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Marek's Disease Is a Threat for Large Scale Poultry Production

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Abstract

Marek's disease (MD) is one of the widespread infectious diseases that causes huge losses in large-scale poultry production. This is due to weight loss, poorer feed conversion and an increased number of deaths among infected birds. The etiological agent is a Marek's disease virus (MDV) belonging to the Herpesviridae family. It is mainly described in poultry, however, it is also found in geese. There are three MDV serotypes, and four pathotypes within serotype 1. Currently, Marek's disease is very rare in its classical form. There are non-specific clinical symptoms, and anatomopathological changes are mainly observed in the liver, spleen and the reproductive system. This may be due to the evolution in the pathogenicity of MDV field strains over the past several decades. The presence of MDV and number of molecular diagnostic tests based on the detection of viral nucleic acids and viral proteins is already found in birds that have several weeks old. Laboratory diagnostics are based mainly on molecular biology (mainly PCR) methods. The only relatively effective method instead of biosecurity measures, of preventing MD is prophylactic vaccination of 1-day-old chickens or *in ovo* vaccination. Nevertheless, Marek's disease is still recorded in poultry flocks around the world, with estimated losses reaching several million dollars.

Keywords: Marek's disease, poultry production, poultry flocks

1. Introduction

One of the greatest threats to large-fledged poultry production besides avian influenza (AI) and infectious bronchitis (IB) is Marek's disease (MD). All these disease entities cause significant economic losses through reduced weight of birds, worse feed conversion and an increased number of dead birds. Marek's disease is a viral lymphoproliferative disease in chickens that was first described in 1907 by a Hungarian researcher - Dr. Joseph Marek [1]. Then, a year later, other researchers, ie Ellerman and Bang, described similar symptoms in hens, marked with nervous symptoms, and found lymphoid tumors in internal organs that were leukemic in nature. At that time, Marek's disease was also associated with various names such as: neuritis, paralysis, and neurophomatosis gallinarum [2, 3].

It should be noted that since the initial description of this disease entity, there have been many discrepancies as to its name with regards to the visible clinical symptoms and anatomopathological changes observed in the internal organs of infected chickens.

Authors such as Biggs and Campbell have suggested keeping the name Marek's disease, instead of using avian leukemia. The disease is closely related to the presence of nervous symptoms and peripheral nerves lesions in birds [4, 5]. In the following years, Marek's disease was undoubtedly the greatest epizootic threat to birds. Within a few years, the percentage of dead birds increased significantly, usually from 10–30%, but, for example, in the case of secondary infections, even up to 60% - 80% [2]. Such a situation contributed to the undertaking of numerous scientific studies, the main aim of which was to characterize the etiological agent, to understand the mechanisms of immunity conducting after natural infection, the routes of infection, and the mechanisms of the lesions including visceral tumors and formation in internal organs and the broadly understood mechanisms of pathogenesis.

After finding that the etiological factor of Marek's disease is a virus from the Herpesviridae family, called Marek's disease virus (MDV), it was hypothesized that Marek's disease virus strains isolated from field cases may differ in their pathogenicity despite a similar clinical signs [6]. In the course of laboratory studies, it was found that the genetic material of MDV is closely related to the genetic material of the host (target-associated). The cell-free form of the virus is only found in feather follicles. This fact turned out to be useful in research on the pathogenesis of Marek's disease, mainly in terms of the mechanisms of virus spreading among birds and between buildings on the farm, but also in developing the principles of proper immunoprophylactic vaccinations. In addition, it was also helpful in determining the influence and role of antibodies raised in birds after both vaccination and natural infection [7].

The first vaccine used in the immunoprophylactic vaccinations of Marek's disease was a vaccine based on a strain of turkey herpes virus (HVT FC126) belonging to serotype 3. It was in the form of a lyophilisate. Then came a vaccine based on the serotype 1 strain Rispens CVI 988, which required liquid nitrogen temperature for storage [8, 9]. The first studies with these vaccines were carried out to determine the efficacy of the vaccine in birds lacking maternal antibodies and in birds with maternal antibodies against Marek's disease. The vaccine used was based on the HVT (FC 126) strain in both cell-bound and cell-free form. The results of the research showed a much lower effectiveness of the vaccine based on the HVT (FC126) strain in the cell-free form. Additionally, different vaccination efficacy was found for 2 persons simultaneously vaccinating. Thus, the influence of the human factor on the process of preparation and vaccination technique on the effectiveness of the vaccination against Marek's disease was proved [10]. In subsequent studies, it was shown that there is a large variation in the incidence of Marek's disease in poultry houses located within one farm, but even in individual sectors of a given poultry houses. It has also been shown that this state of affairs can be greatly influenced by the presence of predisposing factors in the birds and on the farm in the first 8 weeks of bird life, as well as strains with different pathogenicity [11].

In 1984, Witter et al. Began research on the possibility of using a vaccine containing a strain of serotype 1, serotype 2 and serotype 3 in birds. Unfortunately, the results of these studies turned out to be unsatisfactory and the reason for this was the fact that each was the immune response of the birds. They also observed that the incidence of the occurrence of lymphatic leukemia was significantly more frequent in vaccinated/protected birds [12, 13]. Subsequent studies have shown that Marek's disease can be divided into four successive phases: early cytolytic infection, mainly B lymphocytes, latent phase/ infection, late cytolytic infection and the phenomenon of immunosuppression, and tumor transformation of T lymphocytes [14].

Marek's disease virus strains with high pathogenicity, described as vv + (very virulent plus) strains, were found already in the early 1990s. Even then, these strains were responsible for a very high mortality among birds, even those vaccinated against Marek's disease. These studies were based mainly on classical

methods in the form of a biological assay, but also molecular biology methods, mainly reaction of amplification (PCR) have already started to be used [15, 16]. In 1985, Venugopal et al. identified several genes of Marek's disease virus associated with oncogenicity/neoplastic transformation, and additionally the gene meq. They found the sequence of the latter gene only in strains classified within serotype 1. Currently, as seen, the meq gene sequence is perhaps the most frequently used sequence for the diagnosis of Marek's disease and for differentiating Marek's disease virus virulent field strains from vaccine strains [17]. A breakthrough in the research on Marek's disease was the introduction of large-scale methods of molecular biology, partial genome sequencing and then whole genome sequencing. It was mentioned that the exact sequence of individual genes and, indirectly, the mechanisms of the pathogenicity of Marek's disease virus strains and the mechanisms of the emerging immunity, both after natural infection and after vaccination, began to be studied. Efforts were also made to elucidate the full mechanism of immunosuppression caused by Marek's disease virus [18, 19]. It should also be added that the introduction of Real-Time PCR made it possible to accurately determine the viral load in 1 dose of the vaccine, which was an undoubted breakthrough in research on the effectiveness of the vaccines used [20, 21].

Our own (unpublished data) and other authors' observations confirm the fact that previously and currently used vaccines are not able to fully protect birds not only against infection, but mostly against clinical symptoms and pathological changes in internal organs of infected birds. In addition, changes in internal organs and the skin form in broiler chickens may cause confiscation on the slaughter belt up to 90% of carcasses. This is undoubtedly influenced by the progressive evolution in the pathogenicity of virus strains and the significant intensification of the scale of poultry production. That is why it seems so important to intensify research on Marek's disease [22].

It has been shown that the aforementioned meq gene can be a specific milestone by the cloning reaction of its sequence into the genome of the turkey herpes virus or the genome of the avipox virus.

A further step may be research on the sequence of the virus genome, the so-called fragmentation of non-coding RNAs in tumor cells (e.g. RNA telomerase). It is hoped that all these studies will lead to a more effective vaccine against Marek's disease [23, 24].

2. Brief characteristics of the etiological factor of Marek's disease

The etiological agent of Marek's disease is the herpesvirus (MDV) associated with the host cell (herpes virus-associated cell) of the genus *Marbivirus*. According to the principles of the new taxonomy announced by the International Committee on the Taxonomy of Viruses, the division into 3 MDV serotypes is now as follows: Gallid herpesvirus 1 (serotype 1), Gallid herpesvirus 3 (serotype 2) and Meleagrid herpesvirus 1 (serotype 3) [25]. They were also divided into pathotypes of serotype 1 according to the same committee is as follows:

- classic MDV strains with moderate virulence (mMDV-mild MDV)
- virulent strains (vMDV-virulent MDV)
- very virulent strains (vvMDV-very virulent MDV)
- strains with high virulence plus (vv + MDV-very virulent plus MDV) [25].

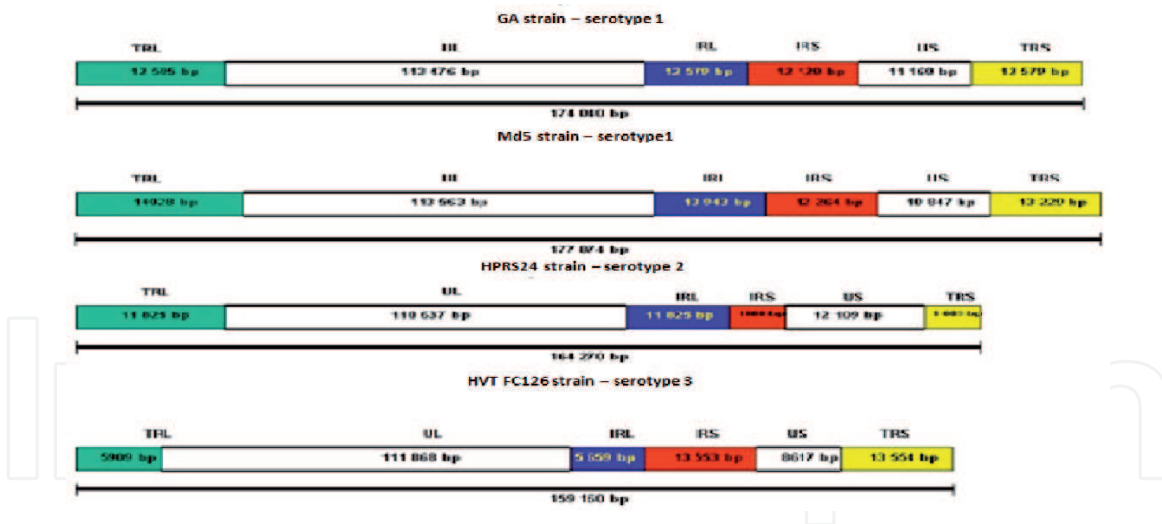


Figure 1. Genome differences between sequences of Marek's disease virus strains within serotypes.

Test the classification of individual strains into appropriate serotypes based on examining with typically specific monoclonal antibodies should be scored. The so-called patotyping of strains within serotype 1 was production based on studies (pathogenicity test) using vaccinated chickens against Marek's disease.

As previously identified with the widespread use of molecular biology, the MDV genome analysis did not define the classical markers of Marek's disease virus strains with the virulence of each of the patotypes in serotype 1 [5, 10, 26].

The MDV virion has hexagonal symmetry with a diameter of approximately 150–160 nm [4]. In turn, the nucleocapsid consists of 162 capsomers, and has a diameter of approximately 85–100 nm. The MDV genome density is approximately 1.706 g / ml (in the CsCl gradient) [4]. The differences between sequences of Marek's disease virus strains within serotypes have been presented in **Figure 1**.

The MDV genome is made of DNA and contains in its composition a unique long sequence (UL), a unique short sequence (US), which are limited by terminal repeat (TRL) sequence and internal repeat sequences (IRS) [27, 28]. Due to the high density, it is extremely difficult to divide the genetic material of the virus from the genetic material of the host. Nevertheless, the genes of the HVT FC126 strain are very often used as a vector in the production of vaccines intended for poultry, e.g. against avian influenza or infectious bursa disease.

3. Brief characteristics of Marek's disease virus genes

MDV genes can be divided into three groups:

- genes related to oncogenicity (oncogenes),
- genes encoding glycoproteins,
- other genes.

The best described and characterized gene is the meq gene. It is a protein that is present only in serotype 1 strains. It plays an important role in oncogenesis and its sequence has been used to develop primers for an amplification reaction in which a single reaction can distinguish field strains from the Rispens CVI 988 vaccine strain [29].

Another important oncogenic gene is the pp38 gene. It occurs in both strains belonged to serotype 1 and serotype 3, where it shows a high homology [30].

4. Virus replication and the pathogenesis of Marek's disease

Marek's disease virus is an infection via the respiratory system. Subsequently, lymphocytes B and macrophages which have been found in the lungs are activated. The virus then moves to the bird's main lymphatic organs, Bursa of Fabricius, thymus, and spleen [31–33]. After replication in lymphocytes B cells, the virus spreads to T cells, mainly CD4 + cells. However, only some of the lymphocytes T are transformed and they are then a source of lymphoma formation. They are located within internal organs, mainly the kidneys, spleen, liver, ovary or testes, and even in the gizzard. They are very rarely found in peripheral nerves, skin and muscles [34]. The virus then enters a latent phase in most transformed lymphocytes T cells. Only a very small percentage of the neoplastic cells approximately (<0.001%) contain viral particles detected by electron microscopy (TEM) [35]. It should also be added that MDV occurs with a latency state only in lymphocytes, and not in neurons, as is the case with other alphaherpesviruses [36]. In the active phase of infection, the virus particles are transferred to the skin cells. Then, along with the exfoliating epithelium, it is spreader into the environment and is a direct source of infection for other birds. Of course, vertical transmission of MDV has not been confirmed, although there are reports of MDV genetic material being found in experimentally infected chicken embryos and egg surfaces [37]. On the basis of research using the immunofluorescence method, it was found that the so-called follicles play the main role in the process of MDV release into the environment of the poultry house. It has been shown that follicles can develop complete and mature virus particles which are capable of infecting other birds. It was also found that the full infectious particles of MDV in the poultry house environment is up to 7–9 months at room temperature, and much longer at lower temperatures. This time is significantly extended if the virus in the poultry house is “surrounded” by biological material such as dust or chicken droppings. That is why it is so important to thoroughly sanitize and disinfect poultry houses, especially before introducing 1-day-old chickens [38]. The results of conducted studies characterizing viral genes and proteins associated with feather follicles were also performed and published. It was confirmed that many viral proteins are expressed at a much higher level in feather follicles compared to expression in cells of internal organs [39].

Some authors believe that the replication of the virus particles in the feather follicles begins as early as 7–10 days after MDV infection. Most likely, the infected cells transmit the virus to the epithelial layer of the epidermal, where the virus replication and infects the neighboring cells of the skin, including fibroblast cells and melanocyte precursors. So far, a marker responsible for virus replication in skin epithelial cells has not been described [40]. In infected birds, 2 types of lesions may occur in the skin: neoplastic lesions in the form of tumors and lesions other than tumors. Many authors suggest that the nature of these changes does not allow them to be called the so-called “skin leukemia”. It has also been shown in some studies that tumor cells do not contain viral antigens, and the research method used was immunofluorescence. Intensive research is underway on this issue [41]. As previously indicated, the genome structure of each of the MDV regions is similar, but the existing differences are nevertheless crucial. The oncogenicity of serotype 1 of MDV strains is determined primarily by the presence of the meq gene, but also the pp38 gene, vIL8 and vTR gene. They influence the oncogenic transformation processes

and activated T lymphocytes. In turn, the dimmer form of the meq gene influences the expression level of cellular apoptotic factors and virus transformation [42, 43].

In molecular studies, it was found that all MDV strains represented three serotypes reduce the expression level of the Major Histocompatibility Complex (MHC). This phenomenon seems to be useful in studies to generate lines of birds resistant to MDV infection, and in particular with regard to cellular immunity, both after natural infection and prophylactic vaccination [44].

There are several unique features in the pathogenesis of Marek's disease. In the early (cytolytic) phase of the infection, lasting approximately 7 to 10 days, the virus causes a "massive destruction" of lymphoid cells and macrophages, resulting in the phenomenon of immunosuppression [45].

After this time, Marek's disease virus enters a latent phase, which lasts until the end of life in CD4 + and CD8 + T cells. Virtually all genes of Marek's disease virus are expressed at a much lower level. This condition is mainly due to neoplastic conversion of CD4 + cells and the formation / development of multiple lymphomas in several internal organs in infected birds. It causes mortality among birds starting from 3 to 4 weeks post infection and which is sometimes not "associated" with Marek's disease by producers /veterinarians. The first nervous symptoms in the form of paralysis may already appear, which results from a significant degree of lymphatic infiltration in the peripheral nerves [46].

After approximately 10 days, the virus spreads through the bloodstream to the skin epithelial cells / feather follicle cells. This is another type of interaction between Marek's disease virus and the host cell [45, 46]. On the basis of its own and many other authors' observations, it causes various symptoms of this disease or even syndromes that can be divided into two groups: oncogenic and non-oncogenic. This division depends on whether or not the birds have maternal antibodies. It should be presumed that most hatched chickens have maternal antibodies which disappear after 3–4 weeks [unpublished data].

The natural route of infection with Marek's disease virus is through the respiratory system by aspiration of the dust containing cell-free virus particles [47].

The role of lung cells in the pathogenesis, however, is not fully understood despite the presence of viral antigen in lung cells. Phagocytes present in lung tissue are believed to "capture" the virus and transmit it to the lymphoid organs (bursa of Fabricius, thymus and spleen). Infection with the virus affects epithelial cells in internal organs and epithelial cells in blood vessels. There are already primary foci of necrosis and symptoms of inflammation in internal organs. At this stage of the infection, the viral antigen can be detected by electron microscopy in all infected cells. This condition causes transitional immunosuppression [48].

5. Clinical symptoms and anatomopathological changes in the course of Marek's disease is most common in poultry

Laying hens, broiler chickens, turkeys and quails [47]. Recent years have also confirmed that it can also occur in geese. Several cases have already been recorded in Poland, both in the flocks of reproductive geese, but also in the flocks of geese intended for fattening [49]. In the case of breeding geese, Marek's disease is found in birds in the 2nd and 3rd laying season, while in geese intended for fattening at the age of 6–7 weeks, these changes are most often found during post-mortem examination [unpublished data].

A common feature of infections in geese is the fact that previously laying hens or broiler chickens have been reared in the same buildings as geese in which clinical

Marek's disease was confirmed (unpublished data). Based on our own observations, Marek's disease conducted with rapid course of the disease in flock at the beginning of the infection, with a large number of dead birds. The infection then gradually "silences" [unpublished data]. In turn, the frequency of Marek's disease also depends on many predisposing factors, including: transport stress, vaccination stress, too high density of birds, sex or the content of undesirable substances in feed and water [47]. In the course of Marek's disease, clinical symptoms are closely related to the location of neoplastic lesions in the internal organs of the birds [47]. General apathy of the birds, stunted growth and even diarrhea are observed (it can be bloody if it becomes infected with coccidia). The paralysis and twist of the neck and head in the case of neurological disorders in the nervous system. One sided paralysis may be present. In the case of changes in the nerves of the eye and inflammation of intraocular structures, we may be dealing with blindness or pathological changes in the eye [50]. Generally, it can be assumed that the pathological changes can be classified into 3 groups: changes in internal organs, skin and in peripheral nerves [47].

Pathological changes in internal organs can be nodular, diffuse or both. If the lesions are diffuse, then the internal organs are significantly enlarged (rarely of normal size) with white or gray discoloration. When the lesions are nodular, the lymphomas appear singly or in small clusters of white or gray color [51].

As previously mentioned, skin lesions are associated with feather follicles and are best seen in featherless carcasses. The follicles are greatly enlarged and in the form of diffuse lesions. Changes can also be seen on combs and bells. Red lesions and cavities on the skin of the thighs are called "Alabama Red Leg" syndrome. Such changes were described in Poland in 2005 in a flock of broiler chickens [52]. Peripheral nerve enlargement can be called the "golden sign" which we can observe during the anatomopathological examinations [53]. Nerve changes can take the form of unilateral or bilateral nerves dysfunction. The altered peripheral nerves can be 2–3 times enlarged, lose their physiological shine and transverse striations, gray or yellow in color, sometimes swollen or with the presence of streaked hyperaemia [54]. In general, anatomopathological changes in Marek's disease occur most frequently in internal organs such as the liver and spleen. There are the changes described above. Of course, some changes can also occur in other organs, but much less frequently. The following can be mentioned here: the reproductive system (especially the ovary and testicle), kidneys (most often manifested significantly enlarged with a marked structure of renal tubules), and glandular stomach (significant thickening of the wall). In addition, there may be complete atrophy of the thymus and bursa of Fabricius [47].

In the course of Marek's disease, in its non-oncogenic form, there are usually three forms: lymphodegenerative syndrome, transient paralysis and panophthalmitis (also known as "gray eye") [55]. Lymphodegenerative syndrome is observed only in unvaccinated birds against Marek's disease and in which no maternal antibodies are present. On the other hand, lymphatic organs such as the bursa of Fabricius and the thymus undergo very quick atrophy, already around 6–8 days after infection, and turn yellow-green. It has been proven that they are more atrophied after infection with Marek's disease virus of low pathogenicity in case of classical strains. If the birds are subsequently infected with highly virulent strain expressing higher pathogenicity, atrophy does not progress. Additionally, necrotic foci may appear [47]. Transient paralysis is most common in birds vaccinated against Marek's disease, mostly in broiler chickens around 40 days of age. It mainly affects the neck muscles in the initial period, and then the paralysis gradually progresses in other parts of the muscles [56].

6. Diagnosis of Marek's disease

When Marek's disease is suspected and the consequences of the outbreak have been confirmed, the differential diagnosis is very important. It is based on finding clinical symptoms and pathological changes during the conduction of anatomico-pathological examination and comparing them with similar symptoms and macroscopic/microscopic lesions which can be suspected/visualized in other diseases (**Table 1**).

1. Lymphoproliferative syndrome in turkeys. In fallen birds, a slight enlargement of the liver is visible with the presence of small, gray-white necrotic lesions resembling tuberculosis lesions. In turn, the spleen is significantly enlarged and resembles a marbled structure [47].
2. Lymphocytic leukemia. The pathological changes occur mainly in the liver in the form of single neoplastic tumors or, much less frequently, in the disseminated form. The changes further affect the spleen. The visible bumps vary in size and consistency of a compact (solid) or lard-like consistency. Sometimes, and especially in reproductive hens over 19–20 weeks of age, neoplastic tumors may completely fill the body cavity. These tumors may be white or cream-white in color [57].
3. Myelogenous leukemia. In this case, the neoplastic tumors are most often yellow - gray or yellow - white. They are usually diffuse, and very rarely in the form of single lesions [58].
4. Reticuloendotheliosis. During the pathological examination, significant enlargement of the liver and spleen is observed with the presence of necrotic foci of various sizes [58].

| Marek's disease | Other diseases |
|-----------------|---|
| | lymphocytic leukemia |
| | myelogenous leukemia |
| | Lymphoproliferative syndrom |
| | Retikuloendotheliosis |
| | Tuberculosis |
| | Histomonozis |
| | Avian Encephalomyelitis AE |
| | Thiamin, Vitamin B1 deficiency |
| | Vitamin B2 - riboflavin deficiency |
| | Pseudopestis avium, Newcastle Disease, ND |
| | Fowl Pox FP |
| | Coligranulomatosis gallinarum, Hjarre's disease |
| | Botulismus avium, botulism |

Table 1.
The most important diseases or disease syndromes included in the diagnostic differentiations of Marek's disease.

5. Tuberculosis. There is dejection, significant deterioration of the condition of birds and emaciation. Tuberculous lesions are visible in sections in the form of large, uniform tumors or scattered small nodules mainly in the gastrointestinal tract, liver and spleen. The liver and spleen are significantly enlarged also with the presence of necrotic foci [59].
6. Histomonosis. Birds are progressively emaciated. Mortality in young birds can be as high as 100%, while in older birds only 10–20%. The pathological examination reveals a significant enlargement of the liver with the presence of yellow or yellow-green necrotic foci of various sizes [60].
7. Avian encephalomyelitis AE. The disease is mainly associated with symptoms related to the nervous system. There are locomotor difficulties mainly caused by paralysis of the legs. In lying hens, tremors of the neck and head are visible. Older birds may have paralytic symptoms similar to Marek's disease [61].
8. Vitamin B1 deficiency. In this case, growth inhibition, unsteady gait and feathering have been observed. In addition, there may be paralysis of the legs, wings and neck. Birds assume a sitting position on jumps with the head tilted back [62].
9. Vitamin B2 deficiency. Birds are kachetic, even dwarfed, and differenced within the flock. Similarly, in this case, the birds sit on their legs and support themselves on the wings. In a long-term state of deficiency, birds usually lie down with their legs stretched out [69].
10. Newcastle disease. In the case of velogenic and mesogenic strains, apathy, swelling of the head and conjunctivitis occur in birds. Birds are depressed, appetite and thirst decrease, and over time the birds differentiate in body weight [63].
11. Fowl pox. In the cutaneous (chronic) form, tumors are visible in the area of the neck and lower abdomen. These changes are largely similar to those found in the skin form in broiler chickens [64].
12. Coligranulomatosis. It is a chronic disease and the incubation period can last up to several weeks. Virtually all internal organs of the bird are damaged. Numerous cocci are visible in the liver and in the lungs, reproductive system and kidneys [65].
13. Botulism (botulism). Apathy of birds, locomotor difficulties (unsteady gait) and paralysis of the neck and wings have been observed. The bird's posture with the head hanging down is a characteristic posture [66].

7. Laboratory diagnosis of Marek's disease

Generally, the diagnostic methods for Marek's disease can be divided into the following methods: serological, histopathological and virological. In the case of the latter, they include methods based on molecular biology.

Live birds (5 to 10 birds per flock) showing symptoms of the disease and an additional 20 to 25 blood or preferably serum samples should be provided for

virological examination. We can also examine feathers collected from birds from the shoulder girdle or the inner thigh surface. These feathers should be protected in such a way that does not dry out during the transport of the samples to the laboratory. Sick birds delivered to the diagnostic examinations should be euthanized using methods compliant with applicable legislation. Then, a thorough anatomopathological examination should be performed, describing the visible changes. During the anatomopathological examination, samples of the internal organs should be collected (in sterile way) for laboratory diagnostic tests, (classical virological and molecular). Most often samples of the liver and spleen have been collected, but also samples of other internal organs with pathological changes were examined to better understand the virus virulence. In turn, for histopathological examinations, apart from liver and spleen sections, also bursa of Fabricius, glandular stomach and peripheral nerves (sciatic and brachial plexus) have been examined. The possible presence of lesions in peripheral nerves is also a diagnostic tool for differentiating between Marek's disease and avian leukemia infections, but also other neoplastic diseases. Homogenates are prepared from the collected internal organs, which are used to infect cell cultures: SPF (Specific Pathogen Free) chicken embryo fibroblast cultures (CEF) or Chicken embryo kidney cultures (CEK) or chicken embryos. The infected cell cultures are incubated at 37.5°C and the cytopathic effect (CPE) in the form of clustered fine light refracting cells is observed on a daily basis. The formation of CPE in infected cell cultures indicates the presence of Marek's disease virus [67]. Sometimes, plaque formation is found, i.e. places where the connectivity of the cell layer has been interrupted by a proliferating Marek's disease virus strain. The cytopathic effect and plaques created by, for example, the vaccine strain HVT FC 126 (serotype 3) usually begins to appear as early as 72 hours after infection of the cell culture. In turn, field strains form CPE only in the 2nd or even 3rd passage. Under the microscope, starting from the 5th day after infection of the culture, a cytopathic effect is visible in the form of clustered small cells, strongly refracting light, and it most often appears between 7 and 9 days after infection. The final results of incubation are read under the microscope after about 12–14 days culture incubation. Clusters of small cells may also be visible, often overlapping each other and forming so-called focuses [68].

Marek's disease virus can also be isolated directly in a sterile culture prepared from kidney cells collected from diseased birds in a similar manner as described above. Isolation of Marek's disease virus strains in chicken embryos is used very rarely, mainly due to the time-consuming nature of this method. Commercial Marek's disease virus antigen and commercial anti-MDV serum are used in serological method. Blood is taken from the examined birds in a sterile manner, from which the serum is obtained after centrifugation. Feathers, are collected from places where there is an intensive replication of complete virus particles (wings, thighs and shoulder pathway). The presence of the specific anti-MDV antibodies in the serum samples collected from infected birds is determined by the agar gel immunodiffusion method (AGID), while the agar gel radial immunodiffusion test (RID) is used to detect the presence of the specific antigen of Marek's disease virus in the feather follicles of sick birds.

Among the molecular methods, the most frequently used technique is the polymerase chain reaction (PCR) is the amplification method with other variations. The amplification reaction allows the detection of the genetic material of Marek's disease virus strains and the differentiation of field and vaccine strains. Organ samples, blood, feather follicles, as well as dust collected from poultry houses serve as a matrix for DNA isolation. Traditional PCR consists in amplifying a fragment of a gene specific for MDV. Primers whose nucleotide sequence is complementary to the amplified fragment of the MDV genome are most often used for this purpose.

The primers used are usually complementary to the sequence of genes such as: meq gene, 132 bp repeat sequence, pp38 gene or fragment of the SORF1 gene specific for turkey herpesvirus FC 126 strain (serotype 3) [69]. The advantage of the PCR method is its high sensitivity and specificity. The high specificity of the PCR allows the differentiation between field strains belonging to serotype 1 and the vaccine strain HVT FC 126 belonging to serotype 3 and the vaccine strain Rispens CVI 988 belonging to serotype 1. The results of PCR methods have been used to answer the question whether the birds have been vaccinated correctly or not, and whether the birds were infected with a virulent, field strains of Marek's disease virus.

Frequently in birds vaccinated with the bivalent vaccine (Rispens CVI 988 and HVT FC 126), in the case of infection with the field strain, DNA of the Rispens CVI 988 strain is absent. This is most often the case in birds over 6 weeks of age. On the other hand, these birds have DNA of the vaccine strain HVT FC 126 in practically every case. Such a PCR result clearly proves that the examined birds were vaccinated against Marek's disease. It should be remembered that vaccination does not protect birds against infection with a field strain, but only against the manifestation of the clinical symptoms and the occurrence of pathological changes.

8. Immunoprophylaxis of Marek's disease

The one effective way of preventing Marek's disease is prophylactic vaccinations, mainly performed in 1-day-old chickens. *In ovo* vaccination is also used, however, due to its high costs, it is used to a very limited extent. At this point, it should be recalled once again that vaccination against Marek's disease does not protect birds against infection, but against clinical symptoms and pathological changes in the internal organs of the birds. Marek's disease continues to cause heavy economic losses on account of these latter aspects. In 2020 and 2021, a slight upward trend was observed in the number of cases of the clinical form of the disease in field conditions. Practically from the beginning, when the immunoprophylaxis against Marek's disease was introduced, vaccines based on Marek's disease virus serotype 1 and serotype 3 have been used. Marek's disease virus vaccines based on serotype 3, contain the turkey herpesvirus strain HVT (FC126), which occurs naturally in turkeys and is a non-pathogenic strain for these birds. This strain comes in two forms: as a target-associated virus and as a cell-free virus. Cell-bound virus must be stored at liquid nitrogen temperature (-196°C) and cell-free virus in lyophilisate form at $2-8^{\circ}\text{C}$. Vaccines based on the serotype 1 (strain Rispens CVI988) are in the form of a liquid suspension and absolutely must be stored at liquid nitrogen temperature (-196°C). There are also vaccines on the market consisting of two strains: HVT (Fc126) and the Rispens CVI988 strain, and here, also, liquid nitrogen temperature is required.

In the United States, a vaccine based on serotype 2 (strain SB1) is additionally used. However, this vaccine is not used in Poland, although the results obtained in several laboratories indicate that this strain may already be present in poultry flocks in Poland [70].

Administration of the vaccine against Marek's disease in the correct manner causes the reduction of the multiplication process in the body of the virulent field strain and the spreading of the infection in the flock horizontally (lower level of replication in feather follicles). As a result, the formation of pathological changes in internal organs, mainly in lymphatic tissues, is limited. The viral load of infected birds is also reduced [24, 71]. A very important aspect of vaccination against Marek's disease is the fact that vaccine immunity significantly reduces the risk of immunosuppression. It is important due to the possibility of contact of birds on the farm with immunosuppressive factors, mainly from the infectious background [72].

There are a few important information's to keep in mind when prophylactic vaccinations against Marek's disease have been conducted:

- birds of one age (usually 1 day old) must be vaccinated
- strictly follow the safety rules
- only birds without any disease symptoms should be vaccinated
- vaccination should be carried out by qualified staff and under the supervision of a veterinarian.

Most often, the birds are vaccinated on the first day of their life in the hatchery or immediately after being placed on the farm.

An important aspect at the time of vaccination is the fact that Marek's disease vaccine should not be combined with other vaccines. The exceptions are, of course, the recommendations of the vaccine recommendations.

The information that in the field conditions very different volumes of the vaccine are used (not to be confused with the dose of virus in 1 dose of the vaccine) arouse much controversy of scientific and field nature (in terms of vaccine effectiveness). The recommended volume for a 1 day old chickens is 0.2 ml, which contains approximately 2000–3000 PFU (focus-forming units in cell cultures).

Research studies indicated that the use of a 0.1 ml volume of vaccine will not provide adequate protection for the birds from clinical Marek's disease. On the other hand, a vaccine volume of 0.5 ml is used in areas where Marek's disease virus may be endemic. Neither one solution nor the other can find any justification [unpublished data]. According to our own observations, there is a very frequent breakdown of post-vaccination (cellular) immunity, and then we are dealing with the classic course of Marek's disease in a given flock. A very important step in the correct vaccination against Marek's disease is the storage and transport of the vaccine. Recently, vaccines based solely on vaccination with HVT (FC 126) strain in the form of lyophilisate have been used. Therefore, vaccines based on the Rispens CVI 988 strain or in combination with HVT (FC 126) must be strictly stored under the conditions recommended by the vaccine protocol [73]. In some cases, after removing the vaccine, the entire contents of the vaccine remain in the upper part of the ampoule. This clearly proves that the contents of such an ampoule were previously thawed and then frozen again. This vaccine is no longer usable and should be disposed of. The fall in the titter of the vaccine virus is then 100% [73]. Storing the vaccine at a temperature of 40°C or over 25°C for more than 1 hour causes a significant decrease in the viral load. After 24 hours, the decrease in titter is close to 80–90% [73]. The vaccine after removing from the container with liquid nitrogen should be dissolved up to 2 minutes, because a longer time causes a significant decrease in the titter of the vaccine virus in 1 dose of the vaccine. We should not use water over 37°C to defrost the vaccine.

After dissolving the contents of the vaccine in a suitable solvent, use the entire contents within 1 hour or a maximum of 2 hours. Research carried out at PIWet-PIB in Puławy showed a decrease in the viral load with the duration of vaccination.

Another important aspect of correct vaccination is the number of chickens vaccinated subcutaneously or intramuscularly. On this point, veterinarians are much divided. It is recommended to vaccinate about 2000–2500 birds within 1 hour. The more vaccinated birds per hour, the lower the number of correctly vaccinated birds should be presumed. You should also take into account the method

of vaccination, whether we vaccinate with an automatic syringe or a special vaccination machine. Many companies offer suitable dyes that are added to the solvent. In this way, the vaccination veterinarian can very easily assess the quality of the vaccination performed. It should also be remembered that birds should not be given any antimicrobials or substances with immunosuppressive activity (e.g. immunostimulators) with the vaccine. In the case of vaccination in breeding flocks and commercial hens it is recommended to use vaccines based on the Rispens CVI 988 strain or vaccines containing the Rispens CVI 988 and the HVT (FC 126) strains. In broiler chicken flocks, vaccines based on the HVT (FC 126) strain are used, however, due to the presence of Marek's disease virus strains with high pathogenicity, it is recommended to use vaccines based on the Rispens CVI 988 strain. For turkey herds, the use of vaccines based on Rispens (CVI 988) is recommended. If vaccination is already performed on the farm, it should be remembered that all proper sanitary and hygienic conditions are maintained. We should apply the very simple but extremely effective "full farm - full empty farm" principle. This means that only one-age birds should be existed on the farm. This is a barrier protecting young birds against the possibility of transmission of pathogens from older birds. Among these pathogens there may be immunosuppressive pathogens, which adversely affect the effectiveness of the vaccination against Marek's disease.

After vaccination, it is very important to deal with chickens for the first 2 weeks of life, i.e. until immunity is developed. In this period, there should be close cooperation between the hatchery, the poultry producer and the veterinarian providing services to the poultry producer [74]. The *in ovo* method is used to inoculate the embryos on the 17.5-19th day of incubation, most often when the embryos are transferred from the incubation chamber to the brood chamber. The site of *in ovo* vaccination is the amniotic, allantoic or yolk sac (extra embryonic - EE), but also the body of the embryo (intra embryonic - IE) [75]. The place where the vaccine is administered depends on the age of the embryo, the egg placement, the size of the egg and the breed of the parent flock. Within one hour, up to 50,000–60,000 embryos can be vaccinated. Properly conducted *in ovo* vaccination reduces the incidence of vaccine breakdown and clinical form/expression of Marek's disease in the field. In addition, it protects or significantly reduces hatched chickens from the possibility of early infection of chickens with virulent, field strain of Marek's disease virus. Like any vaccination method, it has advantages and disadvantages [76].

The advantage of the *in ovo* vaccination is that there is no stress that may occur in vaccinated chickens. There are significantly fewer infections associated with the *in ovo* technique alone compared to the subcutaneous or intramuscular vaccination of 1-day-old chickens. It is important to compare the effectiveness of *in ovo* vaccination compared to that of 1-day-old chickens vaccination. Our own observations at NVRI show that the effectiveness of both methods is probably at a similar level. When *in ovo* vaccination was used, a lower percentage of so-called seizures at the slaughterhouse associated with coetaneous Marek's disease were observed in broiler chickens.

Another controversial issue is the vaccination of chickens already vaccinated on 1 day of life. Immunization is used even in the first day of life after a few hours' break from the first vaccination. However, it seems that there is no scientific justification for this, and moreover, it was not observed in the field that vaccination, e.g. on the 3rd, 7th or even 10/21 days, had an impact on the possible course of Marek's disease in infected birds.

Recently, there has also been a limited amount of data collected on the field (unpublished data) on additional vaccination of birds, especially in breeding flocks 35–37 weeks of age. Also, such a scheme is a bad scheme and should not be used, especially since the vaccination process itself is a great stress for the birds.

9. Conclusion

Despite several decades of immunoprophylactic vaccinations and research on bird breeds resistant to infection, it was not possible to fully combat Marek's disease. The slow evolution in the pathogenicity of MDV strains should accelerate the pace of research into such bird breeds, but above all into other, more effective vaccines. Progress must also be made in laboratory diagnostics.

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