can be detected without a clinical disease or with other disease because all three viruses (MDV, LLV and Reticuloendotheliosis virus) are ubiquitous. So, 'ruling out' an infection will be more helpful than 'ruling in'. Molecular technique of polymerase chain reaction (PCR) cannot be used as a much useful test for confirmation of these diseases due to ubiquitous nature of these viruses [8, 21].

Characterization and morphological characteristics of the cell type (B cells or T cells) have been reported to facilitate differentiation of lymphomas caused by MD from LL [23]. The flaw in this technique is that mere characterization of cell type fails to differentiate normal resident lymphocytes from transformed lymphocytes. Thus a specific marker that could identify transformed lymphocytes can help in such differential diagnosis of these two diseases. One such marker is Marek's EcoRI Q-encoded protein or Meq oncoprotein which unfailingly gets expressed in MD lymphomas [3, 8, 9]. Thus by 'ruling out' or 'ruling in' MD will narrow down the diagnosis of LL. Particularly, in case of LL the transformed B cells express IgM on their surface which could be detected immunocytologically using specific antibodies [5]. Thus a novel

immunocytological technique was adapted and applied to detect both MD specific Meq oncoprotein and LL specific IgM which could be employed to effectively diagnose and differentiate MD and LL while yielding better results than the conventional cytological examination.

Materials and methods

Sample collection

The source of the study material included bird carcasses brought for post-mortem examinations to Avian Diseases Section and Post-Mortem (PM) Facility, Division of Pathology, ICAR-Indian Veterinary Research Institute, Bareilly, Uttar Pradesh. A total of 4520 cases were necropsied and out of which 595 cases suspected for lymphoid tumour cases were collected based on gross observations.

Cytology

Touch-imprints were taken from liver nodules for cytology and immunocytochemistry. Imprints were taken on clean, grease-free slides and wet-fixed in 95% methanol for 4-min for cytological examination. Methanol-fixed air-dried imprints were stained with Giemsa's stain (HiMedia, India). Briefly, imprints were covered with stock Giemsa's stain and kept at room temperature (RT) for 3-min. Then, the stain was diluted with distilled water (DW) in the ratio of 1:10 and incubated at room temperature (RT) for 10-min. Slides were washed in DW and air-dried before

Fig. 1 **a** Swollen and enlarged liver with white or grayish discoloration with numerous focal to coalescing nodules suspected for Lymphoid Leucosis; **b** focal, nodular firm, grayish colored growths of varying size on the surface of the liver suspected for Marek's disease

◀**Fig. 2** Touch imprint from liver **a, b** presence of pleomorphic lymphoid cell population (Indicative of MD), $\times 1000$; **c, d** presence of uniform lymphoid cell population (Indicative of LL), $\times 1000$; **e, f** pleomorphic neoplastic cells with prominent nuclear positive immunostaining with Anti-Meq mAb (Confirmed MD cases). IP-DAB-MH $\times 1000$; **g, h** tumour imprint from liver: pleomorphic neoplastic cells with prominent nuclear positive immunostaining with Anti-IgM pAb (Confirmed LL cases). IP-DAB-MH $\times 400$, $\times 1000$

observing under microscope. Cytoplasmic and nuclear details, and nature of cell population (pleomorphic/uniform) were recorded. The criteria that were used in classifying tumors into MD and LL were mainly based on the type, morphology (size and shape) and homogeneity of tumour cell population. Cytological features of MD tumors consist of a pleomorphic cell population of small to large lymphocytes, lymphoblasts, plasma cells, and macrophages, whereas LL tumors show large uniform lymphoblasts that may vary slightly in size but all are at the same early developmental stage.

Immunocytochemistry

Immunocytochemistry was performed on impression smears as described earlier [8]. Primary antibodies used were mouse anti-Meq mAb (clone FD7, kindly provided by Dr. Venugopal Nair, UK, to detect MDV Meq oncoprotein of MDV transformed cells and Mouse anti-Chicken IgM (#8390-01, clone M4, Southern Biotech, USA) to detect IgM on the surface of transformed B-cells of lymphoid leukaemia. Dilution of the primary antibodies used were anti-Meq (diluted 1:50 in 1% BSA) and anti-IgM (diluted 1:25 in 1% BSA). The smears were then treated with anti-mouse IgG-HRP secondary antibody raised in rabbit (Sigma, USA) at a dilution of 1:100 in 1% PBS, followed by incubation at room temperature in a humidified chamber for 1 h. Finally, the smears were reacted with Diaminobenzidine (DAB) and immunodetection was observed as brownish-red color intranuclear positive signals (Meq protein) and as surface signals (IgM) under the light microscope. In negative control, 1% BSA was added instead of primary antibody. Imprints in duplicate were subjected for immunostaining separately for both the antibodies.

Results and discussion

Gross lesions fails to differentiate MD and LL

Grossly, focal to diffuse lymphomatous lesions were observed in various visceral organs (liver, kidneys, spleen, lungs, heart, proventriculus, pancreas, ovary, nerves, and intestines). The lesions appeared as diffuse enlargements

with white or grayish discoloration of the organ (Fig. 1a) and/or focal, nodular firm, grayish colored growths of varying size with smooth cut surface (Fig. 1b). Grossly it was difficult to differentiate between MD and LL cases.

Cytomorphology aids in initial differentiation of MD from LL in visceral lymphoma cases

In a total of 595 cases examined, 502 cases (84.37%) had pleomorphic lymphocytic cell population (Fig. 2a, b), 53 cases (8.91%) had uniform lymphocytic/lymphoblast cell population (Fig. 2c, d). In few cases the cytoplasm of cells was disintegrated or cytoplasmic staining was indistinct, the nucleus of ruptured erythrocytes was seen rounded-up mimicking lymphocytic nuclear morphology, such cases were marked as inconclusive which accounted for 40 cases (6.72%).

Imprint cytology is a well-recognized simple technique for preparing a surgical specimen for pathological assessment in humans [2] with comparable results to frozen sectioning [14, 16]. Cytology and special staining have been used regularly for detection and differentiation of avian lymphoid tumors in the past [10, 20]. Cytological features of MD tumors consist of a mixed population of small to large lymphocytes, lymphoblasts, plasma cells, and macrophages. The proportion of cell types varies with the stage of the disease and virulence of the virus, and the most aggressive lymphomas may contain high numbers of lymphoblasts. LL tumors show large lymphoblasts that may vary slightly in size but all are at the same early developmental stage [15, 17].

Pleomorphic cells were seen in 502 cases, so they may represent cases of MD. The cell types were small and medium lymphocytes, large lymphoblasts characteristic of MD tumour cell population. Out of these, 53 cases had uniform lymphoblast cells may represent cases of LL. But, the published data suggests that all tumors with uniform lymphoblast population in cytology can be of MD or LL. In such cases, confirmatory diagnosis can be made based on immunocyto/histochemistry (Meq, IgM) and ruling-out virus infection (MDV/ALV) by PCR. Imprints taken from diffuse liver tumors were almost always inconclusive as the hepatocytes, erythrocytes and other resident cell population hindered in the evaluation of tumour cells. In few cases there was mild to moderate autolysis leading to changes in the cell morphology and thus a prompt diagnosis could not be made. The above situation was observed in 40 cases and thus these were also considered as inconclusive cases. In such cases, the application of immunocytochemistry was found to be helpful in arriving at a confirmatory diagnosis. Out of 595 cases examined, MD/LL can be suspected in 555 cases while the rest cases were inconclusive. Hence,

Table 1 Comparative analysis of cytological and immunocytological detection and differentiation of Marek's disease and lymphoid leucosis

Cytology-cell characteristics			Immunocytology-reactivity		
Pleomorphic cells (MD)	Lymphoblast cells (LL)	Inconclusive-indistinct morphology (MD/LL)	Meq positive (MD)	IgM positive (LL)	Meq + IgM (Mixed)
502	–	–	502	–	–
–	53	–	–	53	–
–	–	40	16	24	–
502 (84.37%)	53 (8.91%)	40 (6.72%)	518 (87.06%)	77 (12.94%)	–

cytology can be helpful to some extent to arrive at an apt definitive diagnosis.

Immunocytology aids in confirmatory diagnosis and differential diagnosis of MD and LL

Immunocytochemistry in 595 cases showed 518 cases (87.06%) with immunoreactivity for Meq protein (Fig. 2e, f) and 77 cases (12.94%) showed immunoreactivity for IgM (Fig. 2g, h). A simultaneous immunoreactivity with both Meq and IgM was not observed in any case tested. The outcome of cytology and immunocytology results are presented in Table 1. Out of 40 cases that were inconclusive in cytology, 16 cases showed Meq positive and the remaining 24 were reactive to IgM. Of note, a simultaneous immunoreactivity with both Meq and IgM was not observed in any case tested, which reflected that mixed cases of both the diseases were not present, thus a definitive diagnosis could be achieved at this level. Those cases having reactivity for Meq were confirmed as of MD and the cases with IgM reactivity were of LL.

In humans, touch imprint cytology and cytochemistry have been found useful in making a rapid initial diagnosis of clinical cases [4, 6, 10]. Immunocytology has also been used in the detection of lymph node metastases in dogs with malignant epithelial tumors [7]. In this study, imprints were taken from fresh cases as far as possible to avoid post mortem changes interfering in immunoreactivity. Tumour touch-imprints were taken from a total of 595 cases. The imprints were taken exclusively from the liver tumors, and used for demonstration of MDV Meq oncoprotein and IgM on tumor cell surface transformed by ALV.

In the present study, a total of 518 cases revealed immunoreactivity for Meq protein in the neoplastic cells, and were diagnosed as cases of MD. It is to be noted that demonstration of Meq protein in tumour cells alone can confirm the presence of MD lymphomas [1]. Other 77 cases showed IgM-positive uniform lymphoid cells, and were diagnosed as LL. The findings suggest that touch-imprint immunocytology can yield almost confirmatory diagnosis if done on imprints taken from tumors without

mixed disease. Thus, a combination of cytology and immunocytology can be used effectively to detect and differentiate MD and LL lymphomas in poultry.

Immunocytology as an alternate technique to detect and differentiate MD and LL

Thus immunocytology proved to be novel, simple, rapid and inexpensive technique that could be used as an alternate way to detect and differentiate MD and LL in a very short period of time without the necessity of frozen sections and costly equipments.

In conclusion, the present study reports the application and utility of the technique of immunocytology for the first time as a novel, simple, quick and effective methodology employing MD specific Meq oncoprotein and LL specific IgM to detect and differentiate field cases of MD and LL in poultry while yielding better results than the conventional cytological examination.

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