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Review on Virulence and Diagnostic Methods of Mareks Disease Virus in Chickens

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SUMMARY

Marek's Disease is a highly contagious oncogenic and neuropathic disease of chickens caused by *Marek's Disease Virus* which is a highly cell-associated *Alpha herpes virus* that replicates in chicken lymphocytes and establishes a latent infection within T cells. Evolution of *Marek's Disease Virus* towards higher virulence have been a concern over the years from mild to virulent, very virulent and very virulent plus. Mareks Disease is diagnosed by clinical examination of infected chickens accompanied with demonstration of postmortem lesions. The technique for diagnosis of infection with *Marek's Disease Virus* involves isolation and identification of *Marek's Disease Virus* from infected tissues. Virus isolation is usually by virus propagation in cell culture and identification/quantification by cytopathic changes. Further confirmatory diagnosis of *Marek's Disease Virus* can be done by serological test such as Agar Gel Immuno Diffusion Test and Enzyme Linked Immuno Sorbent Assay and molecular based technique such as Polymerase Chain Reaction. High-throughput sequencing technology which is a second-generation sequencing, provide the possibility to rapidly obtain the full sequence of pathogen genome at once for studying virus evolution and development of novel vaccines. Mareks Disease can be controlled by live-attenuated vaccines. In Ethiopia, reports coming from various regions of the country indicated that Marek's Disease possesses a great threat to the flourishing young poultry industry.

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Introduction

In modern poultry production, Marek's Disease is among the diseases with the highest economic impact worldwide [1]. Marek's Disease is a highly contagious oncogenic and neuropathic disease of chickens [2]. It was first described by a Hungarian Veterinary Pathologist Jozsef Marek in the last century [3].

It is caused by Marek's Disease Virus (MDV) which is a highly cell-associated oncogenic Alpha herpes virus that replicates in chicken lymphocytes and establishes a latent infection within CD4 (+) T cells [4,5]. Following the current nomenclature, MDV belongs to the genus Mardivirus which encompasses three serologically related but distinct species: pathogenic MDV also referred to as Gallid *herpesvirus* type 2; apathogenic Gallid herpes virus type 3 and Meleagrid herpes virus type 1, for which the turkey is the natural host [6].

Chickens are the most important natural host for Marek's Disease, but the disease has been reported in turkeys, Japanese quails, pheasants, owls, ducks, geese [7,8]. The economic losses caused by Marek's Disease are due to lower feed conversion, weight loss, decrease in egg production, and condemnations of carcasses at slaughter [9]. In addition, the virus has an indirect economic impact by increasing the need for farming hygiene, vaccinations, and by inducing immunosuppression, which makes chickens more susceptible to secondary infections [10].

Marek's Disease is primarily controlled by live-attenuated vaccines generated by repeated in vitro serial passage [11].Vaccination has drastically reduced the incidence of Marek's Disease. The MDV serotype 2 and 3 are not pathogenic and are used as candidates for the production of vaccines against Marek's Disease [12].

The virulence of *Gallid herphesvirus*-2 strain has shifted over the years from mild (m) to virulent (v), very virulent (vv) and very virulent + (vv+). Increased virulence and greater fitness of field *Gallid herphes virus*-2 strains were emerging worldwide [13,14]. There is a positive correlation between very virulent MDV quantity and the pathology [15]. The increasing virulence of MDV strains may however compromise the success of control through vaccination and reports of very virulent MDV are spread worldwide [16].

Diagnosis of Marek's Disease depends on an accurate history, epidemiological assessment, and the correct samples for pathology and virus testing. The diagnosis of lymphoid tumor caused by Marek's Disease is complicated due to multiple etiological agents capable of causing very similar tumors, thus one must consider both the diagnosis of the disease/tumors and the virus.

The omission of the proper samples can lead to inconclusive diagnostic testing and delays in developing an effective control response to Marek's Disease [17].

The demonstration of peripheral nerve enlargement along with suggestive clinical signs in chickens that is around three to four months old with visceral tumors is highly suggestive of Marek's Disease. This is done through histological demonstration of lymphomatous infiltration into the affected tissue. In addition to gross pathology and histology, other advanced procedures used for a definitive diagnosis of Marek's Disease include virus isolation and serology to confirm infections and quantitative PCR for identification of the virus [18].

In Ethiopia, research and case reports coming from various regions of the country indicated that Marek's Disease possesses growing threat to the young poultry industry. The intensification and dissemination of exotic breeds to villages has created chicken population, which is susceptible to Marek's Disease [19]. Therefore, the objective of this review was to assess available data on the virulence and diagnostic methods of Mareks disease Virus.

Literature Review

Etiology of Disease

Marek's Disease Virus is the causative agent of Marek's Disease and is classified as *Gallid Herpesvirus* 2, Genus *Mardivirus*, Family *Herpesviridae*, Subfamily *Alpha herpesvirinae*, Order *Herpesvirales* as per the recent classification by the International Committee on Taxonomy of Viruses [20].

Serotypes and Pathotypes of Marek's Disease Virus

The genus *Mardivirus* has three members that are serologically related but distinct species: *Gallid herpesvirus* 2 (serotype 1), *Gallid herpesvirus* 3 (serotype 2), and *Meleagrid herpesvirus* 1 (Herpesvirus of turkey, serotype 3) [21,22]. All the virulent or oncogenic strains of MDV belong to serotype 1 [16]. Serotype 2 includes naturally non-attenuated strains of MDV. Serotype 3 includes Turkey herpesvirus, the non-oncogenic MDV related virus isolated from turkey. Serotype 1 MDV strain viruses are further classified into pathotypes based on induction of lymphoproliferative lesions and severity of disease in vaccinated chickens: mild (m) MDV, virulent (v) MDV, very virulent (vv) MDV, and very virulent plus (vv+) MDV strains [23]. New pathotypes have been emerging indicating continuous evolution of MDV towards greater virulence. The increase in virulence of MDV over the past 50 year is of major significance. The shift from mMDV to vMDV strains in the late 1950s, the shift from vMDV to vvMDV in the late 1970s and, more recently, the appearance of the putative vv+ MDV in the early 1990s have each resulted in the potential for greater disease losses that have persisted until introduction of a more effective vaccine [24].

Marek's Disease Virus Characteristics

Herpesvirus infectious particles comprise more than 30 different proteins, assembled according to a complex architecture including the following: a central capsid containing the viral genome, a protein layer termed tegument, comprising more than 15 proteins, and a lipid bilayer in which about 10 envelope glycoproteins are anchored. The MDV genome is a linear double-stranded DNA of approximately 175 kb which contains a unique long (UL) sequence and a unique short (US) sequence, both flanked with terminal repeat (TR) and internal repeat (IR) sequences [25]. (Figure:1).

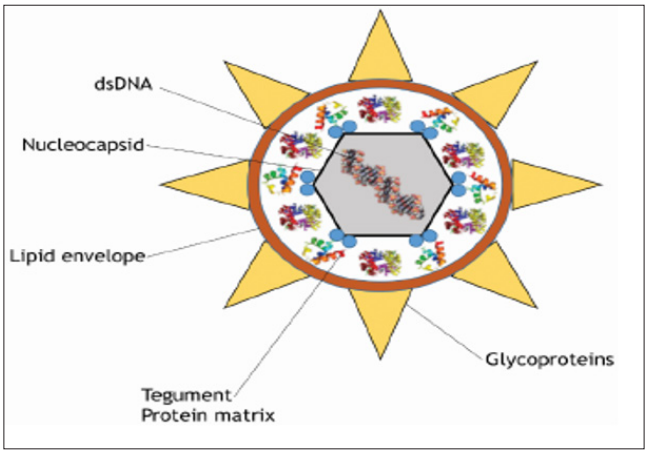

Figure 1: Schematic representation of an MDV [26].

Transmission of the Disease

MDV is transmitted through inhalation of virus-contaminated dust. Once inside a host, the virus goes through an incubation period of about one week, after which new virus particles are first shed from feather follicle epithelial cells. MDV can also be transmitted by direct contact with infected chickens and indirect contact with infected dust and dander, premises litter, and chopped feathers. Marek's disease does not spread vertically [27].

Pathogenesis

The pathogenesis of Marek's Disease is complex, with infection occurring throughout the respiratory route from inhalation of poultry house dust contaminated with the virus to the generation of infectious virus in feather follicle epithelial cells. The MDV pathogenesis can be broken down into four interlacing main phases: Entry, Replication, Latency, and Spread (Figure: 2). Initial virus replication was previously detected in macrophages and B cells in the lung of infected animals. Phagocytic cells, such as macrophages, are thought to transport the virus to regional lymphatic tissues, the bursa of Fabricius, and the spleen, where other immune cells become infected. It has been shown that phagocytes-like macrophages and dendritic cells support MDV replication and cell-to-cell spread in vitro [28].

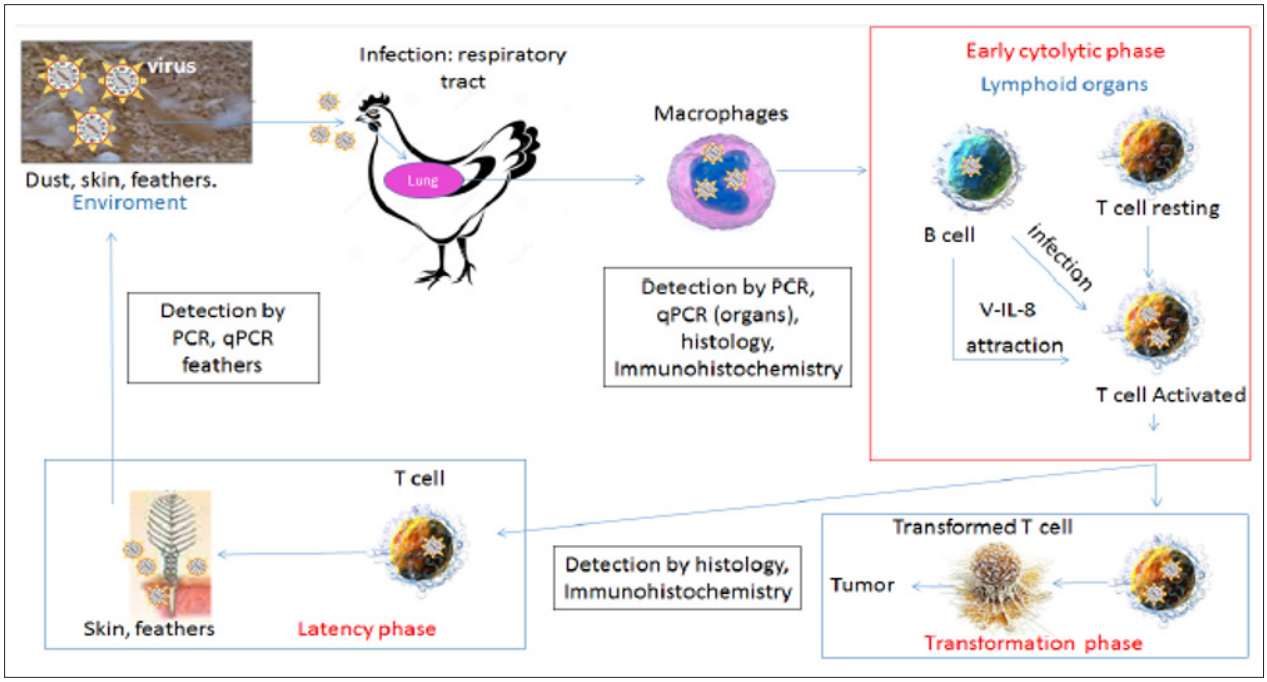

Figure 2: Pathogenesis of MDV in chickens [29].

MDV secretes a viral chemokine that was initially termed vIL-8, but recently changed to vCXCL13 based in its biological properties [30]. This chemokine recruits B cells and a subset of CD4+ T cells, and is crucial for the establishment of infection via the natural route [31]. Infection of chickens is accompanied by virus replication in several cell types, while B cells represent the majority of infected cells. Infected B cells can be readily detected in infected birds and appear to be the most susceptible cells for lytic replication [32].

Latency starts approximately within one week of infection, mainly in T lymphocytes CD4+, although the transition from cytolytic to latent infection is not entirely understood [24]. Latently infected T CD4+ cells in genetically susceptible unvaccinated chickens are transformed and originate tumors [33], and may systemically disseminate MDV through the feather follicle epithelium, where the productive replication may resume, disseminating MDV to the environment and housing in desquamating epithelial cells [34]. During latency in T CD4+, the productive (lytic) infection is suppressed and expression of cellular apoptosis is blocked.

Virulence Factors

MDV encodes more than 100 genes that are involved in various processes of the viral pathogenesis [35]. The gene encoding a major lytic and transformation maintaining phosphoprotein antigen (pp38), Marek's EcoRI-Q-encoded protein (meq) and virus-induced IL-8 homology (vIL-8) genes were virulence factors reported to play a role in viral oncogenicity and pathogenicity. These virulence factors act alone or in concert with each other to drive pathogenesis and tumor formation [32].

The meq protein is one of the most important MDV proteins that are only present in MDV-1 strains; it is highly expressed in MDV-1-transformed cell lines and tumour samples. Meq protein is a 339- amino acid protein expressed abundantly by meq during the lytic and latent phases of cellular interaction, activating transcription and involved in the transformation of T lymphocytes but not needed for replication [36]. Recently, meq gene mutation has attracted attention as a possible cause for the increased oncogenicity , although many other genes also play important roles in the development of lymphomas. MDV lacking meq is not oncogenic, as for serotypes 2 and 3, and its deletion of pathogenic strains will result in loss of oncogenicity [38,33].

Phosphoprotein 38 is required for the induction of cytolytic infection in B lymphocytes and for the production of adequate levels of latently infected T lymphocytes in the lymphoid organs. In addition, pp38 has been shown to play a role in maintaining the transformation of T lymphocytes by preventing their apoptosis [37].

The vIL-8 gene is involved in early cytolytic infections in lymphoid organs, presumably the recruitment of B or T lymphocytes in vivo. It has a less impact on either virus reactivation from latency or virus shedding. Deletion of vIL-8 leads to weak activation of T cells, resulting in reduced numbers of target cells for transformation and significantly decreased pathogenicity and tumour incidence [38].

MDV also encodes a rich repertoire of non-coding RNAs including micro RNAs and a viral telomerase RNA [39]. MDV encodes micro RNAs that play important roles during latency and tumorigenesis [40]. So far, 14 micro RNAs precursors were identified that produce 26 mature micro RNAs. They are involved in the regulation of viral and cellular target genes [41].

Viral telomerase RNA shares 88% sequence identity and the conserved stem-loop structure with its cellular homolog in the chicken. Moreover, viral telomerase RNA is crucial for efficient virus-induced tumor formation. Tumorigenesis of a virus lacking viral telomerase RNA could be restored by the insertion of chicken telomerase RNA into the virus genome. Over expression of a cellular telomerase RNA can drive tumor formation and that the virus likely acquired the gene from its host [42].

During the evolution of MDV, specific changes in the genome have been documented that could contribute to the increase in virulence and allow the virus to overcome vaccine protection [43]. Comparative bioinformatics studies have been performed to identify genes that could contribute to the increase in MDV

virulence. Mutations in several open reading frames, including meq, ICP4 and ICP27 directly correlate to the changes in virulence [14].

Clinical Signs

The presence of the virus in many poultry farms, detection of virus, viral antigens, or nucleic acids in the absence of clinical disease does not confirm the occurrence of Marek's Disease, because of the ubiquitous nature of MDV [13].

Classical form: The characteristic finding is enlargement of one or more peripheral nerves. Those most commonly affected and easily seen at post-mortem are the brachial and sciatic plexuses, coeliac plexus, abdominal vagus and intercostal nerves [18]. It is characterized by progressive paralysis, usually of the leg; a typical leg-paralysis affected bird will have one leg extended forward and one leg tucked under the bird. This is the result of infiltration of lymphocytes in the sciatic nerve. Weight loss, labored breathing, diarrhea, starvation due to an inability to reach feed and water can lead to death [21,22] (Figure: 3).

Figure 3: Leg paralysis of Marek's Disease suspected chickens [44].

Visceral form: The acute form in which tumors are observed in internal organs including heart, ovary, spleen, liver, kidney and lung and enlarged peripheral nerves [45]. Affected birds usually have enlarged peripheral nerves, as in the classical form. In younger birds, liver enlargement is usually moderate in extent, but in adult birds the liver may be greatly enlarged and the gross appearance identical to that seen in lymphoid leukosis, from which the disease must be differentiated. Nerve lesions are often absent in adult birds with Marek's Disease [13].

Cutaneous form: Recognized readily after plucking round, nodular lesions occur at the feather follicles of young birds. The non-feathered area of the legs may have a distinct red coloration, and Marek's Disease is therefore sometimes called "red leg syndrome" [46].

Ocular form: Rare syndrome that leads to graying of the iris of one or both eyes as a result of infiltration of transformed lymphocyte; the pupil is irregular and eccentric, and there is partial or total blindness. Mortality is rare [46].

Diagnosis

Diagnosis of Mareks Disease in poultry is complicated due to multiple etiological agents capable of causing very similar tumors. It is not uncommon that more than one avian tumor virus can be present in a chicken, thus one must consider both the diagnosis of the disease/tumors and of the virus. A step-wise process has been proposed for diagnosis of Marek's Disease which includes history, epidemiology, clinical observations and gross necropsy, characteristics of the tumor cell and virological characteristics [17].

Field Diagnosis

Clinical examination of infected chickens depends on observation of clinical symptoms like depression, loss of weight, raised and roughened skin around feather follicles, flaccid neck, paralysis of wing and leg enabling for tentative diagnosis of Marek's Disease [46]. Marek's Disease gross lesions are characterized by diffuse enlargement of the liver, spleen, presence of lymphomas in liver, kidney, ovary, proventriculus, spleen, lungs, nerves, heart, skin, and atrophy of the bursa of fabricius and thymus [13].

Laboratory Diagnosis

The histopathology of affected organs shows a marked cellular polymorphism, with the presence of lymphocytes, lymphoblasts, fibroblasts and infiltration of tumor cells arranged in circumscribed or diffuse form. In the liver, these lesions are accompanied by degeneration and necrosis of parenchymal liver cells, atrophy of the hepatic ducts and vacuolization. Necrosis and destruction of lymphoid cells are described in the thymus and bursa of fabricius [13].

Viral culture is a technique in which samples of a virus are placed to different cell lines which the virus being tested for is able to infect. If the cells show changes, known as cytopathic effects, then the culture is positive [47]. Primary chicken embryo kidney cells were prepared from 18-day old embryonated specific pathogen free eggs can be used for MDV cell culture. Primary chicken embryo fibroblast cell was also prepared from 10 - 11-day old embryonating specific pathogen free eggs for the isolation of MDV [45].

MDV was detected by isolating the virus from the suspected tissue samples. Accordingly, 10% w/v tissue suspensions were prepared using sterile phosphate buffer saline and inoculated onto monolayer cultures of chicken embryo kidney grown in plastic cell culture flasks for four consecutive passages and then inoculated to chicken embryo fibroblast cell again up to four consecutive passages. Inoculated and non-inoculated control cultures were incubated at 37°C in a humid incubator containing 5% CO2. Culture medium was replaced at every 2-day intervals. Areas of cytopathic effects were appreciated within 6–8 days of incubation [48].

Serological Tests

Agar Gel Immuno Diffusion (AGID), Indirect Fluorescent Antibody (IFA) and Enzyme Linked Immuno Sorbent Assay (ELISA) are serological diagnostic methods for the detection of MDV antibody produced in response to Mareks Disease infection [18].

AGID for the detection of MDV antibody produced in response to Mareks Disease infection is conducted using glass slides coated with 1% agar in phosphate buffered saline containing 8% sodium chloride. Adjacent wells are filled with antigen or serum and these are incubated in a humid atmosphere at 37°C for 24 hours for diffusion to take place. If the serum sample contains antibodies to antigens they bind forming an interlaced antigen antibody complex that precipitates in the agar [18].

Indirect fluorescent antibody detects the presence of antigen-specific antibodies in suspected samples. It uses primary antibody which is unconjugated and a fluorophore-conjugated secondary antibody directed against the primary antibody is used for detection. The fluorescent secondary antibody binds to the antigen-specific antibody rather than the antigen. Although the indirect immunoflourescent technique is more time-consuming than direct immunofluorescence, it has several advantages, of being less expensive, because the secondary antibody can be used for different primary antibodies [18].

Indirect ELISA can be used for the quantitative estimation of antibodies in the serum and other body fluids. The principle of indirect ELISA involves binding process of primary antibody and enzyme labeled secondary antibody. In this method, specimens are added to microtiter plate wells coated with antigen to which specific antibodies are to be detected. After a period of incubation, the wells are washed. If antibody was present in the sample, antigen–antibody complex would have been formed and will not get washed away. On the other hand, if the specific antibody was not present in the specimen, there would not be any complex formation. Then, an anti-isotype antibody conjugated with an enzyme is added and incubated. After another washing step, a substrate for the enzyme is added [18]. Sensitivity of Indirect ELISA is higher as more than one labeled antibody is bound per antigen molecule and fewer labeled antibodies are required [49].

Molecular Based Techniques

Real time - Polymerase Chain Reaction (RT-PCR) is molecular technique used for amplification and quantification of various MDV genes. Amplification is measured in real time during the reaction. As the amplification is progressed the detector detects the amount of fluorescence emitted. It offers faster and more reliable test as diagnostic tool of Marek's Disease. Frozen tissue samples of feather, liver, spleen, kidney, and muscle were homogenized using a dispesing homogenizer. RT- PCR was performed targeting to the amplification of the meq, Gb and ICP4 gene of MDV using forward and reverse primer [50].

Whole Genome Sequencing

Whole-genome sequencing of viruses is a powerful tool for the development of novel treatments and vaccines, for studying virus evolution and genetic association to diseases or for tracking outbreaks. The genome of MDV strain is organized in the same overall manner as long and short unique regions which are bounded by identical inverted repeats. The Guanine and Cytosine content in the repeat regions is higher than that in unique regions [51]. Nucleotide sequences of independent Meq, pp38, vil-8 and Gb genes of MDV strain can be compared with reference viral sequences in Genbank [52].

High-throughput sequencing technologies called the "next-generation" or "second-generation" sequencing, provide the possibility to rapidly obtain the full sequence of pathogen genomes at once. Three main methods based on High-throughput sequencing are currently used for viral whole-genome sequencing: metagenomic sequencing, target enrichment sequencing and PCR amplicon sequencing, each showing benefits and drawbacks [53].

Differential Diagnosis

Lymphoid Leukosis and Reticulo endotheliosis are the most common disease of chickens to be differentiated from Mareks Disease. Unlike Lymphoid Leukosis and Reticuloendotheliosis, Mareks Disease can affect birds of any age, including <16 weeks of age. In Mareks Disease, there is frequent wing and leg paralysis, potential nerve enlargement and CNS involvement compared to lymphoid Leukosis and Reticulo endotheliosis [18].

Control and Prevention

Complete cleaning and disinfection after each crop because of the presence of the MDV in feather dander, it is important to remove all dust and clean-out. Knowing the origin of chickens and avoiding mixing of chickens from different sources is important. If possible there should be no visitors in turn maximizing biosecurity. To avoid stress there should be proper space and provision of feed and water [54].

Before the development of the vaccine for Marek's Disease, the disease caused substantial revenue loss in the poultry industries in different countries of the world. Vaccination is the only known method to prevent the development of tumors when chickens are infected with the virus. The Marek's Disease vaccine is a leaky vaccine, which means that only the symptoms of the disease are prevented. These 'imperfect' vaccines allow the virus to evolve and acquire a higher virulence [55,56]. Emergence of hypervirulent strains with a further increase in the virulence of field virus strains was suggested as the main cause of this vaccination failure. However, the vaccine does reduce the amount of virus shed in the feather dander, hence reduces horizontal spread of the disease [2,16].

The vaccine can be administered to one-day-old chicks through subcutaneous inoculation or by in ovo vaccination when the eggs are transferred from the incubator to the hatcher. In ovo vaccination or embryo vaccination is the administration of vaccines into fertile chicken embryos at incubation day 18 to 19 after drilling a hole via the egg shell. In ovo vaccination is the preferred method, as it does not require handling of the chicks and can be done rapidly by automated methods. Immunity develops within two weeks [57].

Conclusion and Recommendations

Marek's Disease caused by Mareks Disease Virus is a highly contagious oncogenic and neuropathic disease of chickens. Phosphoprotein antigen, Marek's EcoRI-Q-encoded protein and virus-induced interleukin homology are potent genes acting as virulence factors for Mareks Disease Virus oncogenicity and pathogenicity. The virulence of Mareks Disease Virus strain are emerging worldwide and has increased over the years from mild to virulent, very virulent and very virulent plus. Initial diagnosis of Mareks Disease Virus depends on clinical examination of infected chickens accompanied with demonstration of postmortem lesions. Infection by Mareks Disease Virus in a flock can be detected by isolating the virus from the tissues of infected chickens. Further confirmatory diagnosis of Mareks Disease Virus can be done by serological test such as Agar Gel Immono Diffusion, Indirect Fluorescent Antibody test and Enzyme Linked Immune Sorbent Assay. Molecular based diagnosis of Mareks Disease Virus such as Real Time Polymerase Chain Reaction can be performed for amplification and quantification of Mareks Disease Virus genes. High-throughput sequencing technology is second generation sequencing currently applied for viral whole-genome sequencing.

Based on the above conclusion the following recommendations were forwarded:

- Better understanding of the reason for increased virulence of Mareks Disease Virus for development of novel vaccines.
- Application of sophisticated diagnostic methods for diagnosis of Mareks Disease Virus gene.

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