Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE VETERINARIE

Ciclo 34

Settore Concorsuale: 07/H3 – Malattie infettive e parassitarie degli animali

Settore Scientifico Disciplinare: VET/05 - Malattie infettive degli animali domestici

INSIGHTS ON EPIDEMIOLOGY AND CONTROL OF MAREK'S DISEASE THROUGH TRADITIONAL AND NOVEL MOLECULAR TOOLS

Presentata da: Dott.ssa Giulia Mescolini

Coordinatore Dottorato

Prof.ssa Carolina Castagnetti

Supervisore

Prof.ssa Elena Catelli

Esame finale anno 2022

Abstract

Marek's disease (MD) is a contagious, lymphoproliferative and neuropathic disease of poultry caused by a ubiquitous lymphotropic and oncogenic virus, *Gallid alphaherpesvirus 2* (GaHV-2). MD has been reported in all poultry-rearing countries and is among the viral diseases with the highest economic impact in the poultry industry worldwide, including Italy. MD has been also recognized as one of the leading causes of mortality in backyard poultry.

The present doctoral thesis aimed at exploring Marek's disease virus molecular epidemiology in Italian commercial and backyard chicken flocks and, for the first time, in commercial turkeys affected by clinical MD. Molecular biology techniques targeting the full-length *meq* gene, the major GaHV-2 oncogene, were used to detect and characterize the circulating GaHV-2 strains searching for genetic markers of virulence.

A final study focused on the development of rapid, sensitive, and species-specific loop-mediated isothermal amplification assays coupled with a lateral flow device readout for the detection of conventional and recombinant HVT-based vaccines is included in the thesis. HVT vaccines, currently used to protect chickens from MD, are referred to as "leaky", as they do not impede the infection, replication, and shedding of field GaHV-2: vaccinal and field viruses can coexist in the vaccinated host and molecular tests able to discriminate between GaHV-2 and HVT are required. These new simple, fast, and accurate tests for the monitoring of MD vaccination success in the field could be greatly beneficial for field veterinarians, small laboratories, and more broadly for resource-limited settings.

TABLE OF CONTENTS

Introduction

Marek's disease (MD) is a contagious, lymphoprolipherative and neuropathic disease of poultry caused by a ubiquitous lymphotropic and oncogenic virus, *Gallid alphaherpesvirus 2* (GaHV-2) also known as Marek's disease virus (MDV). GaHV-2 infection, which occurs through inhalation of infectious viral particles that are present in the environment, may induce neoplastic transformation of T cells resulting in development of lymphoid tumours, paralysis and immunosuppression (Schat and Nair, 2013) and is responsible for one of the most frequent cancers in animals (Bertzbach et al., 2020).

MD has been reported in all poultry-rearing countries (Dunn & Gimeno, 2013; Mete et al., 2016) and is among the viral diseases with the highest economic impact in poultry industry worldwide (Bertzbach et al., 2020). MD has been recognized as one of the leading causes of mortality in backyard poultry in several countries representing a constant threat for backyard farming too (Pohjola et al. 2015, Demeke et al. 2017, Cadmus et al. 2019, Brochu et al. 2019, Chacón et al. 2019).

Vaccination with live vaccines has represented the central strategy for the prevention and control of MD since 1971 (Schat, 2016), although implemented over the years to cope with the emergence of field GaHV-2 strains with increased virulence (Witter, 1997; Trimpert et al., 2017; Nair, 2018). MD vaccines are referred to as "leaky", as they prevent clinical MD but do not impede the infection, replication, and shedding of wild-type GaHV-2 in the environment (Islam & Walkden-Brown, 2007; Fakhrul Islam et al., 2008; Islam et al., 2014; Read et al., 2015; Ralapanawe et al., 2016a,b) and can promote evolution of increased pathogen virulence (Bailey et al., 2020).

Literature review: Marek's disease

Etiology

Marek's disease (MD) is caused by *Gallid alphaherpesvirus 2* (GaHV-2), traditionally referred to as Marek's disease virus (MDV), member species of the genus *Mardivirus* belonging to the subfamily *Alphaherpesvirinae* of the family *Herpesviridae* based on the International Committee on Taxonomy of Viruses (ICTV) classification (Gatherer et al., 2021).

Two other species of interest are included in the *Mardivirus* genus: *Gallid alphaherpesvirus 3* (GaHV-3), also known as Marek's disease virus serotype 2 (MDV-2), and *Meleagrid alphaherpesvirus 1,* the herpesvirus of turkeys (HVT). GaHV-3 and HVT were shown to be apathogenic for chickens and antigenically related to GaHV-2 offering good protection against MD and have been successfully used as vaccines against MD in chickens since the 1970s (reviewed by Schat, 2016) alone or in combination with attenuated GaHV-2 vaccine strains (e.g CVI988/Rispens vaccine) (Gimeno et al., 2012a).

GaHV-2, as a member of the family *Herpesviridae,* has spherical virions (Figure 1) consisting of an inner core of linear double-stranded DNA, icosahedral capsid, tegument, and a lipid envelope (Denesvre 2013; Gatherer et al., 2021).

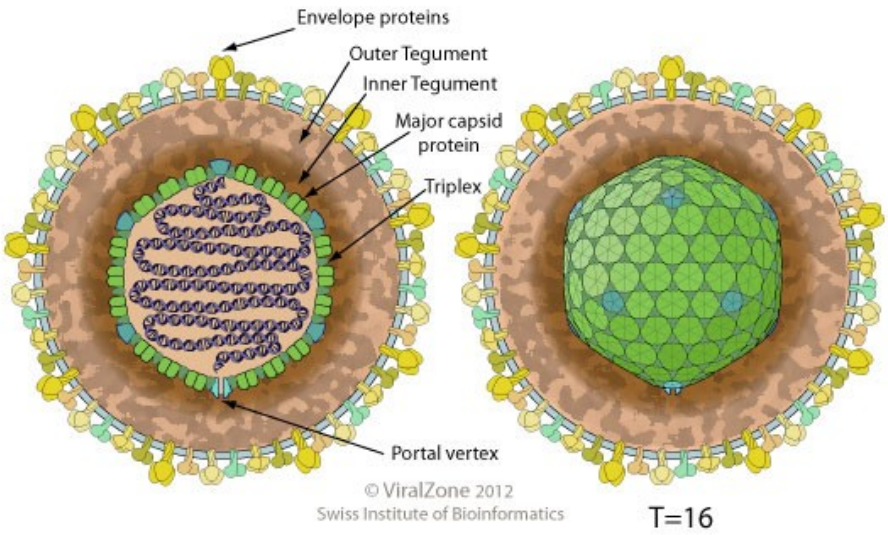

Figure 1. Schematic drawing of the prototype alphaherpesvirinae subfamily virion (https://viralzone.expasy.org/15?outline=all_by_species).

The linear, double-stranded DNA genomes is approximately 160-180 kb long and contains more than 100 genes encoding proteins that are involved in the control of the GaHV-2 life cycle and pathogenesis (Tulman et al., 2000; Bertzbach et al., 2018a; Bertzbach et al., 2020). GaHV-2 genome (Figure 2) is organised into a unique long (U_L) and a unique short (U_S) region flanked by terminal (TR_L and TR_S) and internal (IR_L and IR_S) repeat long and short regions (Tulman et al., 2000).

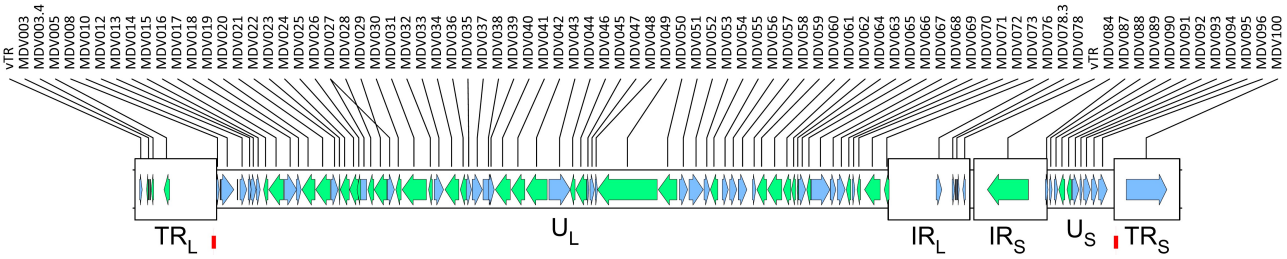

Figure 2. Diagrammatic representation of GaHV-2 genome showing the genomic locations and orientations of each of the recognised open reading frames (Trimpert et al., 2017).

GaHV-2 genomic structure resembles that of alphaherpesviruses, and the U_S and U_L regions of GaHV-2 are collinear and conserved with the corresponding regions of other alphaherpesviruses. In contrast, the repeat regions of the GaHV-2 genome differ among these viruses, containing genes whose products are believed to participate in GaHV-2 virulence and oncogenicity. The complete genome sequences of multiple GaHV-2 strains have now been determined and publicly available on the GenBank database (Tulman et al., 2000; Spatz et al., 2007; Trimpert et al., 2017). At phylogenetic analysis, the complete nucleotide sequences of GaHV-3 strains are more similar to HVT strains than to GaHV-2 strains showing approximately 60% sequence identity with the sequence of the reference GaHV-2 strain Md5 (Spatz and Schat, 2011). HVT resembles other alphaherpesviruses in genome organization and is closely related to GaHV-2 and GaHV-3 within UL and US regions, where homologous genes share a high degree of colinearity and their proteins share a high level of amino acid identity (Afonso et al., 2001). Significant genomic differences occur between HVT and GaHV-2 in and adjacent to repeat long and short regions, and these may account for differences in virulence, making HVT nonpathogenic for chickens.

Among GaHV-2 genes, there are several unique genes that are directly or indirectly involved in pathogenesis and tumorigenesis such as the Marek's Eco RI-Q (*meq*) oncogene, the gene encoding the viral chemokine vIL-8/vCXCL13, RLORF4, RLORF5a, the gene encoding the neurovirulence factor pp14, and the gene encoding the phosphoprotein pp38, as recently reviewed (Osterrieder et al., 2006; Bertzbach et al., 2018a; Bertzbach et al., 2020).

Between virulence-associated genes, the *meq* oncogene, unique to GaHV-2 and highly expressed in latently-infected and transformed T CD4+ cells (Tai et al., 2017), appears to play a key role in the virus-induced transformation process of latently-infected T lymphocytes. The *meq* gene encodes the Meq protein, a protein with homology to the leucine-zipper class nuclear oncogenes, which is composed of an N-terminal basic-leucine zipper (bZIP) domain and a C-terminal proline-rich domain (Qian et al., 1995; Liu et al., 1999; Ross, 1999) (Figure 3). Meq oncogenic activities are mediated by its dimerisation, through the bZIP domain, with itself (homodimers), as well as with c-Jun-like proteins (heterodimers) repressing the expression of several genes or promoting the transcription (Brown et al., 2009; Suchodolski et al., 2010). Meq can also bind to cellular transcription factors (Deng et al., 2010) and interacts with cellular proteins lacking the bZIP domain, such as the cellular tumour suppressors p53, the retinoblastoma protein, the cyclin-dependent kinase 2, and the heat shock protein 70 (Deng et al., 2010; Gennart et al., 2015).

The *meq* gene is highly polymorphic but is generally 1020 base pairs (bp)-long and encodes for 339-amino-acid Meq protein (Chang et al. 2002). *Meq* gene polymorphism is due to the presence of insertions or deletions in the C-terminal proline-rich repeat region and several Meq protein isoforms of various sizes (from 247 to 438 amino acids) have been reported over the years thanks to the sequencing of the entire *meq* gene of the circulating GaHV-2 strains (Chang et al., 2002b; Shamblin et al., 2004; Molouki et al., 2021). The first studies conducted on *meq* associated the variable number of proline-rich repeats (PRR), along with specific point mutations in the PRR, with GaHV-2 virulence: highly virulent GaHV-2 strains showed shorter Meq isoforms due to the presence of a

lower number of PRRs and mutations interrupting the PRRs (Shamblin et al., 2004; Renz et al., 2012).

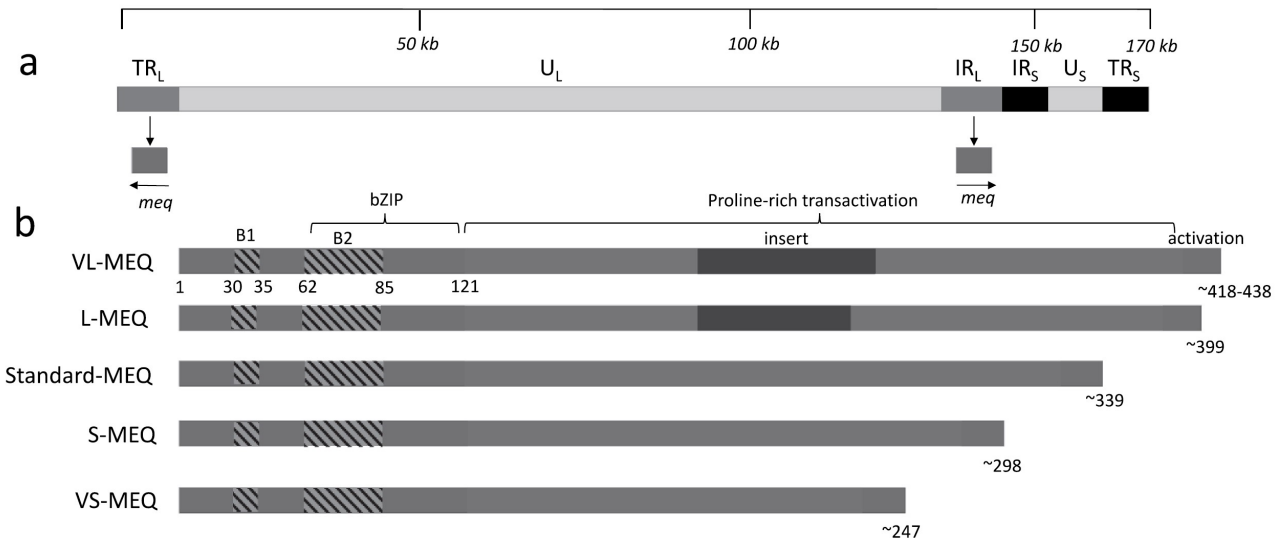

Figure 3. Location in the GaHV-2 genome of the two copies of the *meq* gene (a) and schematic structure of some of the different Meq protein isoforms (b) (Molouki et al 2021).

The *meq* gene is one of the candidate genes associated with the increase of GaHV-2 virulence over the years due to the presence of a greater-than-average number of point mutations found in its sequence in the virulent GaHV-2 strains after whole-genome sequencing (Trimpert et al., 2017). The *meq* gene is evolving at a much faster rate than most dsDNA viruses (Duffy, Shackelton, & Holmes, 2008; Firth et al., 2010), and most of its polymorphisms have evolved under positive selection most likely imposed by vaccination, reflecting viral adaptation against the host immune responses (Padhi and Parcells, 2016; Trimpert et al., 2017). These findings have been definitively corroborated in 2020 when Conradie and colleagues, after the generation of recombinant viruses carrying Meq isoforms coming from reference strains belonging to different pathotypes, confirmed with *in vivo* experiments that even a few point mutations affecting the number of PRRs in the *meq* gene have contributed to GaHV-2 evolution towards a greater virulence by influencing the transactivation activity of Meq towards target cellular and viral genes. These point mutations acquired in the *meq* gene during evolution through the years contributed to an increase in GaHV-2

virulence, to an increase in vaccine resistance, and to enhance the virus shedding into the environment (Conradie et al., 2020).

Strain classification and virus evolution

The clinical features of MD have changed drastically since its first description by Joszef Marek, which dates back to 1907, as a mild paralytic syndrome: MD turned into a highly contagious and severe neoplastic disease throughout the years due to a shift in the virulence of GaHV-2 isolates with the emergence of increasingly virulent strains. For this reason, virus strains have been classified based on their virulence into four pathotypes: mild (m), virulent (v), very virulent (vv), and very virulent + (vv+) (Witter, 1997; Witter et al., 2005).

Mild strains (mGaHV-2), were predominant before the 1950s; virulent strains (vGaHV-2) emerged during the 1950s through the 1960s, the period during which the broiler industry had undergone a drastic increase in the number of birds per square meter; very virulent strains (vvGaHV-2), emerged during the late 1970s, after the introduction and widespread use of HVT-based vaccines, and very virulent plus strains (vv+GaHV-2) emerged in the early 1990s after the introduction in the USA of the bivalent vaccination with HVT + GaHV-3 SB-1 strain (Witter, 1997). After the emergence of vv+GaHV-2 strains, in the mid-1990s, US poultry producers have included CVI988/Rispens in MD immunization protocols: since then and to date no significant shift in virulence has been noted in the US pathotyped field GaHV-2 strains with vv+ pathotype remaining the more virulent pathotype identified (Dunn et al., 2019) (Figure 4).

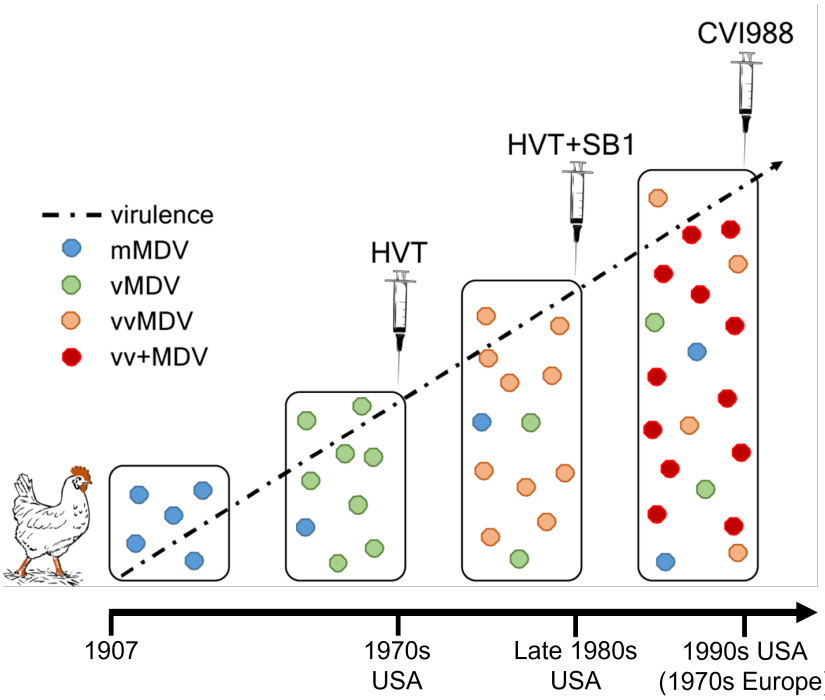

Figure 4. Step-wise evolution of GaHV-2 virulence and relationship with the introduction of different vaccines in the US and Europe (HVT: herpesvirus of turkey; SB1: Gallid herpesvirus 3 strain SB-1; CVI988: non-oncogenic GaHV-2 strain) (modified from Bertzbach et al., 2020).

At present *in vivo* pathotyping studies are mandatory for an accurate inclusion of GaHV-2 strains into one of the known pathotypes for monitoring shifts in virulence of field strains (Dunn et al., 2014). Traditional pathotyping assays designated the virulence of field GaHV-2 strains based on lesion responses in unvaccinated, HVT-vaccinated, and HVT+ GaHV-3-vaccinated specific pathogen free chickens (Witter et al., 2005; Dudnikova et al., 2007). To better standardize the pathotype designations between different laboratories, prototype reference isolates of known pathotype (vGaHV-2 strain JM/102W, vvGaHV-2 strain Md5, and vv+GaHV-2 strain 648A) were introduced as a comparison against field isolates.

The mutational landscape of GaHV-2, whose genome is large and complex, is wide and multiple genotypic pathways underlie GaHV-2 evolution towards greater virulence. The phylogenomic analysis of the whole genome sequences of twenty GaHV-2 strains isolated in 50 years' time (from 1968 to 2015) in the USA, Europe, and China revealed a higher evolutionary rate, in terms of nucleotide substitution per site per year, than expected for dsDNA viruses, which are commonly characterized by relatively low evolutionary rates (Duffy et al., 2008; Firth et al., 2010; Trimpert et

al., 2017). ORFs with mutations that appeared to be specifically associated with virulence were identified. In particular, the *meq* oncogene has been strongly associated with the evolution of GaHV-2 virulence: the *meq* gene sequence evolves at a very fast rate, which is comparable with the evolutionary rates of genes belonging to RNA viruses (Padhi & Parcells, 2016; Trimpert et al., 2017). This acceleration in evolution has been explained by a strong selective pressure undergone by the virus due to the widespread use of imperfect, leaky vaccines in industrially-reared chickens, which are known to affect viral replication (e.g. fitness) and host-to-host transmission (Read et al., 2015) favoring the emergence of field GaHV-2 strains with increased genetic diversity (Padhi & Parcells, 2016). The appearance of the majority of the *meq* polymorphisms as nonsynonymous point mutations indicate that the *meq* gene has evolved under positive selection and the time of genetic divergence coincides with the use of MD vaccines on a massive scale by the poultry industry (Padhi & Parcells, 2016; Trimpert et al., 2017).

Epidemiology

Chicken is the natural host species of GaHV-2. Domestic poultry species such as quails, turkeys, and pheasants are susceptible to natural infection and can develop the disease (Imai et al., 1990; Pennycott et al., 2003; Davidson et al., 2002; Blake-Dyke and Baigent, 2013; Hauck et al., 2020) and horizontal transmission between chickens and quails and between turkeys and chickens was established.

MD is reported worldwide, in all countries where intensive poultry farming is developed, and a 2004 estimate suggested that MD causes annual economic losses of US$1–US$2 billion to the global poultry industry (Morrow and Fehler, 2004). In 2011 an assessment of the global prevalence of the disease was made in 116 countries (Dunn and Gimeno, 2013). The results show how, over a span of ten years, there was an increase in the incidence of the disease in 50% of the countries considered in the study, with most of the cases found in French-speaking Africa, in Eastern Europe, in East Asia, and South America. In 16% of countries, the increase in the incidence of the disease

has been attributed to highly virulent GaHV-2 strains, in the other countries to the contextual presence with other immunosuppressive diseases. In 42% of countries, the incidence of the MD decreased over time, probably due to more widespread use of the CVI988/Rispens vaccine and due to the adoption of management-related improvements. Published reports of MD outbreaks are continuous and their availability through online databases provides valuable updates of MD worldwide status for the different commercial productive types. MD has been also reported as a major cause of mortality in backyard poultry flocks which are composed of birds with different ages, breeds and immune statuses, thus having different susceptibility to GaHV-2 infection and to the development of the clinical disease (Pohjola et al., 2015; Mete et al., 2016).

MD outbreaks have been reported in ornamental and game birds kept as pets such as crested partridges (*Rollulus rouloul*) (Haesendonck et al., 2015; Schock et al., 2016) and white-peafowls (*Pavo cristatus*) (Blume et al., 2016) and in endangered wild species such as red-crowned cranes (*Grus japonensis*) (Lian et al., 2018).

Wild geese and ducks such as mallards (*Anas platyrhynchos*), spot-billed ducks (*Anas poecilorhyncha*), European wigeons (*Anas penelope*), pintails (*Anas acuta*), common teals (*Anas crecca*), and white-fronted geese (*Anser albifrons*) are susceptible to GaHV-2 infection and can act as GaHV-2 carriers or reservoirs (Murata et al., 2012) (Figure 5).

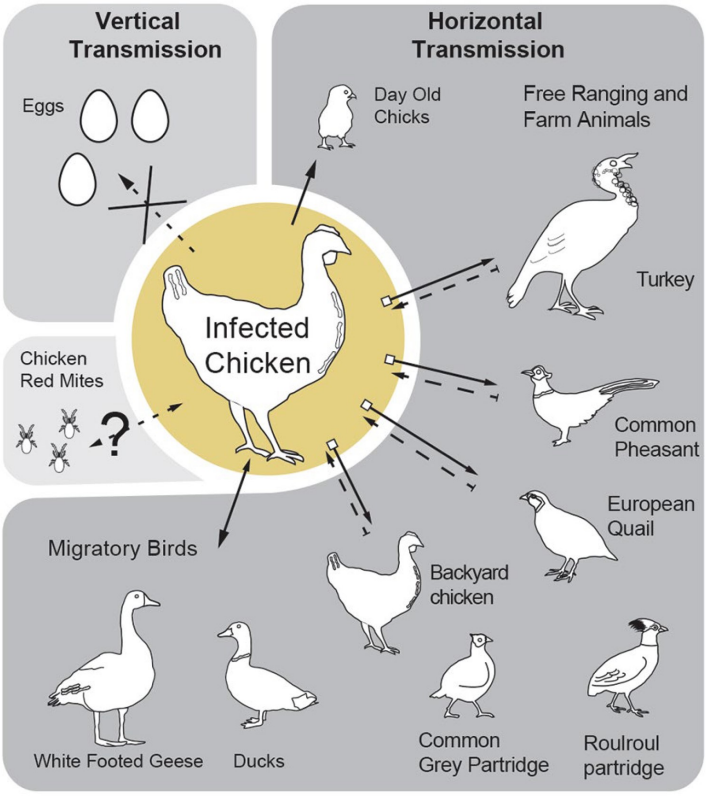

Figure 5. Graphical display of the transmission mode of GaHV-2 between susceptible host species (Boodhoo et al., 2016).

Mammals are refractory to experimental infection with GaHV-2, although in 1991 GaHV-2 was unfairly listed as a cause of multiple sclerosis in humans; this was subsequently disproved as no evidence was found for the spread of GaHV-2 to humans (Hennig et al, 2003).

The transmission of GaHV-2 is horizontal, direct or indirect, by the airborne route. Susceptible host species become infected by inhalation of infected aerosols containing cell-free virus particles (Hao et al., 2014). Infectious GaHV-2 virions are shed through feather follicle epithelium desquamation: desquamated cells are the source of contamination for the poultry house environment and for other individuals (Calnek et al., 1970). The virus eliminated into the environment accumulates in feather dander and poultry dust where it remains infectious for several months at 20-25°C, and for years at 4°C (Jurajda and Klimes, 1970). Chickens become infected when placed in poultry houses harboring contaminated dust or litter from previous GaHV-2-infected flocks or through contaminated equipment and personnel. Vertical transmission of GaHV-2 from hen to eggs has not

been demonstrated (Solomon et al., 1970). The role of Dermanyssus gallinae as a potential carrier of GaHV-2, though suspected, has yet to be proved (Huong et al., 2014).

MD-associated mortality is rather variable and depends on host genetic susceptibility to the disease, vaccination status, and virulence of the GaHV-2 strains involved: GaHV-2 strains of higher virulence more rapidly kill hosts, but vaccination enhances host life expectancy (Atkins et al., 2011).

Pathogenesis

The generally accepted model of the GaHV-2 life cycle, the "Cornell model" of GaHV-2 infection, proposes the starting of GaHV-2 life cycle with host infection through inhalation of infectious viral particles and its ending with the generation of infectious virus in feather follicle epithelial cells and the subsequent environmental spread (Calnek, 2001). GaHV-2 life cycle can be divided into four interlacing phases: entry, replication, latency, and spread (Bertzbach et al., 2020) (Figure 6).

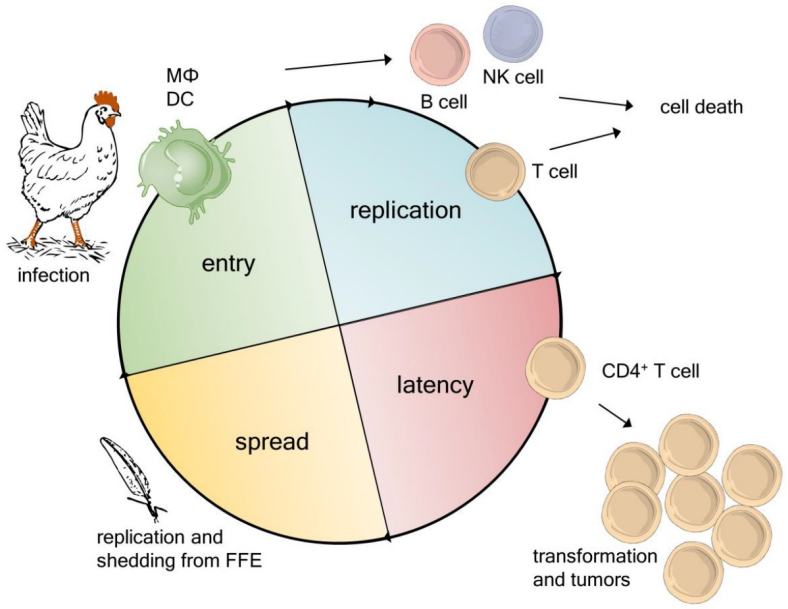

Figure 6. Schematic representation of GaHV-2 life cycle (Bertzbach et al., 2020).

The infection begins with the inhalation of airborne infectious viral particles from the surrounding environment. Phagocytes, such as macrophages and dendritic cells, that have been shown to support GaHV-2 replication *in vitro* (Chakraborty et al., 2017), engulf GaHV-2 virions in the chicken

respiratory tract and transport the virus to lymphoid tissues (spleen, bursa of Fabricius, and thymus) where lymphocytes become infected (Barrow et al., 2003). At 4-6 days post infection the virus causes cytolytic, cell-associated productive infection of B and T lymphocytes (CD4+ T cells and, rarely, CD4–CD8– T cells or CD8+ T cells) and of natural killer cells (Bertzbach et al., 2019) especially in the primary lymphoid organs where the virus is rapidly amplified (Baigent & Davison, 1999; Baigent et al., 1998; Bertzbach et al., 2018b). This first replication phase in lymphocytes is known as early cytolytic phase (Figure 7). From approximately 7 days post infection, the virus establishes latency in CD4+ T lymphocytes (Schat et al., 1991); integrating its genome into the telomeres of latently infected T cells (Kheimar et al., 2017) that act as virus reservoir in the host. This phase is called "latent infection" or "latency", it coincides with the development of the host immune response, and can last for the lifetime of the bird. Latency is defined as the "presence and maintenance of viral genomes without production of infectious progeny virus" (Osterrieder et al., 2006). Infected T lymphocytes transport the virus to the feather follicle epithelial cells, where cell-free, infectious GaHV-2 is assembled and, subsequently, shed into the environment to infect another host. GaHV-2 can reactivate from latency and disseminate to reach tissues of epithelial origin (e.g. liver, proventriculus, kidneys, lungs, oesophagus, adrenal glands, and skin) where a second, late round of productive cytolytic infection can be established by the end of the second week after infection (Calnek, 2001). GaHV-2, in contrast to the other two member species of the Mardivirus genus affecting the chicken, GaHV-3 and HVT, is able to transform latently infected T cells resulting in the development of multifocal T cell lymphomas in visceral organs, skin, and peripheral nerves. This phase is identified as the "neoplastic phase" (Calnek, 1986). GaHV-2 is capable of replicating and establishing latency in T lymphocytes and may induce neoplastic transformation of latently-infected CD4+ T cells, leading to the development of multiple lymphomas in the visceral organs (Nair, 2013). The basic leucine zipper protein Meq and the virus-encoded RNA subunit of telomerase (vTR) have been shown to be directly involved in GaHV-2-induced lymphomagenesis

(reviewed by Osterrieder et al., 2006). The last 3 stages (latency, late cytolytic infection, and neoplastic transformation) can coexist in different cells of the same bird.

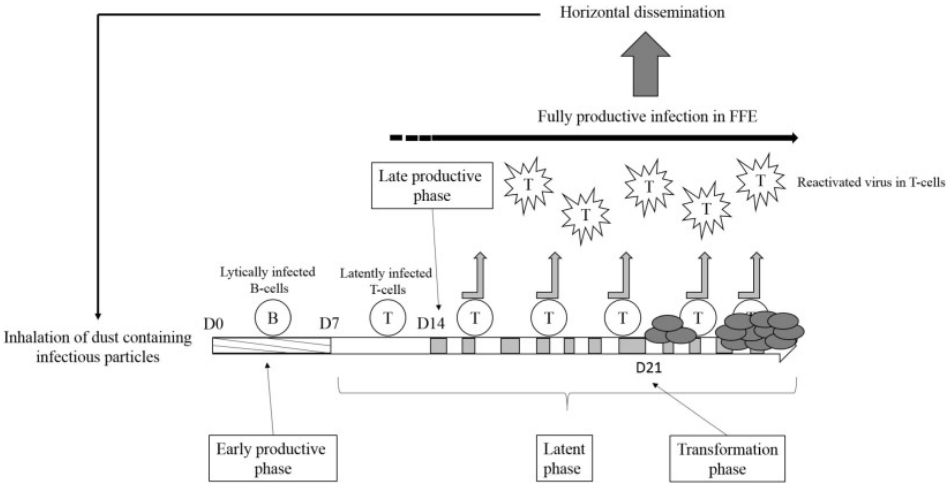

Figure 7. Different phases of oncogenic GaHV-2 infection: (1) early cytolytic/productive phase, (2) latency, (3) late cytolytic/productive phase, (4) neoplastic transformation phase (Gennart et al., 2015).

Pathological syndromes and clinical signs

GaHV-2 can induce multiple pathological syndromes accompanied by specific or aspecific clinical signs. These syndromes can be divided into two broad categories: neoplastic and nonneoplastic; the former being the most relevant for poultry production due to the significant economic repercussions connected to their appearance while the impact of the latter category is less clear (Gimeno, 2014). Lymphoproliferative syndromes (neoplastic), referred to as “acute MD” and caused by virulent oncogenic strains of GaHV-2 (Witter, 1997), appear clinically with signs that vary according to the location of the MD-induced lymphomas. Clinically affected animals can show nonspecific signs such as depression, paralysis, anorexia, weight loss, paleness, and diarrhea (Schat and Nair, 2013) which often precede death.

The classic form of Marek's disease, the so-called "fowl paralysis" or “classical MD”, which occurs in unvaccinated animals infected with mild strains of GaHV-2, is characterized by dysfunction of peripheral nerves. The affected animals develop neurologic signs such as spastic paralysis of a single or multiple extremities (legs, wings, or neck), depending on the affected nerve.

Incoordination and lameness are the first clinical signs of the disease to be recognized in the field. A typical clinical presentation of classical MD is a bird with one leg stretched forward and the other back (Figure 8) as a result of unilateral paralysis of the leg due to involvement of the sciatic plexus or sciatic nerve. Paralysis of one or both wings may appear when brachial plexuses and nerves are involved. When the vagus nerve is involved, the crop can appear distended due to paralysis.

Figure 8. Chicken affected by MD and suffering of unilateral paralysis of a leg (https://partnersah.vet.cornell.edu/avian-atlas/#/disease/Marek's_Disease).

Lymphodegenerative syndromes, transient paralysis, and panophthalmitis fall within the category of nonneoplastic syndromes and usually occur in susceptible unvaccinated chickens lacking maternal antibodies, thus are quite rare to observe in industrial flocks. The productive cytolytic infection established in the lymphoid organs between 5 and 6 days post infection with highly virulent GaHV-2, in susceptible chickens lacking maternal antibodies, may lead to severe necrosis and inflammation of lymphoid organs inducing lymphodegenerative syndromes. Birds are immunosuppressed and become depressed, with ruffled feathers and reluctant to move (Gimeno, 2014).

Transient paralysis is a neurological syndrome causing, in the affected animals and in its classical manifestation, temporary ataxia and flaccid paralysis of the neck or limbs lasting 24-48 hours followed by a rapid and complete recovery (Figure 9). A few weeks later, affected birds can succumb due to visceral lymphomas (Schat and Nair, 2013). This neurological syndrome is occasionally reported in unvaccinated broilers (Gimeno, 2014) and in unvaccinated backyard chickens.

Figure 9. Chicken affected by transient paralysis showing flaccid paralysis of the neck (https://partnersah.vet.cornell.edu/avian-atlas/#/disease/Marek's_Disease).

Some MD-affected animals may present eye damage with uni- or bilateral blindness (Pandiri et al., 2008). The involved eye loses its ability to accommodate when exposed to an intense source of light, the pupil shows irregular margins, and the iris loses its normal pigmentation and acquires a greyish color (Figure 10).

Figure 10. Chickens with ocular abnormalities (https://partnersah.vet.cornell.edu/avian-atlas/#/disease/Marek's_Disease).

Gross and microscopic lesions

The typical gross lesions of the acute lymphoproliferative form of the disease are visceral lymphomas in various organs and tissues: liver, spleen, proventriculus, gonads, kidneys, lungs, heart, mesentery, intestine, bursa of Fabricius, thymus, adrenal glands, pancreas, skeletal muscle, and skin (Figure 11). Lymphomas appear as a diffuse enlargement of the organ, which loses its normal color appearing whitish or greyish, or as focal white or grey nodular lesions of different

sizes, firm and smooth when cut (Schat and Nair, 2013). In the past, skin leukosis represented one of the most important causes of broiler condemnation at slaughterhouse. Skin tumors are generally associated with the feather follicles and are initially whitish and nodular in shape. Later the lesions can take on a crusty and brownish appearance. They can be disseminated throughout the body surface or located in a specific area (Gimeno, 2014).

Histologically MD lymphomas are composed of a pleomorphic population of mononuclear cells such as small or medium T lymphocytes and lymphoblasts, natural killer cells, B lymphocytes, and macrophages (Schat and Nair, 2013) (Figure 12). Usually, lymphomas can be observed microscopically starting from one week post infection; over time they become more and more pronounced and, from about three weeks post-infection, may become visible to the naked eye (Payne, 2004).

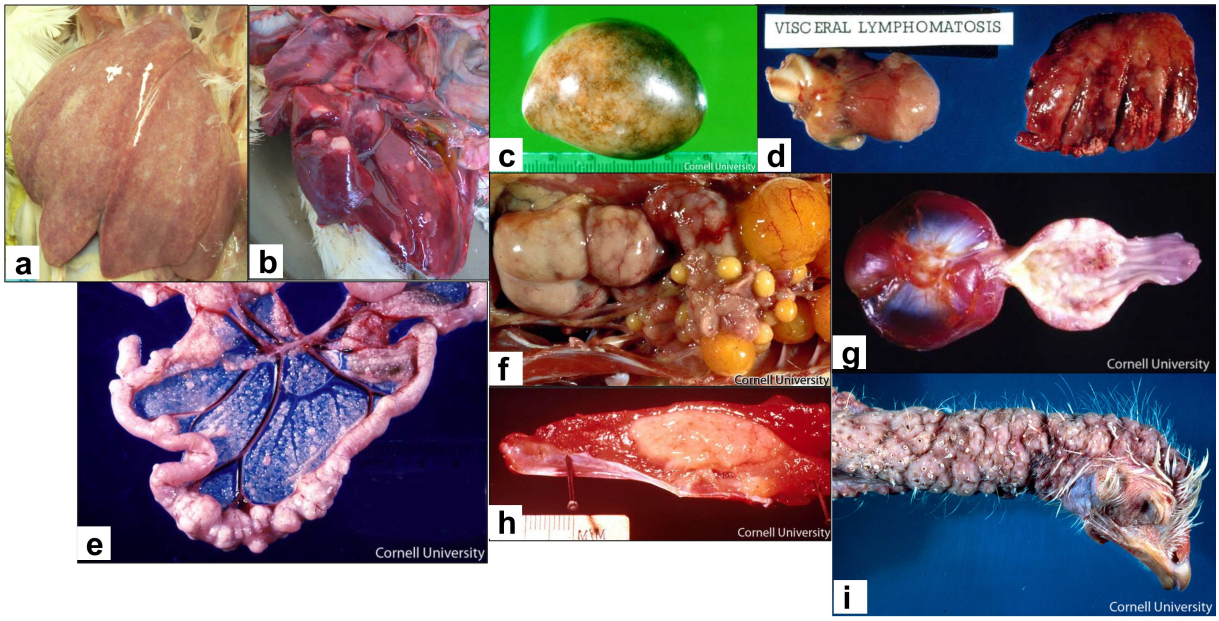

Figure 11. MD-induced lymphomas in different organs and tissues: a) and b) liver (Prof.ssa Piccirillo); c) spleen; d) heart and lung; e) intestine and mesentery; f) kidney and ovary; g) proventriculus; h) deep pectoral muscle; i) skin (https://partnersah.vet.cornell.edu/avian-atlas/#/disease/Marek's_Disease).

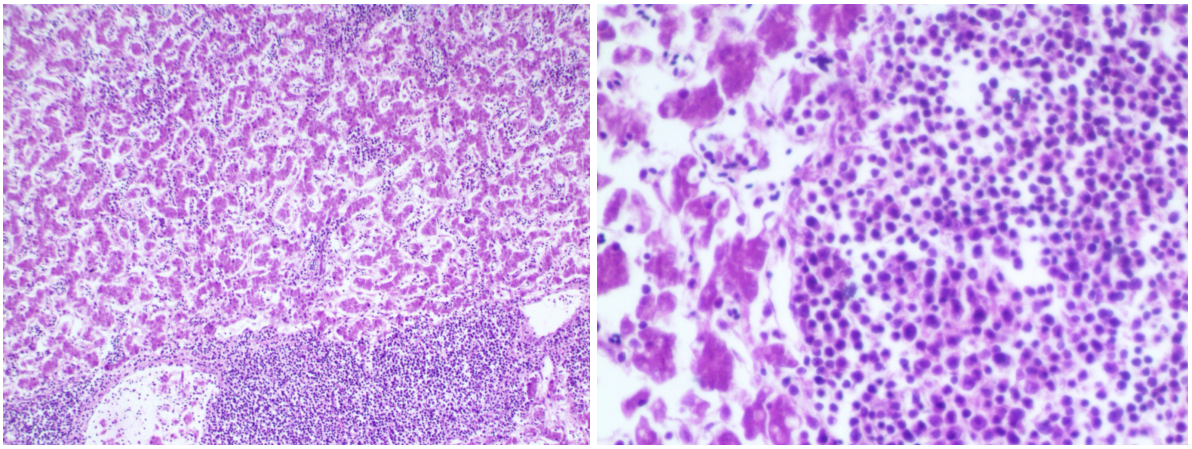

Figure 12. Microscopic appearance of MD visceral lymphomas. Liver. Pleomorphic lymphocyte population in a liver lymphoma. (On the left: 10x; On the right: 40x). Haematoxylin and eosin (HE) (University of Bologna).

Classical MD or "fowl paralysis" is mainly characterized by gross lesions affecting peripheral nerves and nervous plexuses. Involved nerves and root ganglia are enlarged and edematous, can lose their typical cross-striation and their pearly white color may be replaced by a gray-yellow discoloration (Figure 13). Lesions can be uni- or bilateral and can affect a nerve uniformly along its length or only focally involving separate portions of the nerve (Schat and Nair, 2013; Gimeno, 2014). Gross nerve lesions may be found also in acute MD concomitantly with visceral tumors. At microscopic examination, the lesions affecting peripheral nerves have been subdivided into three types: A, B, and C (Payne and Biggs 1967) (Figure 14). Type A lesions, considered neoplastic, consist of massive infiltration of pleomorphic lymphoid cells (small, medium, and large lymphocytes and reticuloendothelial cells) that destroy the architecture of the nerve and that can be associated with demyelination. Type B lesions, which appear after type A lesions, are inflammatory in nature and are characterized by an infiltration of scattered small lymphocytes and plasma cells, accompanied by edema. Type C lesions are less pronounced type B lesions and are characterized by a mild infiltration of plasma cells and small lymphocytes. The coexistence of the three types of lesions can be observed in different nerves of the same animal or in different areas of the same nerve.

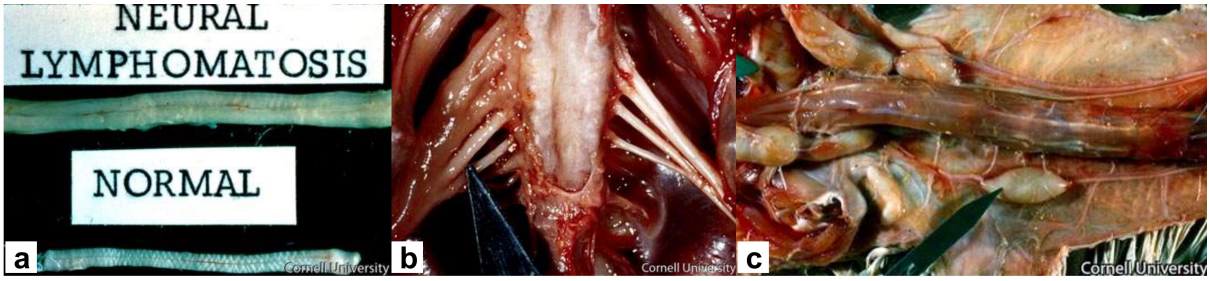

Figure 13. MD-induced gross lesions of peripheral nerves and nervous plexuses: a) sciatic nerve; b) sciatic plexus; c) vagus nerve (https://partnersah.vet.cornell.edu/avian-atlas/#/disease/Marek's_Disease).

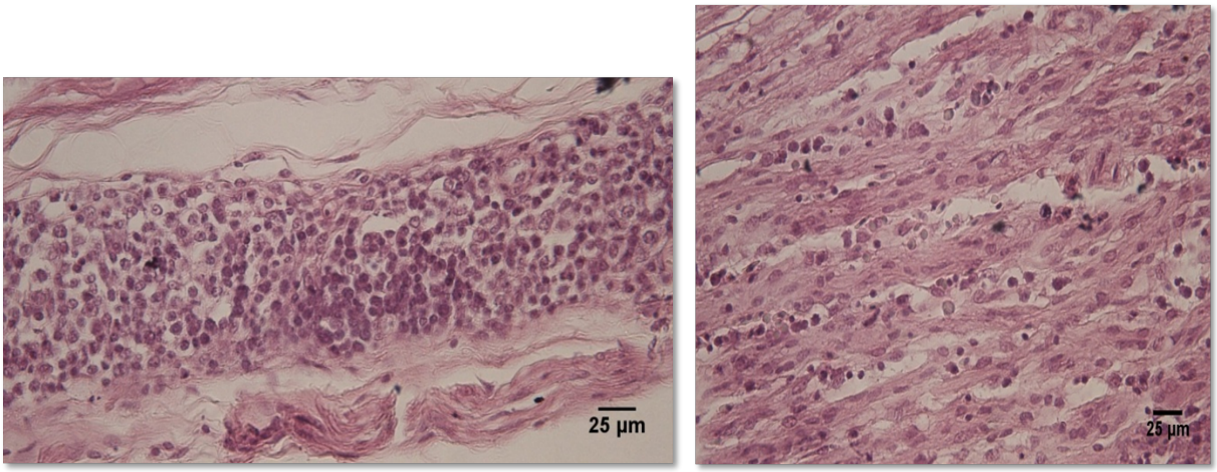

Figure 14. MD-induced lesions in peripheral nerves. On the left: Sciatic nerve. Pleomorphic infiltration of small, medium and large lymphocytes, compatible with A-type lesions (40×). Haematoxylin and eosin (HE); On the right: Sciatic nerve. Interneuritic oedema with diffuse infiltration of small lymphocytes and plasma cells, compatible with B-type lesions (40×). HE (University of Bologna).

Severe thymic and bursal atrophy, sometimes accompanied by splenomegaly, are the gross lesions associated with lymphodegenerative syndromes (Figure 15). These lesions might be reversible after infection with low virulence GaHV-2 strains but persist after infection with highly virulent strains (Gimeno, 2014). At microscopic examination, lymphoid organs have lost their normal architecture due to cytolysis of lymphocytes, hyperplasia of reticulum cells, and infiltration of granulocytes and macrophages.

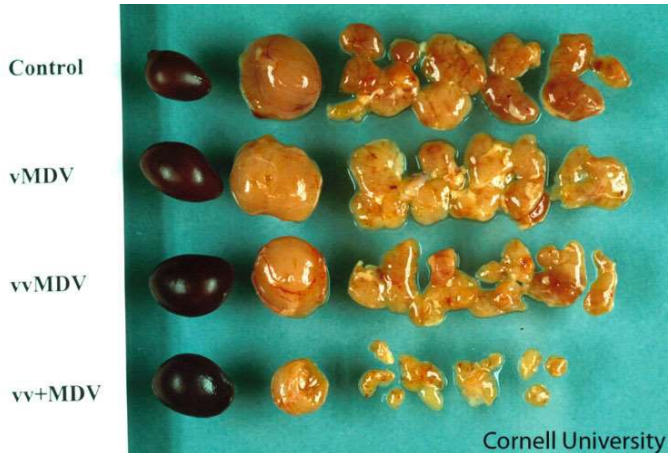

Figure 15. Spleen, bursa and thymus of a healthy animal compared with those of animals infected with of v, vv and vv+ GaHV-2 strains. As virulence increases, splenomegaly and bursal and thymic atrophy increase in severity (https://partnersah.vet.cornell.edu/avian-atlas/#/disease/Marek's_Disease).

There are no gross lesions associated with transient paralysis. On the other hand, nonneoplastic brain lesions such as vasculitis, vasogenic edema, and tissue vacuolization appear microscopically a few hours before or coordinately with clinical flaccid paralysis and resolved in 2–3 days (Swayne et al., 1989; Gimeno et al., 1999).

Loss of pigmentation in the iris and irregularity of the margins of the pupil are the gross lesions affecting the eye during MD (Figure 16). Macroscopic changes are mainly caused by mononuclear cell infiltration of the iris and by lymphohistiocytic inflammatory cell infiltrating other ocular structures (Pandiri et al., 2008).

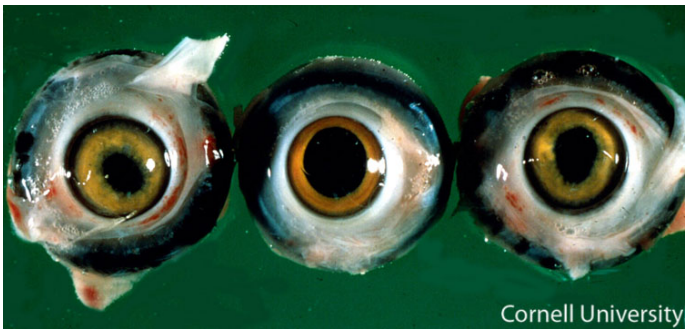

Figure 16. Gross ocular changes due to MD. A normal eye is shown in the centre. The eyes on the left and right exhibit discoloured irises with irregular pupil shape. (https://partnersah.vet.cornell.edu/avian-atlas/#/disease/Marek's_Disease).

Diagnosis

Several techniques may be used to detect GaHV-2 infection and to diagnose MD. MD cannot be diagnosed based solely on the detection of the virus since GaHV-2 is ubiquitous under field

conditions in poultry farming. Infected animals do not always develop clinical disease and vaccinated animals can become infected with field strains without showing clinical disease.

To correctly diagnose the acute form of MD two other viral diseases that induce the formation of lymphomas in chickens must be considered: lymphoid leukosis (LL), caused by an *Alpharetrovirus*, the *Avian Leukosis Virus*, and reticuloendotheliosis (RE), caused by *Reticuloendotheliosis virus*, a *Gammaretrovirus*.

Grossly, the lymphomas caused by the three viruses are indistinguishable and, despite the existence of a certain degree of organ tropism, there are no pathognomonic lesions (Gimeno, 2014). To correctly diagnose MD, it is necessary to proceed step by step (Schat and Nair, 2013; Gimeno, 2014). A first provisional diagnosis of MD can be done directly in the field evaluating epidemiological, clinical, and pathological aspects. Later, the diagnosis should be confirmed in the laboratory through the identification of the causative agent. The most important epidemiological data for the differentiation between MD and retrovirus-induced tumors is the age of the animals at the onset of tumors: LL- or RE-induced lymphomas appear after 14-16 weeks of age; MD-induced lymphomas may appear also in birds younger than 14 weeks of age (Biggs, 1976; Gimeno, 2014). Clinical signs associated with the development of visceral tumors are nonspecific (e.g. depression, weight loss, and ruffled feathers) and may appear in association with each one of the three viral diseases. MD may be suspected when neurological signs are reported in association with lymphomas. Gross pathological lesions can provide useful indications for differential diagnosis of viral neoplastic diseases too. The exclusive presence of peripheral nerve lesions can lead to classical MD or to peripheral neuropathy (PN), a non-neoplastic neurological syndrome of probable autoimmune nature, which induces enlargement of peripheral nerves (Bacon et al., 2001). In the presence of visceral lymphomas and peripheral nerves lesions, PN and LL can be excluded, but MD and RE cannot. On the other hand, the presence of nodular tumors in the bursa of Fabricius is frequently reported in LL and RE, while the involvement of the eye, skin, muscle, and proventriculus is more frequently or exclusively found during an MD outbreak.

The differential diagnosis of viral neoplastic disease is therefore complicated. After acquiring all the information above, the suspicion of Marek's disease may be issued, although the diagnosis needs further laboratory confirmation by histological examination, finding the typical microscopic lesions in affected organs and tissues, and by detecting or isolating GaHV-2 from the same biological matrixes.

Moving on to laboratory diagnosis, portions of tumors and peripheral nerves may be collected and fixed in 10% neutral buffered formalin or in another adequate fixative, embedded in paraffin blocks and stained with haematoxylin and eosin for an adequate histopathological evaluation. Two main aspects must be evaluated in the histological examination: distribution of lesions and characteristics of the tumor cells (Gimeno, 2014). MD tumor lesions may be found in peripheral nerves, tissues, and visceral organs. When lymphomas are detected only in nerves, the diagnosis of MD can be done by histopathology if lesions are type A (proliferative-neoplastic). If type B (inflammatory) lesions are exclusively observed in peripheral nerves it is necessary to rule out PN using more sophisticated laboratory methods such as specific real-time PCR assays to detect oncogenic GaHV-2 strains (Gall et al., 2018). When lymphomas are detected solely in visceral organs, neoplastic diseases induced by retroviruses (LL and RE) must be put in differential diagnosis and further laboratory tests are needed. The morphology of the tumor cells can aid in the diagnosis of MD: the typical MD neoplastic infiltrate is composed of a heterogeneous cell population of lymphoblasts, small, medium, and large lymphocytes and macrophages. LL- and RE-induced lymphomas are formed by a homogeneous population of lymphoblast. Sometimes, however, RE-induced lymphomas can be very similar to those induced by MD. In MD-induced lymphomas most tumor cells are T cells (>70%) while in LL-induced lymphomas are B cells (>90%) (Neumann and Witter, 1979), for this reason, the characterization of the phenotype of tumor cells through immunohistochemistry can be very useful for the differential diagnosis of virus-induced neoplastic diseases of poultry (Hauck et al., 2020). Immunohistochemistry also allows to diagnose MD by

identification of the viral antigen Meq, which is consistently expressed in GaHV-2 transformed tumor cells (Gimeno et al., 2005).

Virus isolation in permissive cell cultures is another valid, but time-consuming, technique to prove GaHV-2 presence for diagnostic purposes and to secure infectious virus stocks for further study. Tissues and organs (e.g. tumors, spleen, kidney) from recently deceases animals containing viable cells, where GaHV-2 infectivity is closely cell-associated, or portions of feathered skin, feather tips, or dust, where cell-free infectious GaHV-2 is present, constitute effective starting substrates for virus isolation (Witter et al., 1969; Calnek et al., 1970; Cui et al., 2016; Machida et al., 2017; Pandey et al., 2016).

Primary cell cultures such as chicken kidney cell (CKC), duck embryo fibroblast (DEF), and chicken embryo fibroblast (CEF) inoculated with viable cell suspensions or with cell-free preparations are preferred substrates for primary GaHV-2 isolation (Witter et al., 1969; Schat, 2005). CEFs are preferable for the isolation of GaHV-3, HVT and for the isolation and propagation of the attenuated vaccine strains CVI988/Rispens (Schat, 2005). The typical cytopathic plaques developing from 4 to 6 days post-infection are evidence for GaHV-2 isolation (Schat, 2005). GaHV-2, GaHV-3, and HVT-induced plaques can be differentiated from each other by immunofluorescence techniques using serotype-specific monoclonal antibodies (Lee et al., 1983) or by species-specific PCR- or LAMP-based molecular methods (Handberg et al., 2001; Walkden-Brown et al., 2003; Islam et al., 2006; Renz et al., 2006; Cortes et al., 2011; Woźniakowski et al., 2013; López-Osorio et al., 2019).

PCR-based molecular methods, which allow through exponential amplification of nucleic acids to search for the viral genome in biological samples (Mullis et al., 1986), are considered the gold standard tests for the detection of GaHV-2, confirming the presumptive MD diagnosis. The best starting samples for detecting GaHV-2 genome by PCR are portions of tumors, spleen, and feathers (Islam et al., 2004; Cortes et al., 2011).

Various end-point and real-time PCR assays targeting different GaHV-2 genes have been developed over time. One of the most commonly used target genes is the *meq* gene, unique to GaHV-2 and directly involved in virus-induced oncogenesis (Hassanin et al., 2013; López-Osorio et al., 2019). More importantly, *meq* gene molecular characterization through sequencing and sequence analysis could give indicative information on GaHV-2 pathogenicity as it carries well-identified virulence markers (Shamblin et al., 2004; Renz et al., 2012).

Loop-mediated isothermal amplification (LAMP) is a rapid, extremely specific, and sensitive molecular method that could overcome most of the drawbacks of PCR-based methods (Notomi et al. 2000; Nagamine et al., 2002). LAMP enables the exponential amplification of the target DNA starting from a peculiar stem-loop DNA structure. LAMP-based assays for the specific detection of GaHV-2, GaHV-3 and HVT genomes have been developed in the past (Woźniakowski et al., 2011; Woźniakowski et al., 2013; Angamuthu et al., 2012; Wei et al., 2012; Wozniakowski & Samorek-Salamonowicz, 2014; Wozniakowski & Niczyporuk, 2015; Adedeji et al., 2017).

Commercial chicken flocks (layers, breeders, and broilers) are normally vaccinated against MD with live attenuated vaccines belonging to the same species of the causative agent of MD (attenuated GaHV-2 strain CVI988/Rispens) or to the other two species of interest included in the genus *Mardivirus* (HVT and GaHV-3). The full-length genome sequences of the three viral species included in the genus Mardivirus are publicly available in online databases (Afonso et al., 2001; Spatz et al., 2007; Spatz et al., 2011; Trimpert et al., 2017; Kim et al., 2020) and many species-specific molecular methods that allow for their differential detection have been developed over time (Handberg et al., 2001; Walkden-Brown et al., 2003; Islam et al., 2004; Islam et al., 2006; Cortes et al., 2011; Woźniakowski et al., 2013; López-Osorio et al., 2019). One of the great advantages offered by PCR and LAMP as molecular methods, in fact, is the ability to differentiate in the light of genetic differences the three viral species, that may coexist within the same sample, in order to correctly diagnose MD and to confirm successful vaccination in the field.

CVI988/Rispens is the most efficacious MD vaccine (Ralapanawe et al., 2016b), it belongs to the same viral species of oncogenic GaHV-2 strains and its genome shows identical overall gene organization and a high degree of identity in the predicted open reading frames with oncogenic strains (Spatz et al., 2007). Having said that, a major effort has been made for the development of sensitive and highly specific assays capable of distinguishing CVI988/Rispens vaccine from oncogenic GaHV-2 strains for the differentiation of GaHV-2 infected or vaccinated animals (DIVA assay). Efficient and reliable DIVA tests have been developed based on real-time PCR and involve the use of differential TaqMan® probes or specifically designed primers for a mismatch amplification mutation assay (MAMA) (Gimeno et al 2014; Baigent et al., 2016, Davidson et al., 2018). All the assays targeted a single nucleotide polymorphism (SNP) found at nucleotide position #320 of the pp38 gene which is consistent between CVI988/Rispens strains and all sequenced virulent MDV-1 strains (Cui et al., 1999; Spatz et al., 2007). Real-time PCR assays were developed which enable quantitation and differentiation between pathogenic GaHV-2 strains and the vaccine CVI988/Rispens based on a DNA sequence variation in the *meq* gene between pathogenic and vaccinal GaHV-2 (Renz et al., 2013). The differentiation of field GaHV-2 strains and vaccine CVI988/Rispens can be reliably achieved also by end-point PCR amplification and sequencing of the whole *meq* gene (Lee et al., 2000; Chang et al., 2002).

Feather tips/pulp represent the optimal starting sample to demonstrate and monitor GaHV-2 infection in chickens: the load of GaHV-2 DNA in feathers is very high both for GaHV-2 strains of field or vaccine origin (Cortes et al., 2011). Furthermore, feathers sampling is easy, non-invasive, and non-lethal for birds and is an advantageous type of sample for monitoring field GaHV-2 infection and also for the evaluation of vaccine uptake in the field (Davidson et al., 2018).

Both vaccine and field viruses, shed into the environment by vaccinated and infected chickens through desquamation of the feather follicle epithelial cell (Islam & Walkden-Brown, 2007), steadily accumulate in environmental dust. Another useful field application of molecular biology is the environmental monitoring of field GaHV-2 contamination or of vaccine virus presence through

molecular-based testing of poultry dust (Walkden-Brown et al., 2013; Pandey et al., 2016; Ralapanawe et al., 2016a; Kennedy et al., 2017).

PCR-based molecular methods are also used for the differential detection of GaHV-2 and the other two major avian oncogenic viruses: *Avian Leukosis Virus* and *Reticuloendotheliosis virus* (Gopal et al., 2012).

Control

Prevention and control of Marek's Disease are achieved by an integrated strategy based on three cornerstones: vaccination of susceptible animals, adoption of optimal biosecurity measures, and selection for genetic resistance. Vaccination represents the central strategy for the prevention and control of MD. Biosecurity and genetic resistance are important adjuncts to properly planned and executed vaccination procedures (Nair, 2018).

Vaccination against MD is a routine practice in intensive poultry-rearing countries, and, protecting the chickens against the disease, has played a major role in the growth of the poultry industry. Live attenuated vaccines have been used against MD on a large scale in commercial chicken flocks starting from the early 1970s (Schat, 2016). Different types of MD vaccines, representing the three species of interest belonging to the *Mardivirus* genus (GaHV-2, GaHV-3, and HVT), are commercially available and are effective either as single vaccines or in combination as multivalent vaccines. The abovementioned vaccines include live attenuated GaHV-2 strains (e.g. CVI988/Rispens) and antigenically related GaHV-3 (e.g. SB-1 or 301B /1) or HVT (e.g. Fc126) strains (Rispens et al., 1972a; Rispens et al., 1972b; Witter, 1987; Okazaki et al., 1970).

The use of MD vaccines has been accompanied over the years with the increase of GaHV-2 virulence (Witter, 1997). At the end of the 1970s in the US, HVT-based vaccine alone, used since the early 1970s, was no longer completely protecting against MD due to the emergence of GaHV-2 strains of greater virulence responsible for an unexplained increase in MD losses in vaccinated flocks (Witter et al., 1980). To control these newly emerged vv strains in the field, a change in

vaccine strategy was made and bivalent vaccines associating HVT with GaHV-3 were used as they showed a protective synergism conferring a higher degree of protection against vvGaHV-2 compared to monovalent vaccines (Schat et al., 1982; Witter, 1982). During the 1990s there was a significant increase in MD incidence in flocks vaccinated with the bivalent vaccine in the USA due to the appearance of the vv+ strains: since then, the introduction of CVI988/Rispens vaccine has been a solution to the MD problem (Witter, 1997; Gimeno, 2008). In Europe, the most widely used vaccine since 1972 was CVI988/Rispens vaccine, which is the most efficacious vaccine currently available and the vaccine of choice worldwide.

Several recombinant vaccines using HVT as a vector (rHVT) to express heterologous immunogenic proteins of chicken viruses causing major diseases such as Newcastle disease (Morgan et al.,1992; Heckert et al., 1996), infectious bursal disease (Darteil et al., 1995), and infectious laryngotracheitis (Johnson et al., 2010) have been developed since the 1990s, and are used worldwide in the control of MD and of the abovementioned poultry diseases. rHVT vaccines are widely used alone in broiler chickens or in combination with CVI988/Rispens vaccine in long-living birds such as layers. Lately, a new live recombinant MD vaccine containing a GaHV-2 chimera (RN1250 strain) genetically modified to contain genomic parts of three different GaHV-2 strains (CVI988/Rispens vaccine strain, vGaHV-2 JM/102W strain in the genome of which two copies of REV LTR were inserted, and vvGaHV-2 Md5 strain in which the genome of which a fragment of GaHV-2 RM1 strain containing the REV LTRs was inserted) has been licensed for use in Europe (Lupiani et al., 2013). Few European countries are currently including this vaccine in MD immunization protocols together with or as an alternative to CVI988/Rispens vaccine.

There are both cell-associated and cell-free formulations available. As most of the vaccine viruses have a strictly cell-associated nature, they have to be injected as a viable infected cell suspension and must be stored and transported in liquid nitrogen and manipulated with particular care to ensure the viability of the cells that must transmit the vaccine virus to birds (Schat, 2016). The cell-associated formulation (GaHV-2, GaHV-3, and recombinant HVT or GaHV-2 vaccines) is the most

used one for its greater effectiveness, especially in the presence of maternal antibodies (Witter and Burmester, 1979). The cell-free formulations, available only for conventional HVT vaccines, could be safely stored between 2°C and 8°C in a lyophilized state. MD vaccines are usually administered to 18-day-old embryos or to day-old chicks immediately after hatch to ensure the development of early immunity, essential for young chicks to face the challenge with GaHV-2 field strains that frequently occur within a few days of being introduced into poultry houses. Revaccination, the practice of administering MD vaccine a second time (e.g. administration of the first vaccine at 18 days of embryonation followed by the administration of a second vaccine at hatch), is frequently used in long-living birds such as future breeders (Kumar et al., 2022). Heterologous revaccination with a second vaccine more protective than the first vaccine provides a beneficial increase in protection (Gimeno et al., 2012b).

MD vaccines are referred to as “imperfect” or “leaky”, as they prevent clinical disease but do not impede the infection, replication, and shedding of wild-type GaHV-2 in the environment (Islam & Walkden-Brown, 2007; Fakhrul Islam et al., 2008; Islam et al., 2014; Read et al., 2015; Ralapanawe et al., 2016a,b). Thus, vaccinal and field viruses can coexist in the vaccinated host (López-Osorio et al., 2019). The widespread use of MD vaccines is thought to have contributed to the evolution of field viruses towards greater virulence promoting the emergence of strains that cause more severe disease in unvaccinated hosts as vaccines keep their host alive thanks to the immunity they elicit but allow the spread and transmission of highly virulent strains that continue to circulate in a susceptible population. Read and colleagues in 2015 experimentally demonstrated that immunization of chickens against Marek's disease virus enhances the fitness of more virulent strains, making it possible for them to readily transmit.

To date, CVI988 / Rispens remains the most effective vaccine available on the market as it provides the highest level of protection even against vv+ GaHV-2 strains, the most virulent strains reported. The evolution of field strains towards greater virulence occurring over time in vaccinated animals raises doubts about what will happen in the future. Since vaccine failures are occurring worldwide

(López-Osorio et al., 2017; Sun et al., 2017; Abdallah et al., 2018; Abd-Ellatieff et al., 2018; Lounas et al., 2021; Yehia et al., 2021), more studies must be conducted to evaluate the protection conferred by Rispens-type vaccines alone or in combination with HVT-based vaccines against more recent, and not yet pathotyped, field strains with a history of high virulence.

The second cornerstone in the control of MD is represented by the adoption of optimal biosecurity measures. It is necessary to associate strict biosecurity measures to the immunization programs adopted to avoid or minimize the early infection of chicks when housed on the farm. The most important source of infection is represented by contaminated environmental dust that remains in the poultry houses between one production cycle and another due to inadequate cleaning and disinfection: infectious virus accumulates in the dust over time and the highest viral loads can be detected at the end of the cycle (Walkden-Brown et al., 2013). It is therefore essential to proceed with a thorough cleaning and disinfection of the walls and pavement of the shed and of the related equipment (e.g. feeders, drinkers, aviaries) before the introduction of a new group. The biosecurity plan must also prevent the virus from entering the farm in the first place and the spread of viruses among the sheds or farms also by controlling the disposal of carcasses and litter at the end of the production cycle (Gimeno, 2004).

Finally, the last cornerstone for MD prevention and control is represented by selection for genetic resistance. The host genetics can influence the effectiveness of MD vaccination in terms of protection (Schat et al., 1982). Major genetics companies implemented their breeding schemes including resistance to Marek's disease among the selection criteria for their genetic lines (Gimeno, 2014). Two main groups of genes that influence MD resistance/susceptibility have been identified: major histocompatibility complex (MHC) genes and non-MHC genes (Bacon and Witter, 1992; Chang et al., 2010). However, infection with highly virulent strains can overcome genetic resistance and therefore selection for genetic resistance is functional when applied in conjunction with vaccination and good biosecurity.

Aim of the thesis

The present doctoral thesis collects the results of three studies exploring Marek's disease virus molecular epidemiology in Italian commercial and backyard chicken flocks and, for the first time, in commercial turkeys. The virulence of GaHV-2 isolates has increased over the years and, even if vaccination keeps the clinical disease under control, field strains of enhanced virulence are supposedly emerging worldwide. The studies aimed to molecularly classify a group of 33 GaHV-2 strains detected in vaccinated and unvaccinated Italian chicken and turkey flocks during suspected MD outbreaks and to scrutinise the ability to predict GaHV-2 virulence according to the *meq* gene sequence. The analysed GaHV-2 strains were detected using end-point PCR targeting the *meq* gene, the major GaHV-2 oncogene. The full-length *meq* genes were amplified and the obtained sequences were analysed searching for genetic markers of virulence.

Finally, a fourth study focused on the development of a rapid, sensitive, and species-specific loop-mediated isothermal amplification assay coupled with a lateral flow device readout for the detection of conventional and recombinant HVT-based vaccines is included in the thesis. HVT vaccines are live vaccines that actively replicate within the host mimicking a natural infection and preventing clinical MD by eliciting a protective immune response and have been used to protect chickens from MD since the early 1970s. Since MD vaccines are "leaky", vaccinal and field viruses can coexist in the vaccinated host and, in case of mixed infection, molecular tests able to discriminate between GaHV-2 and HVT are required. Therefore simple, fast, and accurate tests for monitoring vaccination success in the field could be greatly beneficial for field veterinarians, small laboratories, and more broadly for resource-limited settings.

Published and preliminary papers

I. Molecular characterization of the *meq* gene of Marek's disease viruses detected in unvaccinated backyard chickens reveals the circulation of low and high virulence strains (published paper)

NOTICE: this is post-print (final draft post-refereeing) author's version of a paper that was accepted for publication in the journal "*Poultry Science*". Changes resulting from the publishing process, such as editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. The definitive version of this paper has been published and is available: Mescolini G., Lupini C., Felice V., Guerrini A., Silveira F., Cecchinato M., Catelli E. "Molecular characterization of the *meq* gene of Marek's disease viruses detected in unvaccinated backyard chickens reveals the circulation of low- and high-virulence strains." In Poultry Science, 2019 Aug 1;98(8):3130-3137. doi: 10.3382/ps/pez095.

Molecular characterization of the *meq* gene of Marek's disease viruses detected in unvaccinated backyard chickens reveals the circulation of low and high virulence strains

Giulia Mescolini, [*,1] Caterina Lupini, [*] Viviana Felice, [*] Alessandro Guerrini, [*] Flavio Silveira, [*] Mattia Cecchinato, [†] and Elena Catelli[*]

[*] Department of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra 50, 40064, Ozzano dell'Emilia (BO), Italy

[†] Department of Animal Medicine, Production and Health, University of Padua, Viale dell'Università 16, 35020, Legnaro (PD), Italy

[1] Corresponding author: giulia.mescolini3@unibo.it

Giulia Mescolini

Department of Veterinary Medical Sciences, University of Bologna, via Tolara di Sopra 50, 40064, Ozzano dell'Emilia (BO), Italy.

Full telephone: 0039 0512097560

E-mail address: giulia.mescolini3@unibo.it

Scientific Session: Immunology, Health and Disease

The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database, and accession numbers from MK139660 to MK139678 have been assigned.

ABSTRACT

Marek's disease (MD) is an important lymphoproliferative disease of chickens, caused by *Gallid alphaherpesvirus 2* (GaHV-2). Outbreaks are commonly reported in commercial flocks, but also in backyard chickens. While the molecular characteristics of GaHV-2 strains from the commercial poultry sector have been reported, no recent data are available for the rural sector. To fill this gap, 19 GaHV-2 strains detected in 19 Italian backyard chicken flocks during suspected MD outbreaks were molecularly characterized through an analysis of the *meq* gene, the major GaHV-2 oncogene. The number of four consecutive prolines (PPPP) within the proline-rich repeats of the Meq transactivation domain, the proline content and the presence of amino acid substitutions were determined. Phylogenetic analysis was performed using the Maximum Likelihood method. Sequence analysis revealed a heterogeneous population of GaHV-2 strains circulating in Italian backyard flocks. Seven strains, detected from birds affected by classical MD, showed a unique *meq* isoform of 418 amino acids (aa) with a very high number of PPPP motifs. Molecular and clinical features are suggestive of a low oncogenic potential of these strains. The remaining 12 strains, detected from flocks experiencing acute MD, transient paralysis or sudden death, had shorter Meq protein isoforms (298 or 339 aa) with a lower number of PPPP motifs and point mutations interrupting PPPPs. These features allow us to assert the high virulence of these strains. These findings reveal the circulation of low and high virulence GaHV-2 strains in the Italian rural sector.

Key words: backyard chicken; Marek's disease virus; *meq* gene; molecular characterization

INTRODUCTION

Marek's disease (**MD**) is a worldwide, contagious, lymphoprolipherative disease of chickens caused by a lymphotropic and oncogenic virus, *Gallid alphaherpesvirus 2* (**GaHV-2**); it is also known as Marek's disease virus, belonging to the genus *Mardivirus* of the *Alphaherpesvirinae* subfamily. Genus *Mardivirus* includes two other viral species: *Gallid alphaherpesvirus 3* (**GaHV-3**) and *Meleagrid alphaherpesvirus 1* or Turkey herpesvirus (**HVT**). GaHV-3 and HVT are both non-oncogenic and used as vaccines, being antigenically related to GaHV-2. Four GaHV-2 pathotypes are currently recognized: mild, virulent, very virulent and very virulent plus (Witter, 1997; Witter et al., 2005). Birds become infected by inhalation of infectious viral particles that are present in the environment. GaHV-2 is capable of replicating and establishing latency in T lymphocytes and may induce neoplastic transformation of latently-infected CD4+ T cells, leading to the development of multiple lymphomas in the visceral organs (Nair, 2013). GaHV-2 causes several pathologic syndromes, which can be divided into two types: neoplastic and nonneoplastic (Gimeno, 2014). Neoplastic syndromes, characterized by GaHV-2-induced lymphoproliferative lesions, are the most frequently reported syndromes in the field, having prominent economic significance. Within this category, MD can be subdivided into two forms: classical and acute. Classical MD (also known as fowl paralysis) is characterized by spastic paralysis due to nerve lesions; it was mainly observed prior to the 1950s, concomitantly with infection with low virulence strains (Witter, 1997). The more severe form of the disease, termed acute MD (Biggs et al., 1965), was observed from the late 1950s and is characterized by visceral lymphomas, with or without nerve lesions, and associated with infection with more virulent GaHV-2 strains (Witter, 1997). Nonneoplastic syndromes, such as transient paralysis, panophthalmitis, atherosclerosis and lymphodegenerative syndromes, are rare in the field as they normally occur in unvaccinated, susceptible chickens without specific maternally-derived antibodies (Gimeno, 2014).

Among the more than 200 genes of the GaHV-2 genome, the Marek's *Eco* RI-Q (*meq)* gene, unique to GaHV-2 and highly expressed in latently-infected and transformed T CD4+ cells (Tai et al.,

2017), is proposed to play a key role in the GaHV-2-induced transformation process of latently-infected T lymphocytes. The *meq* gene encodes the Meq protein, a basic leucine zipper transcription factor composed of an N-terminal basic leucine zipper (**bZIP**) domain and a proline-rich C-terminal transactivation domain (Qian et al., 1995). The last 33 carboxy-terminal amino acids are essential for transcriptional transactivation (Qian et al., 1995), whereas the number of proline-rich repeats (PRR) in the transactivation domain seems to be related with repression of transcription (Chang et al., 2002a). *Meq* is a polymorphic gene, with various recognized sizes: long-*meq* (L-*meq*), *meq*, short-*meq* (S-*meq*) and very short-*meq* (VS-*meq*); these encode Meq protein isoforms with 399, 339, 298 and 247 amino acids, respectively (Chang et al., 2002b). The existence of these different length Meq isoforms is due to the presence of insertions or deletions in the transactivation domain, resulting in a variable number of PRR. This number, along with specific point mutations in the PRR, appears to correlate with GaHV-2 virulence (Shamblin et al., 2004; Renz et al., 2012). Moreover, the *meq* gene has been recently included in a list of candidate genes associated with an increase of GaHV-2 virulence due to a greater-than-average number of point mutations found in the virulent Eurasian and North American GaHV-2 strains (Trimpert et al., 2017). This gene is evolving at a fast rate for a dsDNA virus, and most of its polymorphisms have evolved under positive selection (Padhi and Parcells, 2016).

MD is a major cause of mortality in backyard chickens (Pohjola et al., 2015; Mete et al., 2016) and GaHV-2 strains can circulate freely because flocks composed of birds with different immune statuses, ages and breeds, are more susceptible to infection. Backyard farm owners do not generally vaccinate their birds and backyard production methods imply a low biosecurity level (Cecchinato et al., 2011); this facilitates the circulation of infectious agents, including GaHV-2, and constitutes a threat to any commercial poultry holdings nearby. To our knowledge, recent data about molecular characteristics of Marek's disease virus circulating in backyard flocks worldwide is not available. In the present study, we analyzed the complete *meq* gene sequences of 19 GaHV-2 strains detected from suspected MD outbreaks in 19 Italian backyard chicken flocks.

MATERIALS AND METHODS

Backyard Flocks

From 2015 to 2017, 19 Italian backyard chicken flocks were sampled for routine molecular diagnostic activity for MD. All flocks were unvaccinated for MD and showed clinical signs or lesions suggestive of MD. Several chicken breeds were involved in the outbreaks (Table 1). The farms were located in nine different Italian regions (Table 1) and consisted of a variable number of chickens (from 40 to 150), kept mainly for exhibition or hobby and marginally for eggs and meat. Other poultry species, such as turkey, quail, peacock, pigeon, goose, duck, guinea fowl and Roul Roul partridge, were reared alongside the affected chickens on most farms.

Sampling

For GaHV-2 PCR detection, five feathers/bird were collected from the axillary feather tracts, as suggested by Baigent et al. (2013). Feather sampling was chosen because it is easy, fast, non-invasive and non-lethal (Davidson et al., 2018), and is suitable for sampling ornamental chicken breeds that have economic and emotional value.

DNA Extraction

Total DNA was extracted from feather tips using a commercial kit (High Pure PCR Template Preparation Kit, Roche Diagnostics GmbH, Mannheim, Germany), with a subtle adjustment to the manufacturer's instructions. Briefly, five feather tips from each bird were pooled together, cut, ground and digested overnight at 55°C in a digestion buffer containing tissue lysis buffer, proteinase K and DL-Dithiothreitol solution (Sigma-Aldrich, Saint Louis, Missouri, USA). After digestion, binding buffer followed by isopropanol was added and samples were placed in spin columns and centrifuged at 8000 × *g* for 1 min. After two washings, DNA was eluted with 200 µl of elution buffer.

PCR Amplification of the *meq* Gene

The full-length *meq* gene was amplified, according to Shamblin et al. (2004), using the forward primer *Eco*R-Q for 5'-GGT GAT ATA AAG ACG ATA GTC ATG-3' and the reverse primer *Eco*R-Q rev 5'-CTC ATA CTT CGG AAC TCC TGG AG-3'. In a total reaction volume of 25 µl, 3 µl of eluted template DNA was mixed with 0.125 µl of GoTaq G2 Flexi DNA Polymerase (Promega, Madison, Wisconsin, USA), 5 µl of 5X Colorless GoTaq Flexi Buffer, 1.75 µl of $MgCl_2$ solution, 0.5 µl of dNTPs, 13 µl of H_2O for molecular biology, and 1 µl of each primer. Cycling conditions were as follows: 2 min of denaturation at 95°C followed by 35 cycles, each consisting of denaturation at 95°C for 1 min, annealing at 58°C for 1 min and extension at 72 °C for 1.5 min. A final elongation step at 72 °C for 5 min completed the reaction. The PCR products were separated on agarose gel (1%), stained with ethidium bromide and visualized under ultraviolet light after an electrophoretic run at 80Vand 400mA for 50 min.

DNA Sequencing and Sequence Analysis

The amplification products were sequenced using a commercial sequencing service (Macrogen Europe, Amsterdam, The Netherlands). In order to obtain a complete and reliable *meq* gene sequence, primers *Eco*R-Q for, *Eco*R-Q rev (Shamblin et al., 2004) and an internal primer (*meq*-F, 5'-ATG TCT CAG GAG CCA GAG CCG-3') (Hassanin et al., 2013) were used. The obtained sequences were named using the following nomenclature: GaHV-2 / Italy / Chicken (Ck) / ID number / year of detection.

The nucleotide sequences were assembled and edited using Bioedit Sequence Alignment Editor Version 7.2.5.0 (Tom Hall, Ibis Therapeutics, Carlsbad, California, USA), then, aligned and compared, using Clustal W software (Thompson et al., 1994), with the *meq* gene sequences of 32 selected GaHV-2 field and vaccine strains retrieved from the GenBank database (Table 2) and with the sequences of three CVI988/Rispens vaccine strains currently used in Italy. The number of four consecutive prolines (**PPPP**) contained in the proline-rich repeats of the transactivation domain, the

proline content and the amino acid (**aa**) substitutions in the deduced aa sequence of *meq* genes were evaluated.

A phylogenetic tree based on the *meq* gene sequences of Italian and selected GaHV-2 strains from GenBank was generated with the Maximum Likelihood method, using MEGA7 (Kumar et al., 2016). Only the nodes of the tree with bootstrap values equal or greater than 70, calculated based on 1000 replicates, were considered reliable.

RESULTS

All 19 backyard chicken flocks tested in the present study were positive for GaHV-2. The obtained complete *meq* gene sequences were submitted to the GenBank database under the accession numbers listed in Table 3. Sequence analysis revealed that GaHV-2 strains had *meq* gene sequences of variable sizes: 1257 bp, 1020 bp or 897 bp, which were named "very long *meq*", "standard *meq*" and "short *meq*" strains, respectively, based on a slightly modified version of the *meq* open reading frames classification reported by Chang et al. (2002b) (Table 3).

Length, insertion size, number of PPPP motifs within the transactivation domain and the proline content of *meq* deduced amino acid sequences of the Italian GaHV-2 strains and one representative GaHV-2 strain for each pathotype were evaluated (Table 4). Seven GaHV-2 strains showed a long Meq isoform (418 aa, "very long *meq"* strains), with an insertion of 79 amino acids and a high number of PPPP motifs (9–10). Eleven strains had a short Meq isoform (339 aa, "standard *meq*" strains) without insertion in the transactivation domain and a lower number of PPPPs (4–5). Only one strain showed a very short Meq isoform (298 aa, "short *meq*" strain) with two PPPPs in its transactivation domain.

The amino acid substitutions found in the Meq proteins of the analysed strains compared to the vaccine strain CVI988 (Intervet), chosen as reference strain, are reported in Tables 5, 6 and 7. Sequences of "very long *meq*" strains, which differ among themselves with respect to very few amino acid changes, showed 10 to 14 amino acid substitutions when compared with the CVI988

vaccine strain. Five of these mutations, at positions 37 (H37R), 80 (D80E), 98 (H98D), 101 (K101N), and 242 (F242I) of the Meq protein (Table 5), were only found in the Italian strains. The uniqueness of this mutation pattern was further confirmed by a BLAST search. Five to eight amino acid substitutions were found when "standard *meq*" (Table 6) and "short *meq*" (Table 7) strains were compared with the CVI988 vaccine strain. Almost all amino acid changes of "standard *meq*" and "short *meq*" strain-encoded Meqs have already been reported in previously published International sequences.

"Standard *meq*" and "short *meq*" strains contained interruptions of PPPP motifs in the PRR of the transactivation domain, both at the second and third position. In particular, the GaHV-2/Italy/855/17 strain showed a substitution at position 177 (P177S), interrupting a stretch of four prolines at position 3 (PPPP → PPSP). The GaHV-2/Italy/Ck/674/16 strain showed a substitution at position 217 (P217A), interrupting a PPPP sequence at position 2 (PPPP → PAPP). Finally, the strains GaHV-2/Italy/Ck/625/16, GaHV-2/Italy/Ck/689/16, GaHV-2/Italy/Ck/722/16, GaHV-2/Italy/Ck/801/16, GaHV-2/Italy/Ck/810/16, GaHV-2/Italy/Ck/852/16, GaHV-2/Italy/Ck/853/16 and GaHV-2/Italy/Ck/854/16 showed substitutions at position 218 (P218S), interrupting the PPPP sequence at position 3 (PPPP → PPSP).

The phylogenetic tree, based on the Meq amino acid sequences of the Italian strains, the vaccine strains and 32 selected GaHV-2 strains, is shown in Figure 1. The "very long *meq*" Italian strains form an independent cluster, phylogenetically related to a cluster formed by Hungarian and Indian strains. Nine out of eleven Italian "standard *meq*" strains and the "short *meq*" strain clustered together with selected Polish isolates. Two Italian "standard *meq*" strains (GaHV-2/Italy/Ck/674/16 and GaHV-2/Italy/Ck/850/17) did not belong to the above-mentioned group and the GaHV-2/Italy/Ck/674/16 strain appeared to be connected with a recent Tunisian strain.

DISCUSSION

For the first time, the present study provides molecular insights into the GaHV-2 strains currently circulating in backyard chickens, expanding the knowledge on MD in the rural sector. Nineteen strains, detected from 2015 to 2017 in Italian backyard chickens exhibiting typical MD clinical signs or gross lesions, were molecularly characterized on the basis of their *meq* gene sequences, revealing the circulation of a heterogeneous viral population.

Previous studies highlighted a correlation between the *meq* gene sequence and GaHV-2 virulence (Shamblin et al., 2004; Renz et al., 2012). In particular, strains showing a low number of PRR within the transactivation domain, and amino acid substitutions interrupting PPPP motifs within the PRR, exhibit higher virulence. In the sequence analysis, the Italian strains were subdivided, according to *meq* gene length, into three categories: "very long *meq*", "standard *meq*" and "short *meq*".

The "very long *meq*" strains detected in the present study showed a Meq isoform of 418 aa with a high number (from 9 to 10) of PPPP motifs in their transactivation domains. These molecular features could be suggestive of low oncogenic potential. Moreover, all "very long *meq*" strains were detected from birds affected by classical MD, macroscopically not showing visceral tumours and experiencing a complete recovery in three out of seven outbreaks. These strains share diverse and sometimes unique aa substitutions that, in part (H98D, K101N and Q93R), fall within the bZIP domain. This domain is responsible for Meq dimerization with itself or with other dimerization partners, forming homodimers or heterodimers, respectively. The ability to form one interaction or the other is influenced by the bZIP sequence and the presence of mutations in this domain could disrupt the formation of one or both types of dimers (Brown et al., 2009; Suchodolski et al., 2009; Suchodolski et al., 2010). This interaction allows the adjacent basic region of Meq to anchor to specific DNA binding sites with different affinities, depending on the dimer type, consequently transactivating or transrepressing viral and host genes exerting different biological effects, mostly linked to oncogenesis (Qian et al., 1996; Liu et al., 1998; Levy et al., 2005). The three amino acid

substitutions found in the bZIP domain might have altered the Meq binding capacity and contributed to the low oncogenicity of the Italian "very long *meq*" strains.

On the other hand, "standard *meq*" and "short *meq*" strains were detected from flocks experiencing acute MD, transient paralysis or sudden death, occasionally preceded by neurologic signs. These also featured a low number of PPPP motifs in the transactivation domain, and the presence of point mutations in the PRR that interrupted stretches of four prolines in most of the "short *meq*" or "standard *meq*" strains; this allows us to assert, according to Shamblin et al. (2004), the high virulence of these strains. These findings reveal the circulation of both low and high virulence GaHV-2 strains in the Italian rural sector.

The variability of observed MD clinical forms could be also due to different disease susceptibilities amongst the different breeds involved. Genetic resistance to MD is well known and while breeding programs for commercial poultry generally include genetic selection for resistance to MD (Schat and Nair, 2013), selection programs for ornamental chickens are mainly focused on the selection of phenotypic traits compliant with the breed standard.

The heterogeneity of the viral population, supported by the allocation of the analyzed strains into three major clusters, suggests that the introduction of GaHV-2 to Italy could have occurred over multiple occasions. Ornamental chicken owners regularly enter their birds into international 'beauty contests', where chickens are generally kept in adjacent cages, facilitating the transmission of the virus from bird to bird. The national and international trade of live, valuable breeders is another possible route of entry.

Viruses could also have reached the rural context by overcoming the biosecurity measures applied in commercial poultry houses to find a highly variable poultry population with different species, breeds, ages and immune statuses, with unknown susceptibility to MD. The reverse could be also true: backyard flocks could act as reservoir for GaHV-2 strains of various and unknown pathotypes, representing a potential threat for commercial poultry flocks located in the same area. Biosecurity measures are not generally applied to backyard farms (Cecchinato et al., 2011) and, in most cases,

birds have continuous daytime access to open-air pens, and contact with wild birds; these birds have been identified as carriers of presumably pathogenic GaHV-2 strains (Murata et al., 2012), so this may facilitate the introduction of foreign viruses.

Finally, the last detections of low virulence viruses dates back to the 1970s (Smith and Calnek, 1973; Smith and Calnek, 1974), presumably because of the poultry industry's major interest in investigating highly virulent strains responsible for MD outbreaks in vaccinated commercial poultry flocks (López-Osorio et al., 2017; Suresh et al., 2017; Abd-Ellatieff et al., 2018). Weakly virulent viruses are more likely to circulate naturally in backyard flocks, probably due to the absence of vaccine-induced selective pressure and weak biosecurity measures.

Molecular characterization and clinical findings are not sufficient to ascertain the level of virulence of the detected viruses, therefore, in vivo pathotyping assays are needed. For this purpose, viral isolation should be attempted. Moreover, the isolation of weakly virulent strains could offer the opportunity to evaluate their potential as candidate vaccines.

Table 1. Geographical location of the studied backyard flocks, with the observed clinical forms of Marek's disease (MD) and the age and breed of affected chickens.

Flock ID	Italian region	MD form	Chicken breeds	Age range (months)
487/15	Piedmont	Acute	Silkie	4
507/15	Sardinia	Classical - R[1]	Amrock, Millefiori di Lonigo	7 - 24
509/15	Lazio	Classical	Araucana, Marans, Satsumadori	5 - 36
510/15	Lazio	Classical	Campine	36
562/15	Lazio	Classical - R	Sebright	6
599/16	Lazio	Classical	Sebright	24
625/16	Tuscany	Acute	Robusta Lionata	2 - 4.5
674/16	Emilia-Romagna	NS[2]	Padovana, Polish	6 - 12
689/16	Lazio	Acute	Cochin, Padovana	6 - 8
722/16	Tuscany	NS	Sussex	2 - 2.5
801/17	Sicily	NS	Wyandotte	3.5 - 4
810/17	Sicily	Transient paralysis	Padovana	3 - 4.5
847/17	Lombardy	Classical	Brahma	12
848/17	Emilia-Romagna	Classical - R	Silkie	2 - 4
850/17	Tuscany	NS	Brahma, Silkie	6
852/17	Campania	Acute	Australorp, Satsumadori, Sumatra	6 - 9
853/17	Lombardy	Acute	Ayam Cemani	4 - 7
854/17	Trentino-Alto Adige	NS	Serama	9 - 24
855/17	Tuscany	NS	Leghorn, Valdarno	8 - 12

[1] Birds experienced a complete recovery;

[2] Clinical signs and gross lesions were not specific for MD. High mortality is often reported.

Table 2. GaHV-2 strains, retrieved from GenBank, which were included in the molecular analysis.

Strain	Country of origin	Pathotype	Year	GenBank accession number
CVI988 (Intervet)	Netherlands	att[1]	-[2]	DQ534538
814	China	att	1980s	AF493551
3004	Russia	att	-	EU032468
CU-2	USA	m[3]	1970s	AY362708
04CRE	Australia	v[4]	2004	EF523773
MPF57	Australia	v	1994	EF523774
BC-1	USA	v	1970s	AY362707
JM/102W	USA	v	1962	DQ534539
567	USA	v	-	AY362709
571	USA	v	1989	AY362710
617A	USA	v	1993	AY362712
FT158	Australia	vv[5]	2002	EF523771
02LAR	Australia	vv	2002	EF523772
Md5	USA	vv	1977	AF243438
643P	USA	vv	1994	AY362716
L	USA	vv+[6]	-	AY362717
New	USA	vv+	-	AY362719
W	USA	vv+	-	AY362723
648A	USA	vv+	1994	AY362725
ATE	Hungary	-	-	AY571784
24_00	Poland	-	2000	KJ464764
108_11	Poland	-	2011	KJ464831
56_12	Poland	-	2012	KJ464839
Ind/KA12/02	India	-	2012	KP342383
GX14PP03	China	-	2014	KX506775
LZ1309	China	-	2015	KX966280
B2015	India	-	2015	LC195187
GADVASU-M1	India	-	2016	KY651231
MEQ_GIFU_1	Japan	-	2016	LC208801
MEQ_GIFU_2	Japan	-	2016	LC208802
MEQ_GIFU_3	Japan	-	2016	LC208803
TN1014/16	Tunisia	-	2016	KY113150

[1] Attenuated
[2] Unknown
[3] Mild
[4] Virulent
[5] Very virulent
[6] Very virulent plus

Table 3. Lengths of the *meq* genes of Italian GaHV-2 strains, with GenBank accession numbers.

Strain classification	Strain	*Meq* gene length (bp)	GenBank accession number
"Very long *meq*" strain	GaHV-2/Italy/Ck/507/15	1257	MK139661
	GaHV-2/Italy/Ck/509/15	1257	MK139662
	GaHV-2/Italy/Ck/510/15	1257	MK139663
	GaHV-2/Italy/Ck/562/15	1257	MK139664
	GaHV-2/Italy/Ck/599/16	1257	MK139665
	GaHV-2/Italy/Ck/847/17	1257	MK139672
	GaHV-2/Italy/Ck/848/17	1257	MK139673
"Standard *meq*" strain	GaHV-2/Italy/Ck/487/15	1020	MK139660
	GaHV-2/Italy/Ck/625/16	1020	MK139666
	GaHV-2/Italy/Ck/674/16	1020	MK139667
	GaHV-2/Italy/Ck/689/16	1020	MK139668
	GaHV-2/Italy/Ck/722/16	1020	MK139669
	GaHV-2/Italy/Ck/801/17	1020	MK139670
	GaHV-2/Italy/Ck/810/17	1020	MK139671
	GaHV-2/Italy/Ck/850/17	1020	MK139674
	GaHV-2/Italy/Ck/852/17	1020	MK139675
	GaHV-2/Italy/Ck/853/17	1020	MK139676
	GaHV-2/Italy/Ck/854/17	1020	MK139677
"Short *meq*" strain	GaHV-2/Italy/Ck/855/17	897	MK139678

Table 4. Meq protein features of Italian GaHV-2 strains, compared to selected reference strains, with one of each pathotype.

Strain	Meq protein length (aa)	Insertion size (aa)	PPPPs (n°)	Proline content (%)
CVI988 (Intervet) (att)	399	60	8	23.25
CU-2 (m)	398	59	7	23.06
JM/102W (v)	399	60	7	23.06
Md5 (vv)	339	- [1]	4	21.24
648A (vv+)	339	-	2	20.88
GaHV-2/Italy/Ck/847/17	418	79	10	23.87
GaHV-2/Italy/Ck/507/15	418	79	9	23.63
GaHV-2/Italy/Ck/509/15	418	79	9	23.63
GaHV-2/Italy/Ck/510/15	418	79	9	23.63
GaHV-2/Italy/Ck/562/15	418	79	9	23.63
GaHV-2/Italy/Ck/599/16	418	79	9	23.63
GaHV-2/Italy/Ck/848/17	418	79	9	23.63
GaHV-2/Italy/Ck/487/15	339	-	5	21.47
GaHV-2/Italy/Ck/850/17	339	-	5	21.47
GaHV-2/Italy/Ck/625/16	339	-	4	21.18
GaHV-2/Italy/Ck/674/16	339	-	4	21.18
GaHV-2/Italy/Ck/689/16	339	-	4	21.18
GaHV-2/Italy/Ck/722/16	339	-	4	21.18
GaHV-2/Italy/Ck/801/17	339	-	4	21.18
GaHV-2/Italy/Ck/810/17	339	-	4	21.18
GaHV-2/Italy/Ck/852/17	339	-	4	21.18
GaHV-2/Italy/Ck/853/17	339	-	4	21.18
GaHV-2/Italy/Ck/854/17	339	-	4	21.18
GaHV-2/Italy/Ck/855/17	298	-	2	19.40

[1] Absence of insertion.

Table 5. Amino acid substitutions in the Meq proteins of "very long *meq*" Italian GaHV-2 strains, using the CVI988 vaccine strain as consensus sequence. Italian unique mutations, after comparison with all available sequences, are reported in bold.

Strain	Amino acid substitution position													
	37	66	80	93	98	101	139	242	261[1]	352[3]/ 371[4]	373/ 392	386/ 405	390/ 409	391/ 410
CVI988 (Intervet)	H	G	D	Q	H	K	T	F	-[2]	H	L	I	V	W
GaHV-2/ Italy/Ck/847/17	**R**	R	**E**	R	**D**	**N**	A	**I**	I	P	S	T	L	C
GaHV-2/ Italy/Ck/507/15 GaHV-2/ Italy/Ck/562/15 GaHV-2/ Italy/Ck/599/16	**R**	R	**E**	R	**D**	**N**	A	**I**	I	H	L	T	V	W
GaHV-2/ Italy/Ck/510/15	**R**	R	**E**	R	**D**	**N**	A	**I**	I	H	S	T	V	W
GaHV-2/ Italy/Ck/509/15 GaHV-2/ Italy/Ck/848/17	**R**	R	**E**	R	**D**	**N**	A	**I**	F	H	L	T	V	W

[1, 4] Amino acid position with respect to Italian "very long *meq*" strains.
[2] Deletion of CVI988 compared with Italian "very long *meq*" strains.
[3] Amino acid position with respect to CVI988 strain.

Table 6. Amino acid substitutions in the Meq proteins of "standard *meq*" Italian GaHV-2 strains, using the CVI988 vaccine strain as consensus sequence.

Strain	Amino acid substitution position									
	66	71	80	110	115	217[1] / 277[2]	218/ 278	244/ 304	271/ 331	326/ 386
CVI988 (Intervet)	G	S	D	C	V	P	P	C	G	I
GaHV-2/Italy/Ck/850/17	R	A	Y	C	A	P	P	G	G	T
GaHV-2/Italy/Ck/487/15	R	A	Y	S	V	P	P	C	G	T
GaHV-2/Italy/Ck/674/16	R	A	Y	R	A	A	P	C	R	T
GaHV-2/Italy/Ck/625/16 GaHV-2/Italy/Ck/689/16 GaHV-2/Italy/Ck/722/16 GaHV-2/Italy/Ck/801/17 GaHV-2/Italy/Ck/810/17 GaHV-2/Italy/Ck/852/17 GaHV-2/Italy/Ck/853/17 GaHV-2/Italy/Ck/854/17	R	A	Y	S	V	P	S	C	G	T

[1] Amino acid position with respect to Italian "standard *meq*" GaHV-2 stains.
[2] Amino acid position with respect to CVI988 strain.

Table 7. Amino acid substitutions in the Meq protein of "short *meq*" Italian GaHV-2 strain, using the CVI988 vaccine strain as consensus sequence.

Strain	Amino acid substitution position					
	66	71	80	110	177	285[1]/ 386[2]
CVI988 (Intervet)	G	S	D	C	P	I
GaHV-2/Italy/Ck/855/17	R	A	Y	S	S	T

[1] Amino acid position with respect to the Italian "short *meq*" strain.
[2] Amino acid position with respect to CVI988 strain.

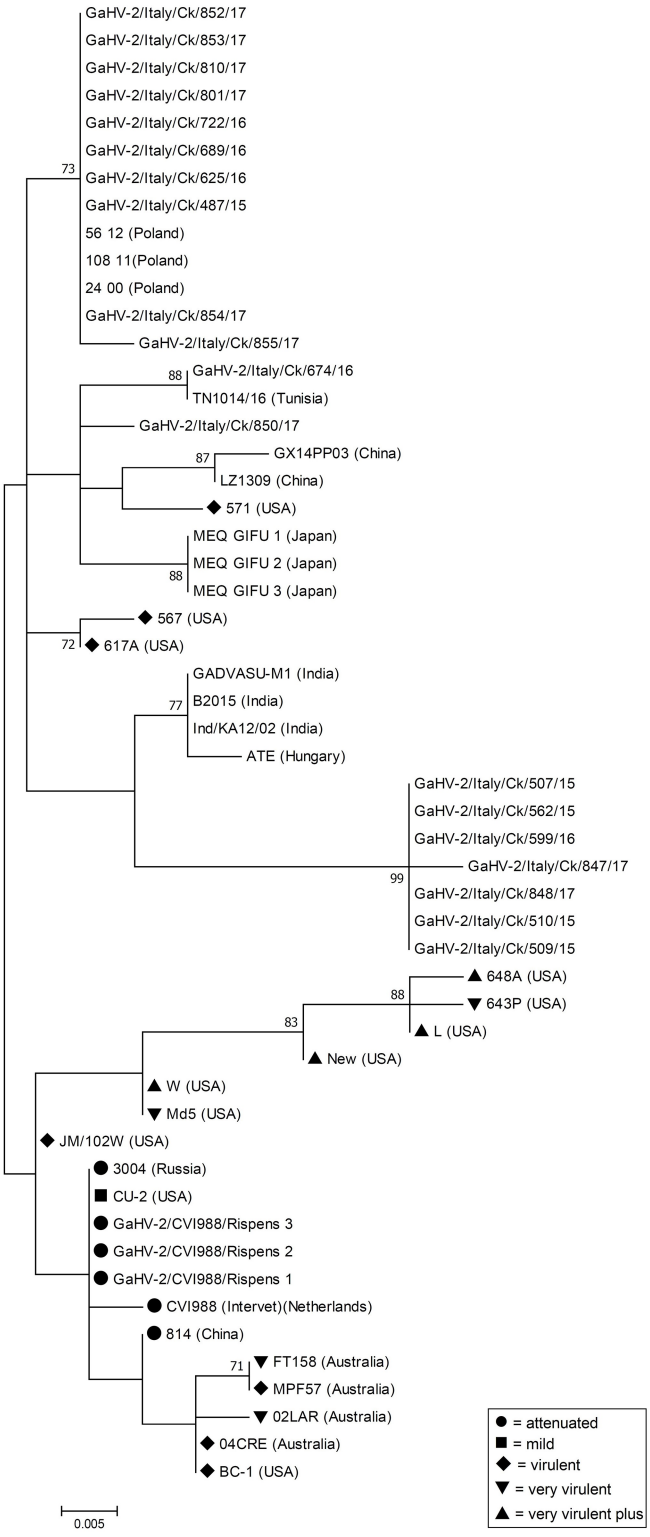

Figure 1. Phylogenetic tree based on Meq amino acid sequences of 19 Italian GaHV-2 strains, 32 international GaHV-2 strains and 3 CVI988/Rispens vaccine strains currently used in Italy. Only bootstrap values ≥ 70 are reported.

REFERENCES

Abd-Ellatieff, H.A., A.A. Abou Rawash, H.F. Ellakany, W.M. Goda, T. Suzuki, and T. Yanai. 2018. Molecular characterization and phylogenetic analysis of a virulent Marek's disease virus field strain in broiler chickens in Japan. Avian Pathol. 47: 47–57.

Baigent, S.J., L.B. Kgosana, A.A. Gamawa, L.P. Smith, A.F. Read, and V.K. Nair. 2013. Relationship Between Levels of Very Virulent MDV in Poultry Dust and in Feather Tips from Vaccinated Chickens. Avian Dis. 57: 440–447.

Biggs, P.M., H.G. Purchase, B.R. Bee, and P.J. Dalton. 1965. Preliminary report on acute Marek's disease (fowl paralysis) in Great Britain. Vet. Rec. 77: 1339–1340.

Brown, A.C., L.P. Smith, L. Kgosana, S.J. Baigent, V. Nair, and M.J. Allday. 2009. Homodimerization of the Meq Viral Oncoprotein Is Necessary for Induction of T-Cell Lymphoma by Marek's Disease Virus. J. Virol. 83: 11142–11151.

Cecchinato, M., A. Comin, L. Bonfanti, C. Terregino, I. Monne, M.Lorenzetto, and S. Marangon. 2011. Epidemiology and Control of Low Pathogenicity Avian Influenza Infections in Rural Poultry in Italy. Avian Dis. 55: 13–20.

Chang, K.-S., K. Ohashi, and M. Onuma. 2002a. Suppression of transcription activity of the MEQ protein of oncogenic Marek's disease virus serotype 1 (MDV1) by L-MEQ of non-oncogenic MDV1. J. Vet. Med. Sci. 64: 1091–1095.

Chang, K.-S., K. Ohashi, and M. Onuma. 2002b. Diversity (polymorphism) of the meq gene in the attenuated Marek's disease virus (MDV) serotype 1 and MDV-transformed cell lines. J. Vet. Med. Sci. 64: 1097–1101.

Davidson, I., A. Natour-Altory, I. Raibstein, E. Kin, Y. Dahan, H. Krispin, and N. Elkin. 2018. Monitoring the uptake of live avian vaccines by their detection in feathers. Vaccine. 36: 637–643.

Gimeno, I.M. 2014. Marek's Disease and Differential Diagnosis with Other Tumor Viral Diseases of Poultry. Pages 156–171 in Encyclopedia of Agriculture and Food Systems, 2nd Edition. N. Van Alfen, ed. Academic Press, Elsevier Science & Technology, San Diego, California, USA.

Hassanin, O., F. Abdallah, and I.E. El-Araby. 2013. Molecular characterization and phylogenetic analysis of Marek's disease virus from clinical cases of Marek's disease in Egypt. Avian Dis. 57: 555–561.

Kumar, S., G. Stecher, and K. Tamura. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol. 33: 1870–1874.

Levy, A.M., O. Gilad, L. Xia, Y. Izumiya, J. Choi, A. Tsalenko, Z. Yakhini, R. Witter, L. Lee, C.J. Cardona, and H.-J. Kung. 2005. Marek's disease virus Meq transforms chicken cells via the v-Jun transcriptional cascade: a converging transforming pathway for avian oncoviruses. Proc. Natl. Acad. Sci. U. S. A. 102: 14831–14836.

Liu, J.-L., Y. Ye, L.F. Lee, and H.-J. Kung. 1998. Transforming potential of the herpesvirus oncoprotein MEQ: morphological transformation, serum-independent growth, and inhibition of apoptosis. J. Virol. 72: 388–395.

López-Osorio, S., D. Piedrahita, M.A. Espinal-Restrepo, G.C. Ramírez-Nieto, V. Nair, S.M. Williams, S.J. Baigent, C. Ventura-Polite, D.A. Aranzazu-Taborda, and J.J. Chaparro-Gutiérrez. 2017. Molecular characterization of Marek's disease virus in a poultry layer farm from Colombia. Poult. Sci. 96: 1598–1608.

Mete, A., R. Gharpure, M.E. Pitesky, D. Famini, K. Sverlow, and J. Dunn. 2016. Marek's Disease in Backyard Chickens, A Study of Pathologic Findings and Viral Loads in Tumorous and Nontumorous Birds. Avian Dis. 60: 826–836.

Murata, S., Y. Hayashi, A. Kato, M. Isezaki, S. Takasaki, M. Onuma, Y. Osa, M. Asakawa, S. Konnai, and K. Ohashi. 2012. Surveillance of Marek's disease virus in migratory and sedentary birds in Hokkaido, Japan. Vet. J. 192: 538–540.

Nair, V. 2013. Latency and Tumorigenesis in Marek's Disease. Avian Dis. 57: 360–365.

Padhi, A., and M.S. Parcells. 2016. Positive selection drives rapid evolution of the meq oncogene of Marek's disease virus. PLoS One 11, e0162180.

Pohjola, L., L. Rossow, A. Huovilainen, T. Soveri, M.L. Hänninen, and M. Fredriksson-Ahomaa. 2015. Questionnaire study and postmortem findings in backyard chicken flocks in Finland. Acta Vet. Scand. 57.

Qian, Z., P. Brunovskis, L. Lee, P.K. Vogt, and H.J. Kung. 1996. Novel DNA binding specificities of a putative herpesvirus bZIP oncoprotein. J. Virol. 70: 7161–7170.

Qian, Z., P. Brunovskis, F. Rauscher 3rd, L. Lee, and H.J. Kung. 1995. Transactivation activity of Meq, a Marek's disease herpesvirus bZIP protein persistently expressed in latently infected transformed T cells. J. Virol. 69: 4037–4044.

Renz, K.G., J. Cooke, N. Clarke, B.F. Cheetham, Z. Hussain, A.F.M. Fakhrul Islam, G.A. Tannock, and S.W. Walkden-Brown. 2012. Pathotyping of Australian isolates of Marek's disease virus and association of pathogenicity with meq gene polymorphism. Avian Pathol. 41: 161–176.

Schat, K.A., and V. Nair. 2013. Neoplastic diseases: Marek's disease. Pages 515–552 in Diseases of Poultry 13th edn. D.E. Swayne, J.R. Glisson, L.R. McDougald, L.K. Nolan, D.L. Suarez, V.L. Nair, ed. Wiley-Blackwell Hoboken, Ames, Iowa, USA.

Shamblin, C.E., N. Greene, V. Arumugaswami, R.L. Dienglewicz, and M.S. Parcells. 2004. Comparative analysis of Marek's disease virus (MDV) glycoprotein-, lytic antigen pp38- and transformation antigen Meq-encoding genes: Association of meq mutations with MDVs of high virulence. Vet. Microbiol. 102: 147–167.

Smith, M.W., and B.W. Calnek. 1973. Effect of virus pathogenicity on antibody production in Marek's disease. Avian Dis. 17: 727–736.

Smith, M.W., and B.W. Calnek. 1974. Comparative features of low-virulence and high-virulence marek's disease virus infections. Avian Pathol. 3: 229–246.

Suchodolski, P.F., Y. Izumiya, B. Lupiani, D.K. Ajithdoss, O. Gilad, L.F. Lee, H.-J. Kung, and S.M. Reddy. 2009. Homodimerization of Marek's Disease Virus-Encoded Meq Protein Is Not Sufficient for Transformation of Lymphocytes in Chickens. J. Virol. 83: 859–869.

Suchodolski, P.F., Y. Izumiya, B. Lupiani, D.K. Ajithdoss, L.F. Lee, H.J. Kung, and S.M. Reddy. 2010. Both homo and heterodimers of Marek's disease virus encoded Meq protein contribute to transformation of lymphocytes in chickens. Virology 399: 312–321.

Suresh, P., J. Johnson Rajeswar, K. Sukumar, T.J. Harikrishnan, and P. Srinivasan. 2017. Complete nucleotide sequence analysis of the oncogene "Meq" from serotype 1 Marek's disease virus isolates from India. Br. Poult. Sci. 48: 111–115.

Tai, S.H.S., C. Hearn, S. Umthong, O. Agafitei, H.H. Cheng, J.R. Dunn, and M. Niikura. 2017. Expression of Marek's Disease Virus Oncoprotein Meq During Infection in the Natural Host. Virology 503: 103–113.

Thompson J.D., D.G. Higgins, and T.J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673–4680.

Trimpert, J., N. Groenke, M. Jenckel, S. He, D. Kunec, M.L. Szpara, S.J. Spatz, N. Osterrieder, and D.P. McMahon. 2017. A phylogenomic analysis of Marek's disease virus reveals independent paths to virulence in Eurasia and North America. Evol. Appl. 10: 1091–1101.

Witter, R.L. 1997. Increased Virulence of Marek's Disease Virus Field Isolates. Avian Dis. 41: 149–163.

Witter, R.L., B.W. Calnek, C. Buscaglia, I.M. Gimeno, and K.A. Schat. 2005. Classification of Marek's disease viruses according to pathotype: Philosophy and methodology. Avian Pathol. 34: 75–90.

II. Marek's disease viruses circulating in commercial poultry in Italy in the years 2015-2018 are closely related by their *meq* gene phylogeny (published paper)

NOTICE: this is post-print (final draft post-refereeing) author's version of a paper that was accepted for publication in the journal "*Transboundary and Emerging Diseases*". Changes resulting from the publishing process, such as editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. The definitive version of this paper has been published and is available: Mescolini G., Lupini C., Davidson I., Massi P., Tosi G., Catelli E. "Marek's disease viruses circulating in commercial poultry in Italy in the years 2015-2018 are closely related by their *meq* gene phylogeny." In Transboundary and Emerging Diseases, 2020 Jan;67(1):98-107. doi: 10.1111/tbed.13327. Epub 2019 Aug 27.

Title

Marek's disease viruses circulating in commercial poultry in Italy in the years 2015-2018 are closely related by their *meq* gene phylogeny.

Running Title

Virulence of GaHV-2 strains circulating in Italian commercial chickens

Giulia Mescolini[1,*], Caterina Lupini[1], Irit Davidson[2], Paola Massi[3], Giovanni Tosi[3], Elena Catelli[1]

[1] Department of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra 50, 40064, Ozzano dell'Emilia (BO), Italy

[2] Division of Avian Diseases, Kimron Veterinary Institute, Bet Dagan P.O. Box 12 5025001, Israel

[3] Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Sezione Diagnostica di Forlì, Via don Eugenio Servadei 3/E-3/F, 47122, Forlì (FC), Italy

[*]Corresponding author: Giulia Mescolini, Department of Veterinary Medical Sciences -University of Bologna, Via Tolara di Sopra, 50; 40064 – Ozzano dell'Emilia (BO) Italy; Phone +39 051 2097560; e-mail: giulia.mescolini3@unibo.it

Summary

Marek's disease (MD) is a lymphoproliferative disease important to the poultry industry worldwide; it is caused by Gallid alphaherpesvirus 2 (GaHV-2). The virulence of the GaHV-2 isolate has shifted over the years from mild to virulent, very virulent and very virulent +. Nowadays the disease is controlled by vaccination, but field strains of increased virulence are emerging worldwide. Economic losses due to MD are mostly associated with its acute form, characterised by visceral lymphomas. The present study aimed to molecularly classify a group of 13 GaHV-2 strains detected in vaccinated Italian commercial chicken flocks during acute MD outbreaks, and to scrutinise the ability of predicting GaHV-2 virulence, according to the *meq* gene sequence. The full-length *meq* genes were amplified and the obtained amino acid (aa) sequences were analysed, focusing mainly on the number of stretches of four proline molecules (PPPP) within the transactivation domain. Phylogenetic analysis was carried out with the Maximum Likelihood method using the obtained aa sequences and the sequences of Italian strains detected in backyard flocks and of selected strains retrieved from GenBank. All the analysed strains showed 100% sequence identity in the *meq* gene, which encodes a Meq protein of 339 aa. The Meq protein includes four PPPP motifs in the transactivation domain and an interruption of a PPPP motif due to a proline-to-serine substitution at position 218. These features are typically encountered in highly virulent isolates. Phylogenetic analysis revealed that the analysed strains belonged to a cluster that includes high-virulence GaHV-2 strains detected in Italian backyard flocks and a hypervirulent Polish strain. Our results support the hypothesis that the virulence of field isolates can be suggested by *meq* aa sequence analysis.

Keywords: commercial chickens; Italy; GaHV-2; *meq* gene; strain virulence

Introduction

Marek's disease (MD) is an economically-important lymphoproliferative disease to the poultry industry worldwide due to its capacity to cause clinical disease, increased mortality and reduced growth, as well as sub-clinical immunosuppression, causing the exacerbation of other diseases and decreased vaccinal immunity (Schat & Nair, 2013). The virus belongs to the genus *Mardivirus*, subfamily *Alphaherpesvirinae*. According to the most recent nomenclature, it consists of three viral species: *Gallid alphaherpesvirus* 2 (GaHV-2) (aetiological agent of MD), *Gallid alphaherpesvirus* 3 (GaHV-3), and *Meleagrid alphaherpesvirus* 1 (MeHV-1) or Herpesvirus of turkeys (HVT) (International Committee on Taxonomy of Viruses, 2017). GaHV-3 and HVT are antigenically related to GaHV-2 and are widely used as vaccines, usually in association with live attenuated GaHV-2 strains (e.g. CVI988/Rispens). In the field, economic losses due to MD are mostly associated with the acute form of the disease, characterised by visceral lymphomas, however virus-induced immunosuppression might be relevant too, even if its impact is very difficult to assess (Gimeno, 2014; Gimeno & Schat, 2018).

The virulence of GaHV-2 isolates has shifted over the years from mild (m) to virulent (v), very virulent (vv) and very virulent + (vv+) (Witter, 1997; Witter et al., 2005). The disease is controlled by vaccination, but field GaHV-2 strains with increased virulence and greater fitness are emerging worldwide (Trimpert et al., 2017; Nair, 2018). Complex factors might be involved in the occurrence of MD outbreaks in vaccinated chicken flocks, such as: the increased virulence of GaHV-2 over time (Witter, 1997); the inability of the vaccine to offer protection; non-optimal vaccine application, due to its labile cell-associated form (Davidson et al., 2018; Davidson, Natour-Altory, & Shimshon, 2018); co-infection with immunosuppressive viruses (Schat & van Santen, 2013); and other factors.

The GaHV-2 genome encodes more than 200 genes, including the *meq* gene, which was the first discovered GaHV-2 oncogene (Jones et al., 1992). The *meq* gene encodes a protein with homology to the leucine-zipper class nuclear oncogenes, which is composed of a trans-activation N-terminal basic-leucine zipper (bZIP) domain and a C-terminal proline-rich trans-repression domain (Qian et

al., 1995; Liu et al., 1999; Ross, 1999). The oncogenic activities of the Meq protein are mediated by its dimerisation, through the bZIP domain, with itself, as well as with c-Jun-like proteins, such as JunB, c-Jun and c-Fos. Meq also binds to cellular transcription factors such as ATF, CREB and C/EBP (Deng et al., 2010) and interacts with cellular proteins without a bZIP domain, such as the cellular tumour suppressors p53, the retinoblastoma protein (pRb) and the cyclin-dependent kinase 2 (CDK-2) or the heat shock protein Hsp70 (Deng et al., 2010; Gennart et al., 2015). The *meq* oncogene encodes a 339 amino acid unspliced open reading frame in vv and in vv+ GaHV-2 pathotypes and a larger form of 398 amino acids in low virulence strains, having amplifications in the C-terminal proline-rich repeat region (Shamblin et al., 2004).

Concurrently with the stepwise evolution of the virulence of GaHV-2 (Schat & Baranowski, 2007), an increased pattern of genetic polymorphism at the C-terminus domain of the *meq*-encoded oncoprotein has been described (Shamblin et al., 2004). High genetic diversity has been reported for the *meq* gene in spite of the relatively low evolutionary rates of change thought to commonly characterise dsDNA viruses (Duffy, Shackelton, & Holmes, 2008; Firth et al., 2010). Findings by Padhi & Parcells (2016) and Trimpert et al. (2017) reveal that the *meq* gene sequence evolves at a much faster rate than most dsDNA viruses, and is comparable with the evolutionary rate of RNA viruses. By analysing the complete *meq* gene sequence of 84 GaHV-2 strains, Padhi & Parcells (2016) estimated the mean evolutionary rate, beginning from the year 1935, to be greater than other dsDNA viruses, namely 1.02×10^{-4} substitutions per site per year, as compared to the range of 10^{-7} to 10^{-5} for other DNA viruses. Trimpert et al. (2017) analysed 18 complete GaHV-2 genomes and calculated that the GaHV-2 had a mean evolutionary rate of 1.58×10^{-5} substitutions per site per year, in which the *meq* open reading frame (MDV076) was identified as one of the loci harbouring the highest number of point mutations over time. The *meq* gene is evolving under positive selection, most likely imposed by vaccination, reflecting viral adaptation against the host immune responses. Shamblin et al. (2004) and Renz et al. (2012) demonstrated that the number of the four-proline stretches (PPPP) in the *meq* gene transactivation domain are an indicative marker for the

pathogenicity of GaHV-2 strains isolated from chickens; the most virulent isolates showed the lowest number of PPPP repeats, unlike the attenuated and the low pathogenicity isolates, which showed a highest number of repeats. The determination of GaHV-2 virulence by molecular sequencing could be valuable compared to the *in vivo* pathotyping assays, which require complex experimental infection trials of specific genetic lines of chickens (Witter et al., 2005; Dudnikova et al., 2007). At the moment *in vivo* studies are mandatory for an accurate inclusion of GaHV-2 strains into one of the known pathotypes. The *meq* gene polymorphism is also useful to create epidemiological molecular linkages between various GaHV-2 strains, according to the numerous studies that have been recently published from various countries: China (Tian et al., 2011; Zhang et al., 2011; Yu et al., 2013), Poland (Woźniakowski & Samorek-Salamonowicz, 2014), U.S.A. (Padhi & Parcell, 2016), Colombia (Lòpez-Osorio et al., 2017), Egypt (Hassanin, Abdallah, & El-Araby, 2013; Abdallah et al., 2018), India (Gupta, Deka, & Ramneek, 2016; Suresh et al., 2017; Prathibha, Sreedevi, Vinod Kumar, & Srilatha, 2018) and Japan (Abd-Ellatieff et al., 2018).

In the present study, we aimed to molecularly classify a group of 13 GaHV-2 strains, detected during acute MD outbreaks in vaccinated Italian commercial flocks, according to the *meq* gene sequence. We also aimed to scrutinise the ability of suggesting GaHV-2 virulence according to the *meq* gene sequence.

Materials and Methods

Commercial flocks and sampling

MD outbreaks occurred between 2015 and 2018 in 13 commercial chicken flocks located in 6 different Italian regions; flock specifics are reported in Table 1. The chicken flocks were different in terms of production type, genetic line and age. MD vaccination status was known for 10 out of 13 flocks. Flocks were vaccinated with the association of CVI988/Rispens and HVT vaccines. The vaccination of broiler breeders was performed *in ovo* and repeated at 1 or 7 days of age. Cockerels were vaccinated with the association of CVI988/Rispens +HVT at 1 day old. All the examined

flocks experienced acute MD with an increased mortality rate due to visceral lymphomas. At necropsy, portions of the spleen, liver or ovary, showing gross lymphomatous lesions, were sampled and stored at -20 °C until analysis.

Genomic DNA extraction

Genomic DNA was extracted separately from lymphomatous liver, spleen or ovary using the commercial kit "NucleoSpin® Tissue" (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany), following the manufacturer's instructions.

Amplification and sequencing of the *meq* gene

The entire *meq* gene was amplified and sequenced as previously described (Mescolini et al., 2019).

Sequence and phylogenetic analysis of the *meq* gene

The obtained nucleotide (nt) sequences were edited using BioEdit Sequence Alignment Editor Version 7.0.5.3 (Tom Hall, Ibis Therapeutics, Carlsbad, California, USA). Similarities between Italian sequences and *meq* gene sequences available in the NCBI database were investigated through the Basic Local Alignment Search Tool (BLAST).

The obtained sequences were aligned against and compared with previously published complete *meq* gene sequences of Italian strains detected in backyard flocks and with 57 selected complete *meq* gene sequences retrieved from GenBank (Table 2), using Clustal W software (Thompson, Higgins, & Gibson, 1994). Deduced amino acid (aa) sequences were analysed focusing on the number of PPPPs within the proline-rich repeats (PRRs) of the transactivation domain and on the presence of aa substitutions. A phylogenetic tree, based on *meq* gene aa sequences, was built using the Maximum Likelihood method under the Jones–Taylor–Thornton model in MEGA version X (Kumar et al., 2018). Nodal supports were estimated with 1000 bootstrap replicates and considered significant when equal to or greater than 70.

Results

Thirteen GaHV-2 strains were detected by the specific PCR protocol in as many investigated chicken flocks. The *meq* genes of the detected strains were genetically identical, being 1020 bp in length; the obtained sequences were deposited into GenBank under the following names and accession numbers: GaHV-2/Italy/Ck/456/15 - MK855054; GaHV-2/Italy/Ck/498/15 - MK855055; GaHV-2/Italy/Ck/513/15 - MK855056; GaHV-2/Italy/Ck/515/15 - MK855057; GaHV-2/Italy/Ck/559/15 - MK855058; GaHV-2/Italy/Ck/561/15 - MK855059; GaHV-2/Italy/Ck/565/15 - MK855060; GaHV-2/Italy/Ck/567/15 - MK855061; GaHV-2/Italy/Ck/757/17 - MK855062; GaHV-2/Italy/Ck/875/18 - MK855063; GaHV-2/Italy/Ck/876/18 - MK855064; GaHV-2/Italy/Ck/921/18 - MK855065; GaHV-2/Italy/Ck/1083/18 - MK855066.

The molecular characteristics of the deduced Meq protein, compared to prototype strains CVI988, CU-2, JM/102W, Md5 and 648A, are presented in Table 3. All the currently-detected-strains showed a Meq protein of 339 aa that contained, in the transactivation domain, four PPPP motifs and, at position 218, a proline-to-serine substitution, interrupting a hypothetical PPPP sequence at the third position (PPPP → PPSP). Distinctive aa substitutions were found in the Italian GaHV-2 strains at positions 110 (C110S) and 218 (P218S).

The BLAST search showed 100% homology of the currently detected *meq* gene sequences with those of the 8 Italian GaHV-2 strains (GaHV-2/Italy/Ck/625/16; GaHV-2/Italy/Ck/689/16; GaHV-2/Italy/Ck/722/16; GaHV-2/Italy/Ck/801/17; GaHV-2/Italy/Ck/810/17; GaHV-2/Italy/Ck/852/17; GaHV-2/Italy/Ck/853/17; GaHV-2/Italy/Ck/854/17) detected between 2016 and 2017 in Italian backyard flocks that experienced acute MD, transient paralysis or sudden death, and with one of a Polish strain named Polen5 (Table 2). The phylogenetic tree (Figure 1) confirmed the previously reported findings, revealing that the strains detected in the present study clustered together with the Italian strains detected in backyard chickens, and with the Polish strain.

Discussion

The present study reports the sequence analysis of the *meq* gene of 13 GaHV-2 strains detected in several cases of MD-related visceral tumours that occurred in Italian vaccinated commercial chicken flocks collected during the years 2015–2018. Surprisingly, all the *meq* gene sequences of the analysed strains were identical, despite the fact that viruses were detected in flocks differing in terms of production type (broiler breeders, layers or cockerels), geographical location and ownership. The viruses coded for Meq proteins of 339 aa possessing features typically encountered in highly virulent isolates (Shamblin et al., 2004; Renz et al., 2012) and were phylogenetically related to the GaHV-2 strains currently circulating in Italian backyard flocks (Mescolini et al., 2019) and with a hypervirulent strain isolated in Poland in 2010 (Trimpert et al., 2017). Common trade routes may have hypothetically served as a source of dissemination for GaHV-2 between European countries and between industrial and rural compartments, where biosecurity breaches may have also occurred.

Our findings are in accordance with previous studies that reported the geographically-restricted evolution of field GaHV-2 strains in China (Yu et al., 2013), India (Suresh et al., 2017), Egypt (Abdallah et al., 2018) and Poland (Woźniakowski & Samorek-Salamonowicz, 2014). Recently, a comprehensive time-scaled phylogeny study, performed on complete genomes, revealed evidence of the geographical structuring of GaHV-2 strains, supporting the emergence of virulent viruses independently in North America and Eurasia (Trimpert et al., 2017).

Although GaVH-2, as a dsDNA virus, was foreseen to possess high genetic stability to mutations, unexpectedly, its *meq* gene sequence is mutating at a high evolutionary rate, namely 10^{-4} substitutions per site per year (Padhi & Parcells, 2016). The GaHV-2 *meq* gene evolutionary rate is typical for highly mutating RNA viruses, ranging between 10^{-2} to 10^{-5} substitutions per site per year; the avian influenza virus, subgroup H9N2 mutate as an example, with a value of $6.1x10^{-3}$ substitutions per site per year (Davidson et al., 2014). High evolutionary rates reflect the strong positive selection that exists for GaHV-2 in commercial flocks, probably resulting from the

vaccination with highly effective, but imperfect vaccines that are increasing viral diversity (Padhi & Parcells, 2016). The fitness and replication of highly virulent strains seems to be favoured in vaccinated flocks (Read et al., 2015), in which strains able to avoid vaccine-induced protection could be selected.

There is a growing need for new MD vaccines that are efficacious against currently circulating viruses, due to the occurrence of breaks in vaccine immunity. Since vaccine failures are occurring worldwide (López-Osorio et al., 2017; Sun et al., 2017; Abdallah et al., 2018; Abd-Ellatieff et al., 2018), more studies must be conducted to evaluate the protection conferred by Rispens-type vaccines against more recent, and not yet pathotyped, field strains with a history of high virulence. Naturally circulating low virulence strains could represent a solution if they offer improved protection over CVI988/Rispens vaccine.

The rapid increase in the sequencing activities of the *meq* gene of field GaHV-2 strains all over the world has made possible to epidemiologically correlate a significant number of molecular data. The *meq* gene aa sequence has been correlated to GaHV-2 strains virulence (Shamblin et al., 2004; Renz et al., 2012). Our data support this last finding, having our strains molecular features of high virulence and having been detected during severe MD outbreaks in vaccinated chickens.

Meq gene sequencing alone is known to be an insufficient method to include field strains into defined pathotypes, therefore, recently, the research is focusing on finding other genetic predictors of GaHV-2 virulence using complete or targeted DNA sequencing (Dunn et al., 2019). This will provide a highly advantageous alternative to the classical "gold standard" method of *in vivo* pathotyping (Witter et al., 2005), which requires the experimental infection of a large number of a specific type of chickens with standard GaHV-2 prototype strains. As that pathotype classification assay is difficult and not feasible worldwide, Dudnikova et al. (2007) developed an alternative "best fit" pathotyping assay. Although the "best fit" pathotyping assay is simplified, it also employs long-term trials using specific pathogen free chicks. *In vivo* pathotyping assays are not generally

accessible, as those experimental infections require the use of chicks vaccinated at 1 day of age, challenged with virulent isolates and housed in poultry isolation units for 56 days post challenge. As *in vivo* pathotyping is not easily achievable, the *meq* gene molecular characterisation would be the most rapid and accessible way to suggest virulence of field strains. However, the molecular findings should be supported by clinical observations, necropsy findings and vaccination status.

Conflict of Interest Statement

The authors declare no conflict of interest.

Ethical Statement

Ethical statement is not applicable.

Table 1. Description of Italian commercial flocks affected by acute Marek's Disease and their vaccination status.

Flock ID	Region of origin	Year	Production type	Genetic line	Age (weeks)	Vaccine strain	1st vaccination	2nd vaccination	MD form
456/15	Emilia-Romagna	2015	Broiler breeders	Ross 708	NA†	CVI988+HVT	*In ovo*	1 day	Acute
498/15	Emilia-Romagna	2015	Broiler breeders	NA	NA	NA	NA	NA	Acute
513/15	Emilia-Romagna	2015	Layers	Hy-Line	31	NA	NA	NA	Acute
515/15	Emilia-Romagna	2015	Layers	NA	NA	NA	NA	NA	Acute
559/15	Veneto	2015	Broiler breeders	Ross 308	41	CVI988+HVT	*In ovo*	1 day	Acute
561/15	Friuli-Venezia Giulia	2015	Broiler breeders	Ross 708	56	CVI988+HVT	*In ovo*	1 day	Acute
565/15	Veneto	2015	Broiler breeders	Ross 708	36	CVI988+HVT	*In ovo*	1 day	Acute
567/15	Marche	2015	Cockerels	Hy-Line	11	CVI988+HVT	1 day	- ‡	Acute
757/17	Emilia-Romagna	2017	Broiler breeders	Ross 308	51	CVI988+HVT	*In ovo*	1 day	Acute
875/18	Emilia-Romagna	2018	Broiler breeders	Ross 308	40	CVI988+HVT	*In ovo*	7 days	Acute
876/18	Emilia-Romagna	2018	Broiler breeders	Ross 308	31	CVI988+HVT	*In ovo*	7 days	Acute
921/18	Abruzzo	2018	Broiler breeders	Ross 308	27	CVI988+HVT	*In ovo*	1 day	Acute
1083/18	Tuscany	2018	Cockerels	Hy-Line	10	CVI988+HVT	1 day	-	Acute

† Not available
‡ Not performed

Table 2. Details of the GaHV-2 strains, retrieved from GenBank, which were used for the phylogenetic analysis.

GAHV-2 strain	Country	Year	Pathotype	Size of Meq (aa)	PPPPs (N°)	GenBank Accession N°	Reference
CVI988	Netherlands	1969	att	398	7	DQ530348	Spatz et al., 2007
814	China	1986	att	398	7	JF742597	Zhang et al., 2012
3004	Russia	NA[†]	att	398	7	EU032468	NA
CU-2	USA	1970s	m	398	7	AY362708	Shamblin et al., 2004
MD70/13	Hungary	1970	v	339	5	MF431495	Trimpert et al., 2017
571	USA	1989	v	339	3	AY362710	Shamblin et al., 2004
617A	USA	1993	v	339	4	AY362712	Shamblin et al., 2004
MPF57	Australia	1994	v	398	5	EF523774	Renz et al., 2012
04CRE	Australia	2004	v	398	5	EF523773	Renz et al., 2012
573	USA	NA	v	339	4	AY362711	Shamblin et al., 2004
567	USA	NA	v	339	4	AY362709	Shamblin et al., 2004
637	USA	NA	v	339	4	AY362713	Shamblin et al., 2004
BC-1	USA	NA	v	398	7	AY362707	Shamblin et al., 2004
JM	USA	NA	v	398	7	AY243331	Shamblin et al., 2004
JM/102W	USA	NA	v	399	7	DQ534539	Spatz & Silva, 2007
Md5	USA	1977	vv	339	4	AF243438	Tulman et al., 2000
549	USA	1987	vv	339	2	AY362714	Shamblin et al., 2004
595	USA	1991	vv	339	2	AY362715	Shamblin et al., 2004
C12/130	UK	1992	vv	339	5	FJ436096	Spatz et al., 2011
Woodlands1	Australia	1992	vv	399	5	EF523775	Renz et al., 2012
643P	USA	1994	vv	339	2	AY362716	Shamblin et al., 2004
02LAR	Australia	2002	vv	398	5	EF523772	Renz et al., 2012
FT158	Australia	2002	vv	398	5	EF523771	Renz et al., 2012
RB1B	USA	NA	vv	339	5	AY243332	Shamblin et al., 2004
648A	USA	1994	vv+	339	2	AY362725	Shamblin et al., 2004
New	USA	1999	vv+	339	2	AY362719	Shamblin et al., 2004

W	USA	1999	vv+	339	4	AY362723	Shamblin et al., 2004
ATE2539	Hungary	2000	vv+	339	5	MF431493	Trimpert et al., 2017
660-A	USA	NA	vv+	339	2	AY362726	Shamblin et al., 2004
686	USA	NA	vv+	339	2	AY362727	Shamblin et al., 2004
L	USA	NA	vv+	339	2	AY362717	Shamblin et al., 2004
N	USA	NA	vv+	339	2	AY362718	Shamblin et al., 2004
RL	USA	NA	vv+	339	2	AY362720	Shamblin et al., 2004
TK	USA	NA	vv+	339	2	AY362721	Shamblin et al., 2004
U	USA	NA	vv+	339	2	AY362722	Shamblin et al., 2004
X	USA	NA	vv+	339	2	AY362724	Shamblin et al., 2004
EU-1	Italy	1992	NA	339	5	MF431494	Trimpert et al., 2017
0093	China	2002	NA	339	3	AF493550	NA
0095	China	2002	NA	339	3	AF493552	NA
0297	China	2002	NA	339	3	AF493553	NA
0304	China	2002	NA	339	2	AF493554	NA
G2	China	2002	NA	339	4	AF493556	NA
YLO40920	China	2005	NA	339	3	DQ174459	Teng et al., 2011
GXY2	China	2007	NA	339	3	EF546430	Teng et al., 2011
GX070060	China	2008	NA	339	3	EU427303	Teng et al., 2011
GX070079	China	2008	NA	339	3	EU427304	Teng et al., 2011
Polen5	Poland	2010	NA	339	4	MF431496	Trimpert et al., 2017
tn-n1	India	2010	NA	339	5	HM749324	NA
tn-n2	India	2010	NA	339	4	HM749325	NA
UDEACO-04/13	Colombia	2013	NA	339	2	KU058701	López-Osorio et al., 2017
UDEACO-06/13	Colombia	2013	NA	339	2	KU058696	López-Osorio et al., 2017
UDEACO-07/13	Colombia	2013	NA	339	3	KU058697	López-Osorio et al., 2017
bd2	USA	2015	NA	339	2	KU173119	Trimpert et al., 2017
bf1	USA	2015	NA	339	2	KU173117	Trimpert et al., 2017
bf2	USA	2015	NA	339	2	KU173118	Trimpert et al., 2017
sd1	USA	2015	NA	339	2	KU173116	Trimpert et al., 2017
sd2	USA	2015	NA	339	2	KU173115	Trimpert et al., 2017

GaHV-2/Italy/Ck/487/15	Italy	2015	NA	339	5	MK139660	Mescolini et al., 2019
GaHV-2/Italy/Ck/507/15	Italy	2015	NA	418	9	MK139661	Mescolini et al., 2019
GaHV-2/Italy/Ck/509/15	Italy	2015	NA	418	9	MK139662	Mescolini et al., 2019
GaHV-2/Italy/Ck/510/15	Italy	2015	NA	418	9	MK139663	Mescolini et al., 2019
GaHV-2/Italy/Ck/562/15	Italy	2015	NA	418	9	MK139664	Mescolini et al., 2019
GaHV-2/Italy/Ck/599/16	Italy	2016	NA	418	9	MK139665	Mescolini et al., 2019
GaHV-2/Italy/Ck/625/16	Italy	2016	NA	339	4	MK139666	Mescolini et al., 2019
GaHV-2/Italy/Ck/674/16	Italy	2016	NA	339	4	MK139667	Mescolini et al., 2019
GaHV-2/Italy/Ck/689/16	Italy	2016	NA	339	4	MK139668	Mescolini et al., 2019
GaHV-2/Italy/Ck/722/16	Italy	2016	NA	339	4	MK139669	Mescolini et al., 2019
GaHV-2/Italy/Ck/801/17	Italy	2017	NA	339	4	MK139670	Mescolini et al., 2019
GaHV-2/Italy/Ck/810/17	Italy	2017	NA	339	4	MK139671	Mescolini et al., 2019
GaHV-2/Italy/Ck/847/17	Italy	2017	NA	418	10	MK139672	Mescolini et al., 2019
GaHV-2/Italy/Ck/848/17	Italy	2017	NA	418	9	MK139673	Mescolini et al., 2019
GaHV-2/Italy/Ck/850/17	Italy	2017	NA	339	5	MK139674	Mescolini et al., 2019
GaHV-2/Italy/Ck/852/17	Italy	2017	NA	339	4	MK139675	Mescolini et al., 2019
GaHV-2/Italy/Ck/853/17	Italy	2017	NA	339	4	MK139676	Mescolini et al., 2019
GaHV-2/Italy/Ck/854/17	Italy	2017	NA	339	4	MK139677	Mescolini et al., 2019
GaHV-2/Italy/Ck/855/17	Italy	2017	NA	298	2	MK139678	Mescolini et al., 2019

† Not available

Table 3. Molecular characteristics of the deduced Meq protein, compared to prototype strains. Amino acid substitutions interrupting PPPPs are underlined. Distinctive aa substitutions of Italian GaHV-2 strains are framed.

Strain	Meq length (aa)	PPPPs (n°)	Amino acid substitutions														
			71	77	80	110	119	153	176	180	216[†]	217	218	277	283	320	326
CVI988	398	7	S	E	D	C	C	P	P	T	P	P	P	L	A	I	I
CU-2	398	7	S	E	D	C	C	P	P	T	P	P	P	L	A	I	T
JM/102W	399	7	A	E	D	C	C	P	P	T	S	P	P	L	A	I	T
Md5	339	4	A	K	D	C	C	P	P	T	P	A	P	L	V	T	T
648A	339	2	A	K	D	C	R	Q	A	A	P	A	P	P	A	I	T
GaHV-2/Italy/Ck/456/15	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/498/15	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/513/15	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/515/15	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/559/15	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/561/15	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/565/15	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/567/15	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/757/17	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/875/18	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/876/18	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/921/18	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/1083/18	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T

[†] Amino acid position according to the 339 aa-long Meq isoform

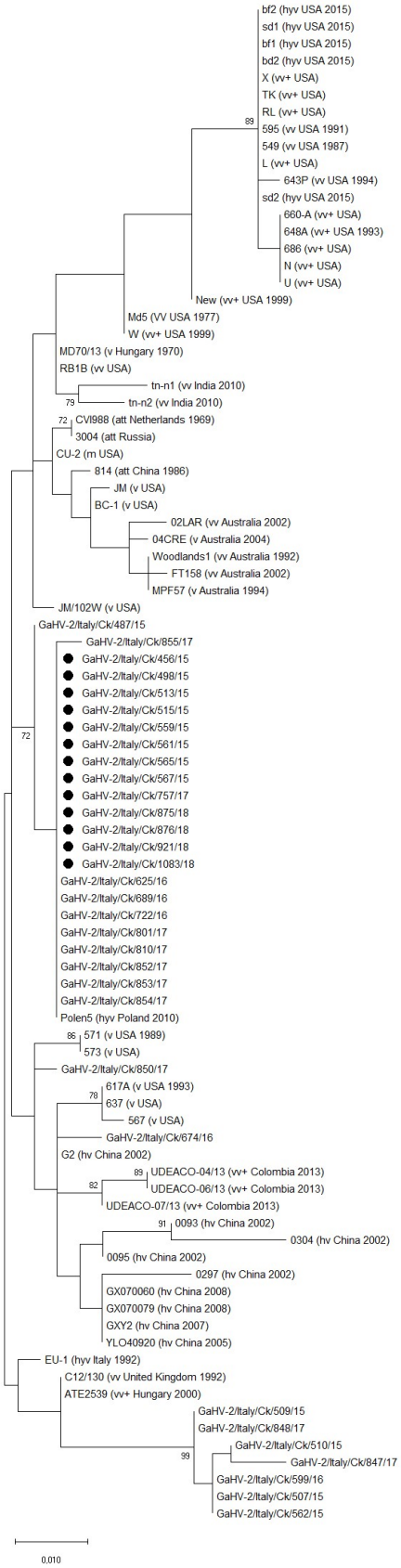

Figure 1. Phylogenetic tree based on *meq* gene complete amino acid sequences of the 13 GaHV-2 strains detected in Italian commercial flocks (marked with a black dot, ●) and of the 76 strains retrieved from GenBank.

References

Abd-Ellatieff, H. A., Abou Rawash, A. A., Ellakany, H. F., Goda, W. M., Suzuki, T., & Yanai, T. (2018). Molecular characterization and phylogenetic analysis of a virulent Marek's disease virus field strain in broiler chickens in Japan. Avian Pathology, 47(1), 47–57. https://doi.org/10.1080/03079457.2017.1362497

Abdallah, F., Hassanin, O., Attar, E., Ali, H., Megahed, M., & Nair, V. (2018). Marek's Disease Virus in Egypt: Historical Overview and Current Research Based on the Major MDV-Encoded Oncogene Meq. Hosts and Viruses, 5, 35–43. http://dx.doi.org/10.17582/journal.hv/2018/5.3.35.43

Davidson, I., Fusaro, A., Heidari, A., Monne, I., & Cattoli, G. (2014). Molecular evolution of H9N2 avian influenza viruses in Israel. Virus Genes, 48(3), 457–463. https://doi.org/10.1007/s11262-014-1037-0

Davidson, I., Natour-Altory, A., Raibstein, I., Kin, E., Dahan, Y., Krispin, H., & Elkin, N. (2018). Monitoring the uptake of live avian vaccines by their detection in feathers. Vaccine, 36, 637–643. https://doi.org/10.1016/j.vaccine.2017.12.052

Davidson, I., Natour-Altory, N., & Shimshon Y. (2018). Evaluation of Live Vaccine Application in Commercial Poultry Flocks Using Feathers – In Practice. Israel Journal of Veterinary Medicine, 73, 8–13.

Deng, X., Li, X., Shen, Y., Qiu, Y., Shi, Z., Shao, D., Jin, Y., Chen, H., Ding, C., LiLi, Chen, P., & Ma, Z. (2010). The Meq oncoprotein of Marek's disease virus interacts with p53 and inhibits its transcriptional and apoptotic activities. Virology Journal, 7: 348. https://doi.org/10.1186/1743-422X-7-348

Dudnikova, E., Norkina, S., Vlasov, A., Slobodchuk, A., Lee, L. F., & Witter, R. L. (2007). Evaluation of Marek's disease field isolates by the "best fit" pathotyping assay. Avian Pathology, 36(2), 135–143. https://doi.org/10.1080/03079450701209857

Duffy, S., Shackelton, L. A., & Holmes, E. C. (2008). Rates of evolutionary change in viruses: Patterns and determinants. Nature Reviews Genetics, 9, 267–276.

https://doi.org/10.1038/nrg2323

Dunn, J.R., Black Pyrkosz, A., Steep, A. & Cheng, H.H. (2019). Identification of Marek's disease virus genes associated with virulence of US strains. Journal of General Virology, 100, 1132-1139. https://doi.org/10.1099/jgv.0.001288

Firth, C., Kitchen, A., Shapiro, B., Suchard, M. A., Holmes, E. C., & Rambaut, A. (2010). Using time-structured data to estimate evolutionary rates of double-stranded DNA viruses. Molecular Biology and Evolution, 27(9), 2038–2051. https://doi.org/10.1093/molbev/msq088

Gennart, I., Coupeau, D., Pejaković, S., Laurent, S., Rasschaert, D., & Muylkens, B. (2015). Marek's disease: Genetic regulation of gallid herpesvirus 2 infection and latency. Veterinary Journal, 205, 339–348. https://doi.org/10.1016/j.tvjl.2015.04.038

Gimeno, I. M. (2014). Marek's Disease and Differential Diagnosis with Other Tumor Viral Diseases of Poultry. In *Encyclopedia of Agriculture and Food Systems* (pp. 156–171). https://doi.org/https://doi.org/10.1016/B978-0-444-52512-3.00193-5

Gimeno, I.M., & Schat K.A. (2018). Virus-Induced Immunosuppression in Chickens. Avian Diseases, 62, 272–285. https://doi.org/10.1637/11841-041318-Review.1

Gupta, M., Deka, D., & Ramneek. (2016). Sequence analysis of Meq oncogene among Indian isolates of Marek's disease herpesvirus. Meta Gene, 9, 230–236. https://doi.org/10.1016/j.mgene.2016.07.009

Hassanin, O., Abdallah, F., & El-Araby, I. E. (2013). Molecular characterization and phylogenetic analysis of Marek's disease virus from clinical cases of Marek's disease in Egypt. Avian Diseases, 57(Suppl 2), 555–561. https://doi.org/10.1637/10337-082912-Reg.1

International Committee on Taxonomy of Viruses, 2017. Master Species List 2017 [WWW Document]. ICTV. Retrieved from https://talk.ictvonline.org/files/master-species-lists/m/msl/7185

Jones, D., Lee, L., Liu, J.L., Kung H. J., & Tillotson, J. K. (1992). Marek's disease virus encodes a basic-leucine zipper gene resembling the fos/jun oncogenes that is highly expressed in

lymphoblastoid tumors. Proceedings of the National Academy of Sciences, 89, 4042–4046. https://doi.org/10.1073/pnas.89.9.4042

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution, 35(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Liu, J. L., Lin, S. F., Xia, L., Brunovskis, P., Li, D., Davidson, I., Lee, L.F., & Kung, H. J. (1999). Meq and V-IL8: Cellular genes in disguise? Acta Virologica, 43, 94–101.

López-Osorio, S., Piedrahita, D., Espinal-Restrepo, M. A., Ramírez-Nieto, G. C., Nair, V., Williams, S. M., Baigent, S., Ventura-Polite, C., Aranzazu-Taborda, D.A., & Chaparro-Gutiérrez, J. J. (2017). Molecular characterization of Marek's disease virus in a poultry layer farm from Colombia. Poultry Science, 96(6), 1598–1608. https://doi.org/https://doi.org/10.3382/ps/pew464

Mescolini, G., Lupini, C., Felice, V., Guerrini, A., Silveira, F., Cecchinato, M., & Catelli, E. (2019). Molecular characterization of the meq gene of Marek's disease viruses detected in unvaccinated backyard chickens reveals the circulation of low- and high-virulence strains. Poultry Science, pii: pez095. doi: 10.3382/ps/pez095. [Epub ahead of print]

Nair, V. (2018). Spotlight on avian pathology: Marek's disease. Avian Pathology, 47, 440–442. https://doi.org/10.1080/03079457.2018.1484073

Padhi, A., & Parcells, M. S. (2016). Positive selection drives rapid evolution of the meq oncogene of Marek's disease virus. PLoS ONE, 11(9). https://doi.org/10.1371/journal.pone.0162180

Prathibha, Y., Sreedevi, B., Vinod Kumar, N., & Srilatha, C. (2018). Molecular characterization and phylogenetic analysis of oncogenes from virulent serotype-1 Marek's disease virus in India. Acta Virologica, 62(3), 277–286. https://doi.org/10.4149/av_2018_221

Qian, Z., Brunovskis, P., Rauscher 3rd, F., Lee, L., & Kung, H. J. (1995). Transactivation activity of Meq, a Marek's disease herpesvirus bZIP protein persistently expressed in latently infected transformed T cells. Journal of Virology, 69(7), 4037–4044.

http://www.ncbi.nlm.nih.gov/pubmed/7769661

Read, A. F., Baigent, S. J., Powers, C., Kgosana, L. B., Blackwell, L., Smith, L. P., Kennedy, D.A., Walkden-Brown, S.W., & Nair, V. K. (2015). Imperfect Vaccination Can Enhance the Transmission of Highly Virulent Pathogens. PLOS Biology, 13(7), e1002198. https://doi.org/10.1371/journal.pbio.1002198

Renz, K. G., Cooke, J., Clarke, N., Cheetham, B. F., Hussain, Z., Fakhrul Islam, A. F. M., Tannock, G.A., & Walkden-Brown, S. W. (2012). Pathotyping of Australian isolates of Marek's disease virus and association of pathogenicity with meq gene polymorphism. Avian Pathology, 41(2), 161–176. https://doi.org/10.1080/03079457.2012.656077

Ross, N. L. J. (1999). T-cell transformation by Marek's disease virus. Trends in Microbiology, 7, 22–29. https://doi.org/10.1016/S0966-842X(98)01427-9

Schat, K. A., & Baranowski, E. (2007). Animal vaccination and the evolution of viral pathogens. Revue Scientifique Et Technique-Office International Des Epizooties, 26(2), 327–338.

Schat, K.A., & Nair V. (2013). Neoplastic diseases: Marek's disease. In D. E. Swayne, J. R. Glisson, L. R. McDougald, L. K. Nolan, D. L. Suarez, V. L. Nair, (Eds.) *Diseases of Poultry* (13th ed., pp. 515–552). Ames, Iowa, USA: Wiley-Blackwell Hoboken.

Schat, K.A., & van Santen, V.L. (2013). Chicken infectious anemia. In D. E. Swayne, J. R. Glisson, L. R. McDougald, L. K. Nolan, D. L. Suarez, V. L. Nair (Eds.) *Diseases of Poultry* (13th ed., pp. 248–275). Ames, Iowa, USA: Wiley-Blackwell Hoboken.

Shamblin, C. E., Greene, N., Arumugaswami, V., Dienglewicz, R. L., & Parcells, M. S. (2004). Comparative analysis of Marek's disease virus (MDV) glycoprotein-, lytic antigen pp38- and transformation antigen Meq-encoding genes: Association of meq mutations with MDVs of high virulence. Veterinary Microbiology, 102(3–4), 147–167. https://doi.org/10.1016/j.vetmic.2004.06.007

Spatz, S. J., Petherbridge, L., Zhao, Y., & Nair, V. (2007). Comparative full-length sequence analysis of oncogenic and vaccine (Rispens) strains of Marek's disease virus. Journal of

General Virology, 88(4), 1080–1096. https://doi.org/10.1099/vir.0.82600-0

Spatz, S.J., & Silva, R.F. (2007). Polymorphisms in the repeat long regions of oncogenic and attenuated pathotypes of Marek's disease virus 1. Virus Genes, 35, 41–53. doi:10.1007/s11262-006-0024-5

Spatz, S.J., Smith, L.P., Baigent, S.J., Petherbridge, L., & Nair, V. (2011). Genotypic characterization of two bacterial artificial chromosome clones derived from a single DNA source of the very virulent gallid herpesvirus-2 strain C12/130. Journal of General Virology, 92, 1500–1507. doi:10.1099/vir.0.027706-0

Sun, G. R., Zhang, Y. P., Lv, H. C., Zhou, L. Y., Cui, H. Y., Gao, Y. L., Qi, X. Le, Wang, Y. Q., Li, K., Gao, L., Pan, Q., Wang, X. M., & Liu, C. J. (2017). A Chinese variant Marek's disease virus strain with divergence between virulence and vaccine resistance. Viruses 9, 71. https://doi.org/10.3390/v9040071

Suresh, P., Johnson Rajeswar, J., Sukumar, K., Harikrishnan, T. J., & Srinivasan, P. (2017). Complete nucleotide sequence analysis of the oncogene "Meq" from serotype 1 Marek's disease virus isolates from India. British Poultry Science, 58, 111–115. https://doi.org/10.1080/00071668.2016.1257780

Teng, L. Q., Wei, P., Song, Z. B., He, J. J., & Cui, Z. Z. (2011). Molecular epidemiological investigation of Marek's disease virus from Guangxi, China. Archives of Virology, 156, 203–206. doi:10.1007/s00705-010-0840-8

Thompson, J.D., Higgins, D.G., & Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22, 4673–4680. doi: 10.1093/nar/22.22.4673

Tian, M., Zhao, Y., Lin, Y., Zou, N., Liu, C., Liu, P., Cao, S., Wen, X., & Huang, Y. (2011). Comparative analysis of oncogenic genes revealed unique evolutionary features of field Marek's disease virus prevalent in recent years in China. Virology Journal, 8, 121.

https://doi.org/10.1186/1743-422X-8-121

Trimpert, J., Groenke, N., Jenckel, M., He, S., Kunec, D., Szpara, M. L., Spatz, S. J., Osterrieder, N., & McMahon, D. P. (2017). A phylogenomic analysis of Marek's disease virus reveals independent paths to virulence in Eurasia and North America. Evolutionary Applications, 10(10), 1091–1101. https://doi.org/10.1111/eva.12515

Tulman, E. R., Afonso, C. L., Lu, Z., Zsak, L., Rock, D. L., & Kutish, G. F. (2000). The Genome of a Very Virulent Marek's Disease Virus. Journal of Virology, 74, 7980–7988. doi:10.1128/jvi.74.17.7980-7988.2000

Witter, R. L. (1997). Increased Virulence of Marek's Disease Virus Field Isolates. Avian Diseases, 41(1), 149. https://doi.org/10.2307/1592455

Witter, R. L., Calnek, B. W., Buscaglia, C., Gimeno, I. M., & Schat, K. A. (2005). Classification of Marek's disease viruses according to pathotype: Philosophy and methodology. Avian Pathology, 34, 75–90. https://doi.org/10.1080/03079450500059255

Woźniakowski, G., & Samorek-Salamonowicz, E. (2014). Molecular Evolution of Marek's Disease Virus (MDV) Field Strains in a 40-Year Time Period. Avian Diseases, 58(4), 550–557. https://doi.org/10.1637/10812-030614-Reg.1

Yu, Z.-H., Man, T., Lou, J., Wang, X.-W., Ding, K., Yu, L-L, Su, J-W, Chi, J.-Q., Zhao, P., Hu, B., Zhang, G.-P., & Liu, J.-X. (2013). Molecular characteristics and evolutionary analysis of field Marek's disease virus prevalent in vaccinated chicken flocks in recent years in China. Virus Genes, 47, 282–291. doi: 10.1007/s11262-013-0942-y

Zhang, F., Liu, C. J., Zhang, Y. P., Li, Z. J., Liu, A. L., Yan, F. H., Cong, F., & Cheng, Y. (2012). Comparative full-length sequence analysis of Marek's disease virus vaccine strain 814. Archives of Virology, 157, 177–183. doi:10.1007/s00705-011-1131-8

Zhang, Y. P., Liu, C. J., Zhang, F., Shi, W., & Li, J. (2011). Sequence analysis of the meq gene in the predominant Marek's disease virus strains isolated in China during 2006-2008. Virus Genes, 43(3), 353–357. https://doi.org/10.1007/s11262-011-0645-1

III. Molecular characterisation of a Marek's disease virus strain detected in tumour-bearing turkeys (published paper)

NOTICE: this is post-print (final draft post-refereeing) author's version of a paper that was accepted for publication in the journal "*Avian Pathology*". Changes resulting from the publishing process, such as editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. The definitive version of this paper has been published and is available: Mescolini G., Lupini C., Davidson I., Massi P., Tosi G., Fiorentini L., Catelli E. "Molecular characterization of a Marek's disease virus strain detected in tumour-bearing turkeys." In Avian Pathology 2020 Apr;49(2):202-207. doi: 10.1080/03079457.2019.1691715. Epub 2019 Nov 22.

Molecular characterisation of a Marek's disease virus strain detected in tumour-bearing turkeys

Giulia Mescolini[1,*], Caterina Lupini[1], Irit Davidson[2], Paola Massi[3], Giovanni Tosi[3], Laura Fiorentini [3], Elena Catelli[1].

[1] Department of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra, 50, 40064, Ozzano dell'Emilia (BO), Italy.

[2] Division of Avian Diseases, Kimron Veterinary Institute, Bet Dagan P.O. Box 12 5025001, Israel.

[3] Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Sezione Diagnostica di Forlì, Via don Eugenio Servadei 3/E-3/F, 47122, Forlì (FC), Italia.

*Corresponding Author: Giulia Mescolini, Department of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra, 50, 40064, Ozzano dell'Emilia (BO), Italy. e-mail: giulia.mescolini3@unibo.it

Abstract

Marek's disease (MD) is a lymphoproliferative disease caused by *Gallid alphaherpesvirus 2* (GaHV-2), which primarily affects chickens. However, the virus is also able to induce tumours in turkeys, albeit less frequently than in chickens. This study reports the molecular characterisation of a GaHV-2 strain detected in a flock of Italian meat-type turkeys exhibiting visceral lymphomas. Sequencing and phylogenetic analysis of the *meq* gene revealed that the turkey GaHV-2 has molecular features of high virulence and genetic similarity with GaHV-2 strains recently detected in Italian commercial and backyard chickens. GaHV-2 is ubiquitous among chickens despite the vaccination, and chicken-to-turkey transmission is hypothesised due to the presence of broilers in neighbouring pens.

Keywords: Marek's disease, turkey, *Gallid alphaherpesvirus 2*, *meq* gene, molecular characterisation, *Turkey herpesvirus*.

Research highlights

- A GaHV-2 strain from Italian turkeys was molecularly characterised;
- The turkey strain presented molecular characteristics of high virulence in its *meq* gene;
- The turkey strain was closely related to previously detected chicken strains.

Introduction

Marek's disease (MD) virus or *Gallid alphaherpesvirus 2* (GaHV-2), the causative agent of MD, is a herpesvirus belonging to the subfamily *Alphaherpesvirinae*, genus *Mardivirus*. Two other viral species are included in this genus: *Gallid alphaherpesvirus 3* (GaHV-3) and *Meleagrid alphaherpesvirus 1* (MeHV-1) or *Turkey herpesvirus* (HVT), frequently used as vaccines against MD in chickens. GaHV-2 isolates can be classified into four pathotypes: mild, virulent, very virulent and very virulent plus (Witter, 1997). GaHV-2 has been extensively studied and described in chickens, whether experimentally or naturally infected. In contrast, studies have seldom focused on GaHV-2 infections in turkeys, and scientific reports remain limited.

The first report on a Marek's disease-like condition in turkeys was from Florida, where two wild turkeys exhibited lymphoid visceral tumours resembling the MD-related tumours of the chicken (Busch & Williams, 1970). Subsequently, field cases were reported from the Netherlands (Voute & Wagenaar-Schaafsma, 1974), France (Coudert *et al*., 1995), Germany (Voeckell *et al*., 1999), Israel (Davidson *et al*., 2002) and the United Kingdom (Pennycott & Venugopal, 2002; Deuchande *et al*., 2012, Blake-Dyke & Baigent, 2013).

Susceptibility to GaHV-2 infection and tumour development has been demonstrated in experimentally infected turkeys with GaHV-2 isolates of chicken or turkey origin (Paul *et al*., 1977; Elmubarak *et al*., 1981; Powell *et al*., 1984; Davidson *et al*., 2002).

At post-mortem examination, GaHV-2-induced tumours in turkeys resemble tumours induced by either the Reticuloendotheliosis virus (REV) (Nair *et al*., 2013) or the Lymphoprolipherative disease virus (LPDV) (Biggs, 1997).

Some of these studies have primarily diagnosed MD based on histopathology, but this is not a decisive assay because even microscopically the neoplastic infiltrate can prove very similar across these lymphoproliferative diseases (Schat & Nair, 2013).

Relatively few studies have employed the PCR to confirm the GaHV-2 tumour's aetiology (Voechell *et al*., 1999, Davidson *et al.*, 2002, Deuchande *et al*., 2012; Blake-Dyke & Baigent, 2013). In our study, the *meq* gene was selected to serve for turkey GaHV-2 identification and classification, having been described as carrying virulence-specific markers (Shamblin *et al*., 2004). Indeed, Shamblin *et al.* (2004) and Renz *et al.* (2012) have observed that the number of sequences of four proline molecules (PPPP) is significantly correlated with the viral pathotype. Isolates of lower virulence present greater PPPP number than higher virulence isolates, which contain the lowest number of four-proline repeats or disrupted PPPP motifs due to point mutations. The determination of GaHV-2 virulence by molecular sequencing is only able to suggest the viral pathotype, as *in vivo* pathotyping assays (Witter *et al.*, 2005) using susceptible chickens are mandatory for an exact inclusion of GaHV-2 strains into one of the known pathotypes.

The aim of the present study is to report the description of GaHV-2-caused visceral tumours in Italian commercial turkeys, alongside with the first molecular characterisation of the detected GaHV-2 strain through *meq* gene sequence analysis and phylogeny.

Materials and Methods

Commercial turkeys. During the year 2016, three-to-four-month-old white meat turkeys, unvaccinated against MD and reared on a commercial free-range farm located in the Lazio region of Italy, experienced mortality. At post-mortem examination livers were enlarged and contained whitish lesions of lymphoproliferative nature. The flock had been reared indoors up to 50 days of age, before moving into outdoor pens until slaughter at five months old. On the same farm, HVT-vaccinated broiler chickens were reared outdoors in neighbouring pens.

DNA extraction. A selected tumour-bearing liver served for the genomic DNA extraction using the commercial kit NucleoSpin® Tissue (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany), according to the manufacturer's instructions.

PCRs for GaHV-2 *meq* gene amplification and HVT detection. The full-length *meq* gene of GaHV-2 was amplified with a previously described PCR protocol (Mescolini *et al*., 2019a). DNA was subjected to a further PCR protocol employing an oligonucleotide set specifically designed to amplify the US3 gene of HVT (Handberg *et al*., 2001). PCR was conducted by adding 3 µL DNA to a 22 µL reaction mixture containing 0.125 µL GoTaq G2 Flexi DNA Polymerase (Promega, Madison, WI), 5 µL 5X Colorless Go-Taq Flexi Buffer, 1.75 µL MgCl2 solution, 0.5 µL dNTPs, 13 µL H2O for molecular biology, 1 µL primer forward HVT-1 (5'-ATG GAA GTA GAT GTT GAG TCT TCG-3') and 1 µL primer reverse HVT-2 (5'-CGA TAT ACA CGC ATT GCC ATA CAC-3'). Cycling conditions were as follows: 2 min of denaturation at 95°C followed by 35 cycles, each consisting of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. A final elongation step at 72°C for 5 min completed the reaction. The PCR products were separated on agarose
gel (2%), stained with ethidium bromide, and visualized under ultraviolet light after an electrophoretic run at 110 V and 400 mA for 35 min.

Sequencing and sequence analysis. PCR products were purified using ExoSAP-IT *Express* PCR Product Cleanup (Thermo Fisher Scientific, Massachusetts, USA) and sequenced by a commercial sequencing service (Macrogen Spain, Madrid, Spain).

In order to obtain the whole *meq* gene sequence, PCR primers *Eco*R-Q for (5'-GGT GAT ATA AAG ACG ATA GTC ATG-3') and *Eco*R-Q rev (5'-CTC ATA CTT CGG AAC TCC TGG AG-3') (Shamblin *et al*., 2004) as well as an additional and internal primer (meq-F, 5'-ATG TCT CAG GAG CCA GAG CCG-3') (Hassanin *et al*., 2013) were used for sequencing. The sequence was edited and assembled using BioEdit Sequence Alignment Editor, Version 7.2.5.0 (Tom Hall, Ibis Therapeutics, Carlsbad, California, USA), before being aligned against selected complete *meq* gene sequences of 36 reference GaHV-2 strains of known pathotype and 32 GaHV-2 strains recently detected during MD outbreaks in Italian backyard (Mescolini *et al.*, 2019a) and commercial chickens (Mescolini *et al.*, 2019b) (Table 1). The number of PPPP motifs contained in the proline-

rich repeats (PPRs) of the transactivation domain, the proline content (%) and the amino acid (aa) substitutions in *meq* gene-deduced amino acid sequence were evaluated.

A phylogenetic tree based on the *meq* gene aa sequences was constructed with the maximum likelihood (ML) method using MEGAX (Kumar *et al*., 2018). Nodes of the tree with bootstrap values obtained with 1,000 replicates equal to or greater than 70 were considered significant.

The HVT US3 gene amplicon was sequenced in both directions using the PCR primers and was submitted to the basic local alignment search tool (BLAST) for a similarity search.

Accession numbers. Sequences were submitted to the GenBank database and are available under the following accession numbers: MN017102 (*meq* gene of GaHV-2) and MN017103 (US3 gene of MeHV-1).

Results

The analysed sample was positive at PCR for the GaHV-2 *meq* gene, producing an amplicon of the expected size. The detected strain was named GaHV-2/Italy/Turkey/601/16. Sequence analysis revealed a *meq* gene encoding for a 339 aa-long Meq protein isoform with a proline content of 21.18% and a 100% nucleotide sequence identity with Italian GaHV-2 strains recently detected in commercial (Mescolini *et al.*, 2019b) and rural chicken flocks (Mescolini *et al.*, 2019a).

Four PPPPs were identified in the transactivation domain together with a PPSP sequence, in which a serine replaced a proline at position 218 (P218S). The overall molecular characteristics of the detected strain are reported in Table 2. GaHV-2/Italy/Turkey/601/16 showed an aa substitution (S71A) that is typically found in all *in vivo* pathotyped vv+ strains and other three aa substitutions (D80Y, C110S and P218S) found in field strains from Italy (Mescolini *et al.*, 2019a, b) and Poland (Woźniakowski *et al*., 2010; Woźniakowski & Samorek-Salamonowicz, 2014; Trimpert *et al.*, 2017) with an history of elevated virulence in the field. The phylogenetic analysis (Figure 1) confirmed the close relationship of the turkey strain with GaHV-2s recently detected in Italy from MD outbreaks in chickens, as they belong to the same cluster.

Finally, an amplicon of the expected size (505 bp) was obtained when the specific PCR for the US3 gene of HVT was applied. The BLAST search confirmed the detection of an HVT strain (MeHV-1/Italy/Turkey/601/16), presenting a 100% sequence identity with the US3 gene of the HVT strain FC126 (GenBank accession number AF291866), commonly used as MD vaccine in chickens.

Discussion

The present report, which molecularly identifies a GaHV-2 strain in free-range commercial turkeys, builds upon the few existing studies of turkeys infected by GaHV-2, which is primarily a chicken's pathogen. The *meq* gene, the main GaHV-2 viral oncogene, was selected for the molecular characterisation of the GaHV-2/Italy/Turkey/601/16 strain owing to its molecular variability, which correlates with the level of virulence of the strain (Lee *et al*., 2000; Shamblin *et al*., 2004). The GaHV-2 strain showed molecular features suggestive of high virulence due to the presence, in the transactivation domain of the Meq protein, of a low number of four-proline repeats, of a disrupted PPPP motif and of aa substitutions typically found in all vv+strains (S71A) and in Italian and Polish strains (D80Y, C110S and P218S) with an history of high virulence in the field (Woźniakowski *et al*., 2010; Woźniakowski & Samorek-Salamonowicz, 2014; Trimpert *et al.*, 2017; Mescolini *et al.*, 2019a, b). In order to report the original turkey GaHV-2 sequence without any possible molecular changes that may have occurred during tissue culture propagation (Shamblin *et al*., 2004), this study employed the original turkey tissue for amplification and sequencing, as advocated by Davidson *et al.* (1995) and Davidson and Silva (2008).

For the first time a turkey GaHV-2 *meq* gene sequence was obtained and compared with *meq* gene sequence GaHV-2 strains of known pathotype and GaHV-2 strains recently detected during MD outbreaks in Italian chickens.

A resemblance of the turkey GaHV-2 to chicken GaHV-2 strains with molecular characteristics suggestive of high virulence previously detected in Italy was evident from the *meq* gene sequence characterisation and phylogenetic analysis.

This report strengthens the previously sporadic observation of the potentially detrimental effects of virulent GaHV-2 strains infecting the turkey. In particular, turkeys reared with the possibility of contact with GaHV-2-affected chickens are prone to infection by circulating GaHV-2 strains. Whereas Davidson *et al.* (2002) reported MD in commercial turkey flocks reared in poultry houses previously occupied by MD affected chickens, the present report describes free-range birds of both species located in neighbouring pens. Due to the high and efficient horizontal environmental spread of GaHV-2 by means of desquamated feather follicle epithelial cells, which harbour infectious viral particles, it can be assumed that the affected turkey flock has been subjected to considerable risk of infection due to the continuous and close presence of broilers. Unfortunately, the neighbouring broiler flock was not tested for GaHV-2 presence, but the virus is ubiquitous in chickens and might infect vaccinated chickens asymptomatically.

Although the susceptibility of turkeys to GaHV-2 infection has been recognised, reports on MD in this species are rare. This can be attributed to a lack of awareness, to different degrees of MD genetic resistance, or to the widespread presence of HVT in this species, which as hypothesised by Witter and Solomon (1971) may confer a certain degree of protection against the disease. Nevertheless, the latter possibility has been contested by Elmubarak *et al.* (1981), who have found that HVT vaccination is ineffective in protecting turkeys against MD under experimental conditions, and Blake-Dyke and Baigent (2013), who report that an early infection with HVT may prove unable to provide adequate immunity and protect turkeys from the challenge with a field GaHV-2 strain. The moment at which the birds in our investigation became infected with HVT is unknown, because the virus was detected simultaneously with the MD outbreak, and so the role of HVT in protecting turkeys from MD remains unclear. The genetic similarity of the detected HVT strain with the FC126 vaccine strain suggests that the virus probably came from the neighbouring broilers, but it cannot be excluded that the examined turkey flock naturally harboured the detected HVT strain.

The protection of turkeys against MD is at present heavily reliant on management procedures, namely the effective separation from GaHV-2-affected chickens. Further studies are required to understand whether the associations of currently available vaccines are able to prevent MD in turkeys.

Disclosure statement

No potential conflict of interest was reported by the authors.

Table 1. Chicken GaHV-2 strains included in the analysis.

GAHV-2 strain	Country	Year	Pathotype	Size of Meq (aa)	PPPPs (N°)	GenBank Accession N°	Reference
CVI988	The Netherlands	1969	att[a]	398	7	DQ530348	Spatz *et al*., 2007
814	China	1986	att	398	7	JF742597	Zhang *et al*., 2012
3004	Russia	NA[b]	att	398	7	EU032468	NA
CU-2	USA	1970s	m[c]	398	7	AY362708	Shamblin *et al*., 2004
MD70/13	Hungary	1970	v[d]	339	5	MF431495	Trimpert *et al*., 2017
571	USA	1989	v	339	3	AY362710	Shamblin *et al*., 2004
617A	USA	1993	v	339	4	AY362712	Shamblin *et al*., 2004
MPF57	Australia	1994	v	398	5	EF523774	Renz *et al*., 2012
04CRE	Australia	2004	v	398	5	EF523773	Renz *et al*., 2012
573	USA	NA	v	339	4	AY362711	Shamblin *et al*., 2004
567	USA	NA	v	339	4	AY362709	Shamblin *et al*., 2004
637	USA	NA	v	339	4	AY362713	Shamblin *et al*., 2004
BC-1	USA	NA	v	398	7	AY362707	Shamblin *et al*., 2004
JM	USA	NA	v	398	7	AY243331	Shamblin *et al*., 2004
JM/102W	USA	NA	v	399	7	DQ534539	Spatz & Silva, 2007
Md5	USA	1977	vv[e]	339	4	AF243438	Tulman *et al*., 2000
549	USA	1987	vv	339	2	AY362714	Shamblin *et al*., 2004
595	USA	1991	vv	339	2	AY362715	Shamblin *et al*., 2004
C12/130	UK	1992	vv	339	5	FJ436096	Spatz *et al*., 2011
Woodlands1	Australia	1992	vv	399	5	EF523775	Renz *et al*., 2012
643P	USA	1994	vv	339	2	AY362716	Shamblin *et al*., 2004
02LAR	Australia	2002	vv	398	5	EF523772	Renz *et al*., 2012
FT158	Australia	2002	vv	398	5	EF523771	Renz *et al*., 2012
RB1B	USA	NA	vv	339	5	AY243332	Shamblin *et al*., 2004
648A	USA	1994	vv+[f]	339	2	AY362725	Shamblin *et al*., 2004

New	USA	1999	vv+	339	2	AY362719	Shamblin *et al*., 2004
W	USA	1999	vv+	339	4	AY362723	Shamblin *et al*., 2004
ATE2539	Hungary	2000	vv+	339	5	MF431493	Trimpert *et al*., 2017
660-A	USA	NA	vv+	339	2	AY362726	Shamblin *et al*., 2004
686	USA	NA	vv+	339	2	AY362727	Shamblin *et al.*, 2004
L	USA	NA	vv+	339	2	AY362717	Shamblin *et al.*, 2004
N	USA	NA	vv+	339	2	AY362718	Shamblin *et al*., 2004
RL	USA	NA	vv+	339	2	AY362720	Shamblin *et al*., 2004
TK	USA	NA	vv+	339	2	AY362721	Shamblin *et al*., 2004
U	USA	NA	vv+	339	2	AY362722	Shamblin *et al*., 2004
X	USA	NA	vv+	339	2	AY362724	Shamblin *et al*., 2004
GaHV-2/Italy/Ck/487/15	Italy	2015	NA	339	5	MK139660	Mescolini *et al*., 2019a
GaHV-2/Italy/Ck/507/15	Italy	2015	NA	418	9	MK139661	Mescolini *et al*., 2019a
GaHV-2/Italy/Ck/509/15	Italy	2015	NA	418	9	MK139662	Mescolini *et al*., 2019a
GaHV-2/Italy/Ck/510/15	Italy	2015	NA	418	9	MK139663	Mescolini *et al*., 2019a
GaHV-2/Italy/Ck/562/15	Italy	2015	NA	418	9	MK139664	Mescolini *et al*., 2019a
GaHV-2/Italy/Ck/599/16	Italy	2016	NA	418	9	MK139665	Mescolini *et al*., 2019a
GaHV-2/Italy/Ck/625/16	Italy	2016	NA	339	4	MK139666	Mescolini *et al*., 2019a
GaHV-2/Italy/Ck/674/16	Italy	2016	NA	339	4	MK139667	Mescolini *et al*., 2019a
GaHV-2/Italy/Ck/689/16	Italy	2016	NA	339	4	MK139668	Mescolini *et al*., 2019a
GaHV-2/Italy/Ck/722/16	Italy	2016	NA	339	4	MK139669	Mescolini *et al*., 2019a
GaHV-2/Italy/Ck/801/17	Italy	2017	NA	339	4	MK139670	Mescolini *et al*., 2019a
GaHV-2/Italy/Ck/810/17	Italy	2017	NA	339	4	MK139671	Mescolini *et al*., 2019a
GaHV-2/Italy/Ck/847/17	Italy	2017	NA	418	10	MK139672	Mescolini *et al*., 2019a
GaHV-2/Italy/Ck/848/17	Italy	2017	NA	418	9	MK139673	Mescolini *et al*., 2019a
GaHV-2/Italy/Ck/850/17	Italy	2017	NA	339	5	MK139674	Mescolini *et al*., 2019a
GaHV-2/Italy/Ck/852/17	Italy	2017	NA	339	4	MK139675	Mescolini *et al*., 2019a
GaHV-2/Italy/Ck/853/17	Italy	2017	NA	339	4	MK139676	Mescolini *et al*., 2019a
GaHV-2/Italy/Ck/854/17	Italy	2017	NA	339	4	MK139677	Mescolini *et al*., 2019a
GaHV-2/Italy/Ck/855/17	Italy	2017	NA	298	2	MK139678	Mescolini *et al*., 2019a
GaHV-2/Italy/Ck/456/15	Italy	2015	NA	339	4	MK855054	Mescolini *et al*., 2019b

GaHV-2/Italy/Ck/498/15	Italy	2015	NA	339	4	MK855055	Mescolini *et al*., 2019b
GaHV-2/Italy/Ck/513/15	Italy	2015	NA	339	4	MK855056	Mescolini *et al*., 2019b
GaHV-2/Italy/Ck/515/15	Italy	2015	NA	339	4	MK855057	Mescolini *et al*., 2019b
GaHV-2/Italy/Ck/559/15	Italy	2015	NA	339	4	MK855058	Mescolini *et al*., 2019b
GaHV-2/Italy/Ck/561/15	Italy	2015	NA	339	4	MK855059	Mescolini *et al*., 2019b
GaHV-2/Italy/Ck/565/15	Italy	2015	NA	339	4	MK855060	Mescolini *et al*., 2019b
GaHV-2/Italy/Ck/567/15	Italy	2015	NA	339	4	MK855061	Mescolini *et al*., 2019b
GaHV-2/Italy/Ck/757/17	Italy	2017	NA	339	4	MK855062	Mescolini *et al*., 2019b
GaHV-2/Italy/Ck/875/18	Italy	2018	NA	339	4	MK855063	Mescolini *et al*., 2019b
GaHV-2/Italy/Ck/876/18	Italy	2018	NA	339	4	MK855064	Mescolini *et al*., 2019b
GaHV-2/Italy/Ck/921/18	Italy	2018	NA	339	4	MK855065	Mescolini *et al*., 2019b
GaHV-2/Italy/Ck/1083/18	Italy	2018	NA	339	4	MK855066	Mescolini *et al*., 2019b

[a] Attenuated
[b] Not available, the strain has not been subjected to the *in vivo* pathotyping test.
[c] Mild
[d] Virulent
[e] Very virulent
[f] Very virulent plus

Table 2. Molecular characteristics of the *meq* protein aa sequence of GaHV-2/Italy/Turkey/601/16 strain, compared to prototype strains. Amino acid substitutions interrupting PPPPs are underlined.

Strain	Meq length (aa)	PPPPs (n°)	Amino acid substitutions														
			71	77	80	110	119	153	176	180	216[a]	217	218	277	283	320	326
CVI988	398	7	S	E	D	C	C	P	P	T	P	P	P	L	A	I	I
CU-2	398	7	S	E	D	C	C	P	P	T	P	P	P	L	A	I	T
JM/102W	399	7	A	E	D	C	C	P	P	T	S	P	P	L	A	I	T
Md5	339	4	A	K	D	C	C	P	P	T	P	A	P	L	V	T	T
648A	339	2	A	K	D	C	R	Q	A	A	P	A	P	P	A	I	T
GaHV-2/Italy/Turkey/601/16	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T

[a] Amino acid position according to the 339 aa-long Meq isoform

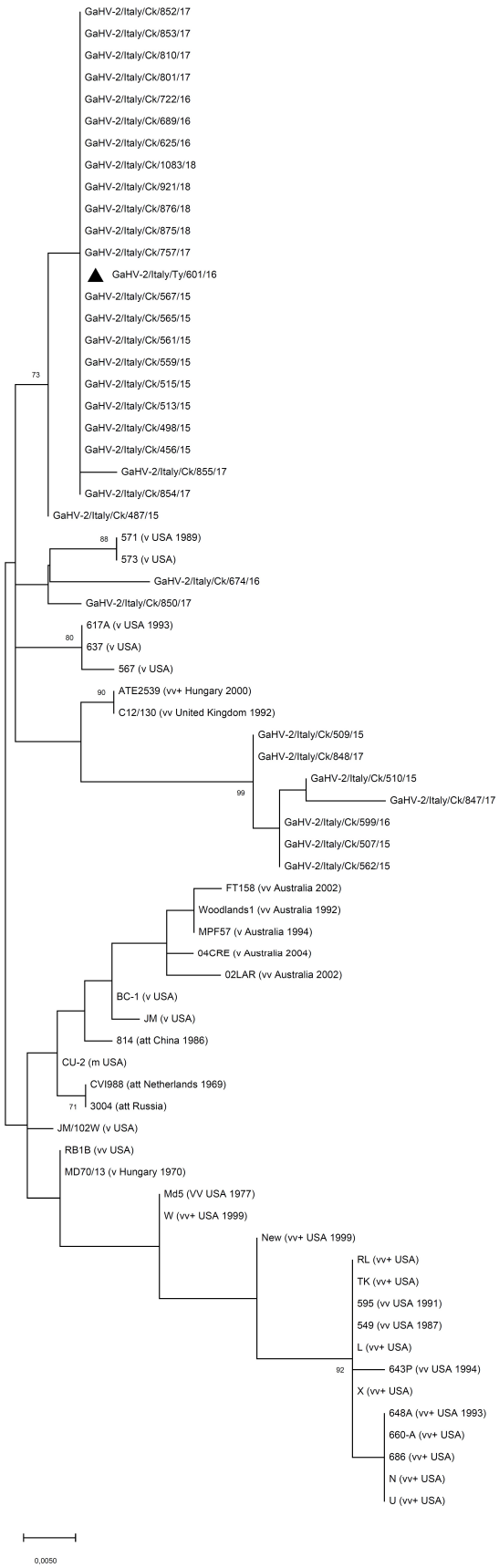

Figure 1. Phylogenetic tree based on *meq* aa sequences of GaHV-2/Italy/Turkey/601/16 (marked with a black triangle), reference GaHV-2 retrieved from GenBank, Italian GaHV-2 and three vaccine strains (CVI988, 814 and 3004).

References

Biggs, P.M. (1997). Lymphoproliferative disease of turkeys. In: B.W. Calnek, H.J. Barnes, C.W. Beard, L.R. McDougald, & Y.M. Saif (Eds.). *Diseases of Poultry* 9th edn (pp.485-488). Ames: Iowa State University Press.

Blake-Dyke, C. & Baigent, S. (2013). Marek's disease in commercial turkey flocks. *Veterinary Record*, 173, 376.

Busch, R.H. & Williams, L.E. Jr. (1970). A Marek's disease-like condition in Florida turkeys. *Avian Diseases*, 14, 550-554.

Coudert, F., Vuillaume, A., Wyers, M. & Legros, F.X. (1995). Une nouvelle pathologie chez la dinde-la maladie de Marek. In: *Proceedings of the leres Journées de la Recherche Avicole* (pp.164-166). Angers, France.

Davidson, I., Borowski, A., Perl, S. & Malkinson, M. (1995). Use of the polymerase chain reaction for the diagnosis of natural infection of chickens and turkeys with Marek's disease virus and reticuloendotheliosis virus. *Avian Pathology*, 24, 69-94.

Davidson, I., Malkinson, M. & Weisman, Y. (2002). Marek's disease in turkeys. I. A seven-year survey of commercial flocks and experimental infection using two field isolates. *Avian Diseases*, 46, 314-321.

Davidson, I. & Silva, R.F. (2008). Creation of diversity in the animal virus world by inter-species and intra-species recombinations: Lessons learned from poultry viruses. *Virus Genes*, 36, 1-9.

Deuchande, R., Murphy, A., Otter, A., Baigent, S., Wood, A. & Irvine, R. M. (2012). Marek's disease in turkeys. *Veterinary Record*, 171, 602.

Elmubarak, A.K., Sharma, J.M., Witter, R.L., Nazerian, K. & Sanger, V.L. (1981). Induction of lymphomas and tumor antigen by Marek's disease virus in turkeys. *Avian Diseases*, 25, 911-926.

Handberg, K.J., Nielsen, O.L. & Jørgensen, P.H. (2001). The use of serotype 1- and serotype 3-specific polymerase chain reaction for the detection of Marek's disease virus in chickens. *Avian Pathology*, 30, 243-249.

Hassanin, O., Abdallah, F. & El-Araby, I. E. (2013). Molecular characterization and phylogenetic analysis of Marek's disease virus from clinical cases of Marek's disease in Egypt. *Avian Diseases*, 57, 555-561.

Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, 35, 1547-1549.

Lee, S.-I., Takagi, M., Ohashi, K., Sugimoto, C. & Onuma, M. (2000). Difference in the meq Gene between Oncogenic and Attenuated Strains of Marek's Disease Virus Serotype 1. *Journal of Veterinary Medical Science*, 62, 287-292.

Mescolini, G., Lupini, C., Felice, V., Guerrini, A., Silveira, F., Cecchinato, M. & Catelli, E. (2019a). Molecular characterization of the meq gene of Marek's disease viruses detected in unvaccinated backyard chickens reveals the circulation of low- and high-virulence strains. *Poultry Science*, 98, 3130-3137.

Mescolini, G., Lupini, C., Davidson, I., Massi, P., Tosi, G. & Catelli, E. (2019b). Marek's disease viruses circulating in commercial poultry in Italy in the years 2015-2018 are closely related by their *meq* gene phylogeny. *Transboundary and Emerging Diseases,* resubmitted after minor revisions, waiting for acceptance.

Nair, V., Zavala, G. & Fadly, A.M. (2013). Reticuloendotheliosis. In: D.E. Swayne, J.R. Glisson, L.R. McDougald, L.K. Nolan, D.L. Suarez, & V. Nair (Eds.). *Diseases of Poultry* 13th edn (pp.593-604). Ames: Wiley-Blackwell Publishing.

Paul, P.S., Sautter, J.H. & Pomeroy, B.S. (1977). Susceptibility of turkeys to Georgia strain of Marek's disease virus of chicken origin. *American Journal of Veterinary Research*, 38, 1653-1656.

Pennycott, T.W. & Venugopal, K. (2002). Outbreak of Marek's disease in a flock of turkeys in Scotland. *Veterinary Record*, 150, 277-279.

Powell, P.C., Howes, K., Lawn, A.M., Mustill, B.M., Payne, L.N., Rennie, M. & Thompson, M.A. (1984). "Marek's disease": The induction of lesions and the establishment of lymphoid cell lines. *Avian Pathology*, 13, 201-214.

Renz, K.G., Cooke, J., Clarke, N., Cheetham, B.F., Hussain, Z., Fakhrul Islam, A.F.M., Tannock, G.A. & Walkden-Brown, S.W. (2012). Pathotyping of Australian isolates of Marek's disease virus and association of pathogenicity with meq gene polymorphism. *Avian Pathology*, 41, 161-176.

Schat, K.A. & Nair, V. (2013). Marek's disease. In: D.E. Swayne, J.R. Glisson, L.R. McDougald, L.K. Nolan, D.L. Suarez, & V. Nair (Eds.). *Diseases of Poultry* 13th edn (pp.515-552). Ames: Wiley-Blackwell Publishing.

Shamblin, C.E., Greene, N., Arumugaswami, V., Dienglewicz, R.L. & Parcells, M.S. (2004). Comparative analysis of Marek's disease virus (MDV) glycoprotein-, lytic antigen pp38- and transformation antigen Meq-encoding genes: Association of meq mutations with MDVs of high virulence. *Veterinary Microbiology*, 102, 147-167.

Spatz, S. J., Petherbridge, L., Zhao, Y. & Nair, V. (2007). Comparative full-length sequence analysis of oncogenic and vaccine (Rispens) strains of Marek's disease virus. *Journal of General Virology*, 88, 1080-1096.

Spatz, S.J. & Silva, R.F. (2007). Polymorphisms in the repeat long regions of oncogenic and attenuated pathotypes of Marek's disease virus 1. *Virus Genes*, 35, 41-53.

Spatz, S.J., Smith, L.P., Baigent, S.J., Petherbridge, L., & Nair, V. (2011). Genotypic characterization of two bacterial artificial chromosome clones derived from a single DNA source of the very virulent gallid herpesvirus-2 strain C12/130. *Journal of General Virology*, 92, 1500-1507.

Trimpert, J., Groenke, N., Jenckel, M., He, S., Kunec, D., Szpara, M.L., Spatz, S.J., Osterrieder, N. & McMahon, D.P. (2017). A phylogenomic analysis of Marek's disease virus reveals independent paths to virulence in Eurasia and North America. *Evolutionary Applications*, 10, 1091-1101.

Tulman, E.R., Afonso, C.L., Lu, Z., Zsak, L., Rock, D.L., & Kutish, G.F. (2000). The genome of a very virulent Marek's disease virus. *Journal of Virology*, 74, 7980-7988.

Voeckell, K., Bertram, E., Gimeno, I., Neumann, U. & Kaleta, E.F. (1999). Evidence for Marek's disease in turkey in Germany: detection of MDV-1 using the polymerase chain reaction. *Acta Virologica*, 43, 143-147.

Voute, A.J. & Wagenaar-Schaafsma, A.F. (1974). Een op de ziekte van Marek lijkende afwijking bij mestkalkoenen in Nederland. *Tijdschr. Diergeneesk*, 99, 166-169.

Witter, R.L. (1997). Increased virulence of Marek's disease virus field isolates. *Avian Diseases*, 41, 149-163.

Witter, R. L., Calnek, B. W., Buscaglia, C., Gimeno, I. M., & Schat, K. A. (2005). Classification of Marek's disease viruses according to pathotype: Philosophy and methodology. *Avian Pathology*, 34, 75-90.

Witter, R.L. & Solomon, J.J. (1971). Epidemiology of a herpesvirus of turkeys: possible sources and spread of infection in turkey flocks. *Infection and Immunity*, 4, 356-361.

Zhang, F., Liu, C.J., Zhang, Y.P., Li, Z.J., Liu, A.L., Yan, F.H., Cong, F. & Cheng, Y. (2012). Comparative full-length sequence analysis of Marek's disease virus vaccine strain 814. *Archives of Virology*, 157, 177-183.

Woźniakowski, G., Samorek-Salamonowicz, E., Kozdruń, W. (2010). Sequence analysis of meq oncogene among Polish strains of Marek's disease. *Polish Journal of Veterinary Sciences*, 13, 263-267.

Woźniakowski, G., & Samorek-Salamonowicz, E. (2014). Molecular Evolution of Marek's Disease Virus (MDV) Field Strains in a 40-Year Time Period. *Avian Diseases*, 58, 550–557.

IV. Rapid, sensitive and species-specific detection of conventional and recombinant herpesvirus of turkeys (HVT) vaccines using loop-mediated isothermal amplification coupled with a lateral flow device readout (LAMP-LFD) (preliminary paper)

NOTICE: this is a preliminary paper, not yet submitted for publication.

Rapid, sensitive and species-specific detection of conventional and recombinant herpesvirus of turkeys (HVT) vaccines using loop-mediated isothermal amplification coupled with a lateral flow device readout (LAMP-LFD)

Giulia Mescolini[1], Susan J. Baigent[2], Elena Catelli[1], Venugopal K. Nair[2]

1 Department of Veterinary Medical Sciences, University of Bologna, Ozzano dell'Emilia (BO), Italy

2 Viral Oncogenesis Group, The Pirbright Institute, Pirbright, Ash Road, Guildford, Surrey, GU24 0NF, United Kingdom

Abstract

Marek's disease, an economically important disease of chickens caused by virulent serotype 1 strains of the *Mardivirus* Marek's disease virus (MDV-1), is effectively controlled in the field by live attenuated vaccine viruses including herpesvirus of turkeys (HVT) – both conventional HVT (strain Fc126) and, in recent years, recombinant HVT viruses carrying foreign genes from other avian viruses to protect against both Marek's disease and other avian viral diseases. Testing to monitor and confirm successful vaccination is important, but any such test must differentiate HVT from MDV-1 and MDV-2, as vaccination does not prevent infection with these serotypes. Endpoint and real-time PCR tests are widely used to detect and differentiate HVT, MDV-1 and MDV-2 but require expensive specialist laboratory equipment and trained operators. Here, we developed and validated two tube-based loop-mediated isothermal amplification tests coupled with detection by

lateral flow device readout (LAMP-LFD): a 'global HVT' test to detect both conventional and recombinant HVT strains, and a second test using novel LAMP primers to specifically detect the Vaxxitek recombinant HVT. Specificity was confirmed using DNA extracted from virus-infected cultured cells, and limit of detection was determined using plasmid DNA carrying either the HVT or Vaxxitek genome. The LAMP-LFD tests accurately detected global HVT or Vaxxitek in crude DNA as well as purified DNA extracted from field samples of organs, feathers, or poultry house dust that were confirmed positive for HVT by real-time PCR. These LAMP-LFD tests have potential for specific, rapid, simple, and inexpensive detection of HVT vaccines in the field.

Introduction

Meleagrid alphaherpesvirus 1, the herpesvirus of turkeys (HVT), is a member of the genus *Mardivirus* of the *Alphaherpesvirinae* subfamily (Gatherer et al., 2021), together with *Gallid alphaherpesvirus 2*, traditionally referred to as Marek's disease virus serotype 1 (MDV-1), the aetiological agent of Marek's disease (MD), and with *Gallid alphaherpesvirus 3*, or Marek's disease virus serotype 2 (MDV-2). HVT was isolated for the first time in 1968 from healthy turkeys by two different research groups (Kawamura et al., 1969; Schat, 2016) and was shown to be apathogenic for chickens, and antigenically related to MDV-1 offering good protection against MD (Witter et al., 1970; Okazaki et al., 1970). For these reasons HVT has been successfully used as a vaccine against MD in chickens worldwide since it was first licensed in 1971 in the United States (reviewed by Schat, 2016) alone or in combination with MD vaccines of other serotypes (i.e. attenuated serotype 1 CVI988/Rispens strain or serotype 2 naturally apathogenic strain SB-1) (Witter, 1984; Gimeno et al., 2012). Conventional HVT vaccines, namely the FC126 strain, have been used to successfully protect chickens from MD since the early 1970s (Purchase et al., 1971). Several recombinant vaccines using HVT as a vector (rHVT) to express heterologous immunogenic proteins of chicken viruses causing major diseases such as Newcastle disease (Morgan et al.,1992; Heckert et al., 1996), infectious bursal disease (Darteil et al., 1995), and infectious laryngotracheitis

(Johnson et al., 2010) have been developed since the 1990s, and are used worldwide in the control of MD and of the abovementioned poultry diseases. The Vaxxitek® range of vaccines (Boehringer Ingelheim) and Innovax® range of vaccines (MSD Animal Health) use HVT as a vector to express single genes from other pathogenic avian viruses: infectious bursal disease virus (IBDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), and Newcastle disease virus (NDV), or combinations thereof. The Vaxxitek® range includes VAXXITEK® HVT+IBD, VAXXITEK® HVT+IBD+ND, and VAXXITEK® HVT+IBD+ILT. The Innovax® range includes INNOVAX® -ND-ILT, INNOVAX® -ND-IBD, INNOVAX® -ND, and INNOVAX® -ILT.

The efficacy of HVT vaccination has decreased over time due to increased virulence of MDV-1 strains (Witter, 1997). Nowadays the use of HVT vaccine alone is restricted to the vaccination of broilers; long-living birds such as breeders and layers, on the other hand, are usually vaccinated with a bivalent HVT and CVI988/Rispens vaccine (Gimeno, 2008). Cell-free lyophilized HVT vaccine, which is cheaper and easier to handle than cell-associated formulations where liquid nitrogen is needed for storage, is frequently adopted for the protection of valuable ornamental chicken flocks with a history of MD, and also some backyard chicken flocks (Mescolini et al., 2019).

Both conventional and recombinant HVT vaccines are live vaccines that actively replicate within the host mimicking natural infection and eliciting a protective immune response. HVT vaccinal viruses replicate in the feather follicle epithelium and are persistently shed into the environment through physiological desquamation of epithelial cells (Rémy et al., 2020). Thus, feather tips taken from vaccinated birds represent a non-invasive sample to confirm HVT vaccine administration and uptake for monitoring success of HVT-based MD vaccination in the field (Cortes et al., 2011). Furthermore, HVT genome can be detected in dust collected from the poultry house environment (Islam et al., 2006; Fakhrul Islam et al., 2008; Islam and Walkden-Brown, 2007), often in combination with MDV-1 and MDV-2 (Walkden-Brown et al., 2013).

MD vaccines are referred to as “imperfect” or “leaky”, as they prevent clinical MD but do not impede the infection, replication, and shedding of wild-type MDV-1 in the environment (Islam & Walkden-Brown, 2007; Fakhrul Islam et al., 2008; Islam et al., 2014; Read et al., 2015; Ralapanawe et al., 2016). Thus, vaccinal and field viruses can coexist in the vaccinated host (López-Osorio et al., 2019) and, in case of mixed infection, molecular tests able to discriminate between MDV-1, MDV-2 and HVT are required.

The full-length genome sequences of the three viral species included in the genus *Mardivirus* are publicly available in online databases (Afonso et al., 2001; Spatz et al., 2007a; Spatz et al., 2007b; Spatz et al., 2011; Trimpert et al., 2017; Kim et al., 2020) and many species-specific molecular methods that allow for their differential detection have been developed over time.

Such molecular methods include end-point and real-time PCR assays (Handberg et al., 2001; Walkden-Brown et al., 2003; Islam et al., 2006; Renz et al., 2006; Cortes et al., 2011; López-Osorio et al., 2019) and have one or more of the following drawbacks: are labour intensive, require time-consuming post-PCR handling such as gel electrophoresis to visualise the outcome, need expensive specialised equipment, and need to be performed by highly trained personnel. A simple, fast, and accurate test for monitoring of vaccination success in the field could be greatly beneficial for field veterinarians and small laboratories.

Loop-mediated isothermal amplification (LAMP) first described by Notomi et al. (2000) and improved by Nagamine et al. (2002) is a rapid, extremely specific, and sensitive molecular method that could overcome most of the drawbacks of PCR-based methods. The outstanding specificity is obtained using six primers (two outer primers, two inner primers and two loop primers) that specifically recognize eight different regions in the target genome. A DNA polymerase with strand displacement activity, working under isothermal conditions (temperature between 60°C and 65°C) combined with the suitably designed primers, enables, starting from the target DNA sequence, the formation of a stem-loop DNA structure, which is the starting point for exponential amplification of the target DNA.

LAMP-based assays for the specific detection of HVT, MDV-1 or MDV-2 genomes have been developed in the past (Woźniakowski et al., 2011; Woźniakowski et al., 2013; Angamuthu et al., 2012; Wei et al., 2012; Wozniakowski & Samorek-Salamonowicz, 2014; Wozniakowski & Niczyporuk, 2015; Adedeji et al., 2017). In all the above-mentioned methods the detection of LAMP products was achieved by sequence-independent methods, such as the utilization of agarose gel electrophoresis or intercalating fluorescent dyes. Sequence-specific detection methods, that enable the exact identification of specific amplicons without being affected by unspecific products (Becherer et al. 2020), are available and, between them, the use of immunochromatographic lateral flow devices (LFDs) is one of the most often used. LFDs are designed to specifically detect dual labelled LAMP DNA amplicons that are captured on a lateral flow test strip, allowing their rapid and direct visualisation. Lateral flow tests are low cost, easy to handle, do not require additional equipment and give an unequivocal positive or negative result that can be interpreted by non-specialist personnel.

Here, we describe the modification of a previously reported HVT-specific LAMP assay (Wozniakowski et al., 2011), to allow detection of dual labelled LAMP products with commercially available LFDs.

In addition, a novel LAMP assay able to specifically detect the recombinant HVT vaccine Vaxxitek® was developed and validated.

Finally, crude DNA extracted from samples of chicken organs, feathers and poultry house dust subjected to a heat treatment, bypassing the extraction of genomic DNA with commercial extraction kits, was shown to be suitable for serotype-specific virus detection in HVT LAMP-LFD assays.

Material and methods

DNA samples from virus stocks and field samples

DNA stocks of HVT strain FC126, MDV-2 strain SB-1, very virulent MDV-1 strain RB-1B, and attenuated MDV-1 vaccine strain CVI988/Rispens of known provenance prepared from virus-

infected chicken embryo fibroblast cells (CEF) (Baigent et al., 2016), and DNA from commercial stocks of rHVT vaccine Vaxxitek® HVT+IBD (Boehringer Ingelheim), and rHVT vaccine Innovax® HVT+IBD (MSD Animal Health), were already available.

DNA stocks from field samples of chicken feathers, organs, tumours, and poultry house dust, submitted to the Marek's Disease Virus Reference Laboratory (MDVRL) of the Pirbright Institute were also available. These included samples taken from vaccinated chickens and from chickens diagnosed with MD, and from both commercial flocks and backyard flocks. These field samples had already been tested by serotype-specific MDVRL real-time PCR assays to determine CT values for HVT, MDV-2 (López-Osorio et al., 2019) CVI988/Rispens, and virulent MDV-1 (Baigent et al., 2016).

DNA from virus BAC clones

Bacterial-artificial-chromosome (BAC) clones, stable infectious clones of the whole virus genome, generated in the Viral Oncogenesis Group of The Pirbright Institute, were available for HVT FC126 (Baigent et al., 2006) and Vaxxitek® (unpublished). These BAC stocks were named pHVT-BAC3, and pVaxxitek-BAC, respectively. The number of viral genome copies in BAC DNA can be easily quantified by determining the mean DNA concentration by spectrophotometry and, subsequently, the number of molecules per µl. Therefore, 10-fold serial dilutions of these BAC DNA stocks (10^0 - 10^6 virus genome copies/3 µl), were used to determine the limit of detection (LoD) of each assay.

Design of LAMP primers

Primers for the HVT specific assay were those previously designed and published by Wozniakowski et al. (2013) to target eight distinct regions of the HVT070 gene according to the sequence of HVT strain FC126. This gene is conserved between all published HVT sequences available in the GenBank database, and between the two rHVT vaccine strains available. Basic Local Alignment Search Tool (BLAST) search confirmed the specificity of the six primers for the HVT genome.

Primers for the rHVT (Vaxxitek® HVT + IBD)-specific assay were designed using Primer Explorer V5 online software (Eiken Chemical Co. LTD, Tokyo, Japan) with manual adjustments to sequences to improve specificity or sensitivity of the method. The cloning vector sequence and insertion sites of the foreign genes differ between Vaxxitek® and Innovax®, so these vaccine types can be distinguished on sequence. Vaxxitek® HVT + IBD expresses the VP2 gene of infectious bursal disease virus (IBDV) which is inserted at a specific site in the HVT genome. A new set of LAMP primers was designed to target a distinctive genomic region encompassing the HVT065 gene and intergenic region plus the cloning vector sequence of Vaxxitek®. The sequence necessary for the primer design was available from previous studies conducted by the Viral Oncogenesis Group of The Pirbright Institute. Each primer was evaluated for GC content, secondary structures, and 3' or 5' end stability. Primer specificity was verified in silico by BLAST analysis both for the HVT genome and for the inserted cloning vector sequence. LAMP primers, both unlabelled and 5'-labelled with Biotin (5'-Biosg) or 6-Carboxyfluorescein (5'-6-FAM), were manufactured by Integrated DNA Technologies, Inc. (Leuven, Belgium). The sequences for each specific set of primers are given in Table 1. Each primer set consisted of six primers: two outer primers (F3 and B3), two unlabelled or 5'-labelled inner primers (FIP and BIP), and two loop primers (LF and LB).

Real-time LAMP assays and tube LAMP assays with LFD readout (LAMP-LFD assay).

Primer sets were tested in LAMP assays with two different types of result readout, detailed in the following sections. Initially, primers were tested in real-time LAMP as a rapid way to check primer specificity and sensitivity using unlabelled, therefore non-expensive, LAMP primers. Subsequently, 5' labelled primers were used in LAMP-LFD using in-tube amplification followed by result readout on inexpensive housed lateral flow test strips.

The real-time LAMP reactions were set up in 96-well PCR plates. Each reaction (total volume: 25 µl) contained the six specific LAMP primers for the virus to be detected (1 µl of 5 µM outer primers, 1 µl of 50 µM inner primers and 1 µl of 25 µM loop primers), 15 µl of GspSSD2.0 Isothermal Mastermix (ISO-004) (OptiGene Limited, Horsham, West Sussex, UK), 4 µl of water

and 3 µl of template DNA. An ABI 7500FAST® Real-Time PCR system (Applied Biosystems, Waltham, Massachusetts, USA) was used to amplify and detect the reaction products, under the following thermal cycling conditions: 30 cycles for 1 min at 65ºC. The master mix contained a ds-DNA binding dye read by the machine through the SYBR green/FAM detection channel allowing the generation of amplification plots used to identify positive samples. Melt curve analysis was performed at 98°C (15 s), 80°C (1 min), 98°C (1 min), 98°C (30 s) and 80°C (15 s), to confirm reaction specificity for positive samples. ABI 7500 v2.3 software was used to analyse the results.

Once the assays in real-time LAMP were validated, 5'-labelled FIP and BIP primers were ordered for each set of virus-specific LAMP primers. FIP primers were labelled with 6-FAM and BIP primers were labelled with Biosg (Table 1). The 'three-stripe LFD strips' used (Abingdon Health, York, UK) have three lines: Test line 1 (T1), Test line 2 (T2), and Run Control line (C). T1 contains antibodies that specifically bind 6-FAM and Biosg to give a chromogenic product to detect 6-FAM/Biosg-labelled amplicons; T2 contains antibodies that specifically bind Digoxigenin (DigN)/Biosg to give a chromogenic product to detect DigN/Biosg-labelled amplicons; C confirms successful running of the reaction solution through the LFD strip. Only two out of three stripes, T1 (marked with a "T" on the plastic housing cassette) and C line, were used in our LAMP-LFD assays. LAMP reactions were run in individual tubes. Each reaction (total volume of 25 µl) contained the six specific LAMP primers for the virus to be detected (1 µl of 5 µM outer primers, 1 µl of 50 µM 5' labelled inner primers and 1 µl of 25 µM loop primers), 15 µl of GspSSD2.0 Isothermal Mastermix (ISO-004), 4 µl of water and 3 µl template DNA. Reactions were run by placing the tubes in a heating block at 65ºC for 30 min. LFD strips were assembled into the plastic housing cassettes. Reaction tubes were only opened in a laminar flow cabinet in a designated laboratory, to avoid the risk of laboratory contamination with LAMP amplicons. The whole volume of LAMP reaction (25 µl) was mixed with 100 µl running buffer and added to the sample application well of the plastic housing cassette. Results were read after 10 minutes of incubation at ambient temperature.

Sensitivity and specificity of the LAMP assays

The sensitivity of the LAMP assays was expressed as LoD and defined as the lowest analyte amount in a sample that can be detected by the assay (Real-time LAMP or LAMP-LFD) in at least 50% of the replicates. LoD was expressed as absolute copy number.

To determine the specificity of the assay, MDV-1 (CVI988/Rispens and RB-1B), MDV-2 (SB-1), HVT (FC126) and rHVT (Vaxxitek® and Innovax®) strains were used as templates for the real-time LAMP and LAMP-LFD assays.

Crude DNA

Crude DNAs were extracted from samples of chicken organs, feather tips and poultry house dust. The samples were weighed and prepared as 4% (organs and dust) or 8% (feather tips) w/v suspensions in sterile PBS. The suspensions were then vortexed and subjected to a heat treatment in a heating block at 95°C for 10 min, centrifuged at 1000 xg for 3 min and the supernatant was tested in LAMP-LFD assay as a crude extract.

Results

HVT-specific LAMP assay

Using target DNA from virus-infected CEF cells, the HVT LAMP primer set previously published by Wozniakowski et al. (2013), targeting the HVT070 gene of HVT, was confirmed to be specific for the amplification of conventional HVT vaccine (strain FC126) and the recombinant HVT vaccines Vaxxitek® and Innovax® in real-time LAMP (Figure 1) and in LAMP-LFD (Figure 2). Negative results were obtained using DNA from MDV-2 strain SB-1, MDV-1 vaccine strain CVI988/Rispens, and very virulent MDV-1 strain RB1B.

The limits of detection (LoD), determined using serial dilutions of pHVT-BAC3 DNA, are shown in Table 2, and the determination of LAMP-LFD LoD is shown in Figure 3.

Field samples, all shown to be positive for HVT using MDVRL HVT real-time PCR assay (CT values ranging from 24 to 38), were all positive in the HVT LAMP-LFD test (Figure 4).

Vaxxitek®-specific LAMP assay

Using target DNA from virus-infected CEF cells, the assay detected only Vaxxitek® (not conventional HVT vaccine or Innovax®) in real-time LAMP (Figure 5) and LAMP-LFD (Figure 6). LoD values, determined using serial dilutions of pVaxxitek-BAC DNA, are shown in Table 3, and the determination of LAMP-LFD LoD is shown in Figure 7.

Crude DNA

Crude DNA prepared from different matrixes (tissues, feathers, and poultry house dust) was used as template in HVT-specific LAMP-LFD assay. HVT DNA was successfully amplified and detected through LFD readout (results not shown).

Discussion

The present study reports the development of two LAMP-LFD assays for the rapid, sensitive, and species-specific detection of conventional and recombinant HVT-based vaccines, the most commonly used vaccines worldwide to prevent and control MD in commercial poultry flocks (Gimeno et al., 2016; Dunn et al., 2019), expanding the diagnostic capabilities, especially in resource-limited settings. The LAMP-LFD technique has proved to be a valuable alternative to the more complex, expensive, and time-consuming PCR-based molecular methods allowing to achieve reliable results in less than 60 min.

Prior to developing the LAMP-LFD assay, the primer sets were tested in real-time LAMP with fluorescent detection of the LAMP amplicons through the SYBR green fluorescence acquisition channel of the ABI 7500FAST® system. The dsDNA-binding dye included in the master mix used intercalates non-specifically into dsDNA, making this method of detection of LAMP products non-sequence specific. For this very reason, post-amplification melting-curve analysis was performed to check real-time LAMP reactions for primer-dimer artifacts and to ensure reaction specificity. The perfected real-time LAMP assays were then transposed in LAMP-LFD. Methods for sequence-specific detection, such as LAMP assays coupled with LFD readout, have gained increasing

importance in the last few years, because, unlike sequence-independent detection methods used in the previously developed LAMP assays for HVT (Wozinakowski et al., 2011; Adedeji et al., 2017), LAMP-LFD assays are highly specific towards the target DNA (Becherer et al., 2020).

The HVT-specific LAMP-LFD assay was proven to be specific for HVT detection alone and did not cross-react with the other two member species of interest included in the Mardivirus genus: MDV-1 and MDV-2. The LAMP primer set used in this assay was previously published by Wozniakowski et al. (2013) and further tested by Adedeji et al. (2017) which demonstrated that LAMP was a successful alternative to end-point PCR for the detection of HVT in vaccinated and unvaccinated poultry having much higher sensitivity compared to the end-point PCR assays. This study revealed that the newly developed HVT-specific LAMP-LFD assay was 10-fold less sensitive than the MDVRL real-time PCR assay, which detects the HVT sORF1 gene (López-Osorio et al., 2019). Despite this, all the tested samples (tissues, feathers, and environmental dust) resulted to be positive for HVT in LAMP-LFD regardless of the Ct value obtained for HVT in real-time PCR proving the reliability of the LAMP-LFD assay.

The results of the Vaxxitek®-specific LAMP-LFD assay confirmed that the assay was specific for VAXXITEK® HVT-IBD detection alone and did not detect MDV-1, MDV-2, and, more importantly, conventional HVT vaccines or other rHVT vaccines (e.g Innovax®). The expression cassettes with foreign genes encoding immunogenic viral proteins inserted in the HVT genome and their sequences differ between recombinant vaccines produced by different pharmaceutical companies and are not present in conventional HVT vaccine strains ensuring the differentiation of the different vaccine strains based on their sequence (Ingrao et al., 2017; Hein et al., 2021). The three-in-one vaccines recently added to the Vaxxitek® range (VAXXITEK® HVT+IBD+ND, and VAXXITEK® HVT+IBD+ILT) use the same bioengineering platform as VAXXITEK® HVT+IBD, so we predict that our Vaxxitek®-specific LAMP-LFD assay will detect all Vaxxitek® vaccines.

The LAMP-LFD assay for the detection of Vaxxitek® can reliably detect as few as 100 copies of

pVaxxitek-BAC DNA per reaction. Unfortunately, no Vaxxitek®-specific real-time PCR assay was available for comparison of analytical sensitivity.

LAMP-LFD was found to be an effective, sensitive and 100% specific technique for HVT detection even in field samples harbouring mixed Mardivirus infections, that are very common in the field. In fact, multiple MD vaccines of different serotypes are frequently administered in combination to achieve optimal protection against MD and, furthermore, these imperfect vaccines are unable to prevent superinfections with field MDV strains (Islam & Walkden-Brown, 2007; Fakhrul Islam et al., 2008; Islam et al., 2014; Read et al., 2015; Ralapanawe et al., 2016; López-Osorio et al., 2019). Therefore, the absolute specificity of these HVT LAMP-LFD assays is crucial for their effective application in the field.

It has been previously shown that LAMP amplification tolerates higher levels of inhibitors present in biological samples than PCR (Curtis et al., 2008; Francois et al., 2011; Kiddle et al., 2012). For poultry samples, these inhibitors include melanin pigment in feathers from coloured birds, and particles of dried litter, faeces, and feed in poultry house dust. The HVT-specific LAMP-LFD assay developed in this study efficiently amplified and detected DNA from crude tissue, feather, and dust samples processed by direct heating obtaining the same molecular results as compared to standard DNA extraction of the same samples and showing robustness to sample-derived inhibitors. This treatment of field samples allows to further reduce the overall procedure time by eliminating the need for nucleic acid extraction with commercial kits and demonstrating LAMP-LFD suitability for field use.

In summary, these novel HVT LAMP-LFD assays are simple, cost-effective, specific and sensitive alternatives to PCR-based methods for the rapid and reliable detection of HVT from chicken tissues and feathers and from poultry dust.

To our knowledge, this is the first time that LAMP technology coupled with LFD readout has been used for the rapid detection of HVT and Vaxxitek®. These new rapid and easy-to-read assays could

be used for monitoring Marek's disease vaccination directly in the field or in small laboratories with few resources.

Further research will be aimed to develop new LAMP-LFD assays to detect the remaining HVT recombinant vaccines and to ultimately determine the performance of the HVT LAMP-LFD assays in analysing field samples obtained from poultry flocks vaccinated with different vaccines and vaccination protocols.

Acknowledgements

This research was funded by a ISCG Agri-food Technology Seeding Catalyst Award (grant reference BB/SCA/Pirbright/17). We thank Dr. Claudio Busato and Dr. Mar Fernandez de Marco (Business Development Unit, Animal and Plant Health Agency, UK), Dr. Elen Withycombe (Abingdon Health, UK) and Dr. Donald King and Dr. Emma Howson (Vesicular Disease Reference Laboratories, The Pirbright Institute) for their guidance, advice and troubleshooting on LAMP-LFD assays. We also thank Dr. Yaoyao Zhang and Mr. Weicheng Li (Viral Oncogenesis Group, The Pirbright Institute) for provision of DNA samples, and laboratory assistance.

Table 1. LAMP primer sets used in this study.

Target	Primer name	Primer sequence and label	Reference
HVT HVT070 gene	HVT-F3	5'-ATAAATTATATCGCTAGGACAGAC-3'	Wozniakowski et al., 2013
	HVT-B3	5'-ACGATGTGCTGTCGTCTA-3'	
	HVT-FIP	5'-6-FAM-CCAGGGTATGCATATTCCATAACA GTTTTCCAAACGACCTTTATCCCA-3'	
	HVT-BIP	5'-Biosg-CCAGAAATTGCACGCACGAGTTTT AGAATTTGTGCATTTAGCCTT-3'	
	HVT-LF	5'-TTGAGAAGAGGATCTGACTG-3'	
	HVT-LB	5'-GCGTCATTGGTTTTACATTT-3'	
Vaxxitek HVT065 gene and intergenic region + cloning vector	Vaxxitek-F3	5'-CCGAACAAACTTCATCGCTA-3'	This study
	Vaxxitek-B3	5'-GCTATTGCTTTATTTGTAACCAT-3'	
	Vaxxitek-FIP	5'-6-FAM-CCCAAAGACCTCTATGAACATTTAT TTTTGCAAAGAGATGCGTGTG-3'	
	Vaxxitek-BIP	5'-Biosg-TGTCGACTCTAGAGGATCCGAAAATT TTGTTAACAACAACAATTGCATTCA-3'	
	Vaxxitek-LF	5'-TACTCAACGGCGCGTGTA-3'	
	Vaxxitek-LB	5'-CACACCTCCCCCTGAACCTG-3'	

Table 2. HVT LAMP assay sensitivities compared with MDVRL real-time PCR sensitivity.

Assay	LoD (number of virus genomes)		
	Real-time LAMP	LAMP-LFD	MDVRL real-time PCR
HVT (HVT070 gene)	100	100	10 (HVT gene sORF1)

Table 3. Vaxxitek®-specific LAMP assay sensitivities.

Assay	LoD (number of virus genomes)		
	Real-time LAMP	LAMP-LFD	MDVRL real-time PCR
HVT Vaxxitek®	100	100	No assay available

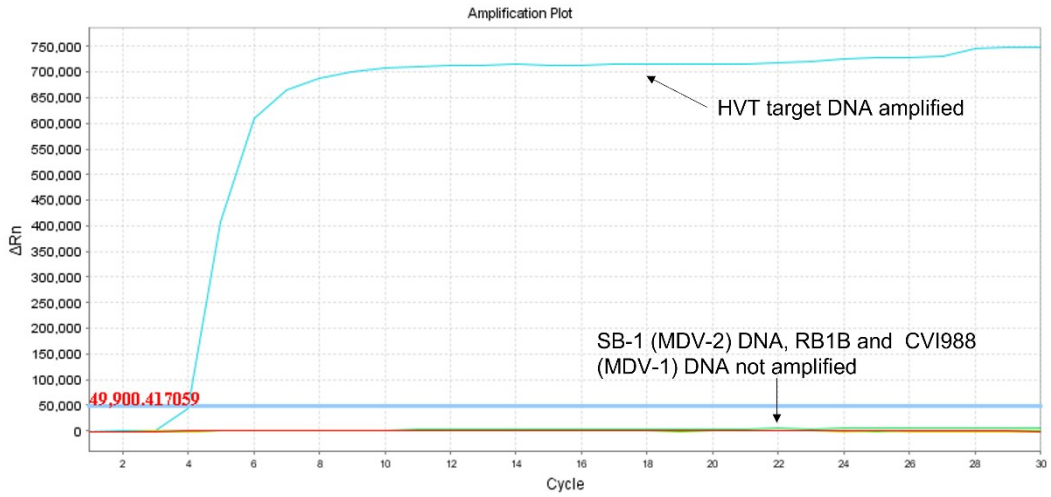

Figure 1. Specificity of HVT LAMP assay in real-time LAMP (amplification plot of fluorescence change vs cycle number).

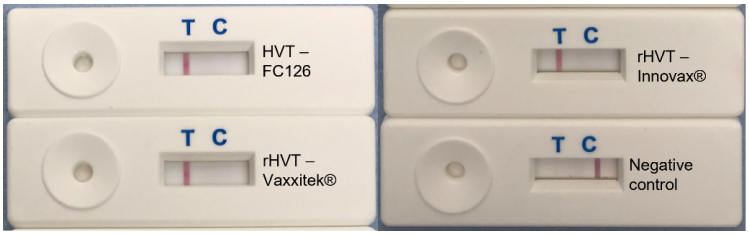

Figure 2. HVT-specific LAMP-LFD assay detects conventional HVT Fc126, and the rHVT vaccines Vaxxitek® and Innovax®.

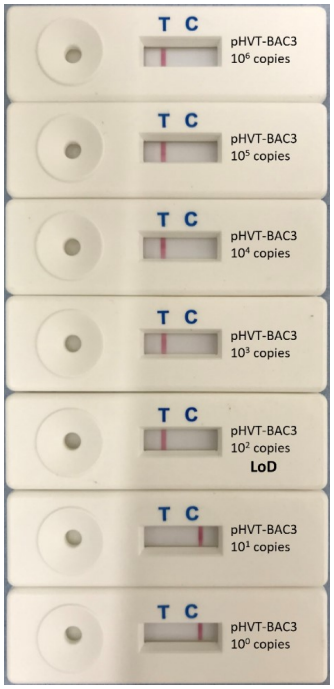

Figure 3: Limit of detection of HVT LAMP-LFD assay. Positive test line (T) shows detection of 6-FAM/Biosg -labelled LAMP amplicons. Replicates not shown.

Figure 4: HVT LAMP-LFD assay results: all the tested field sampled resulted positive for HVT (presence of the T line).

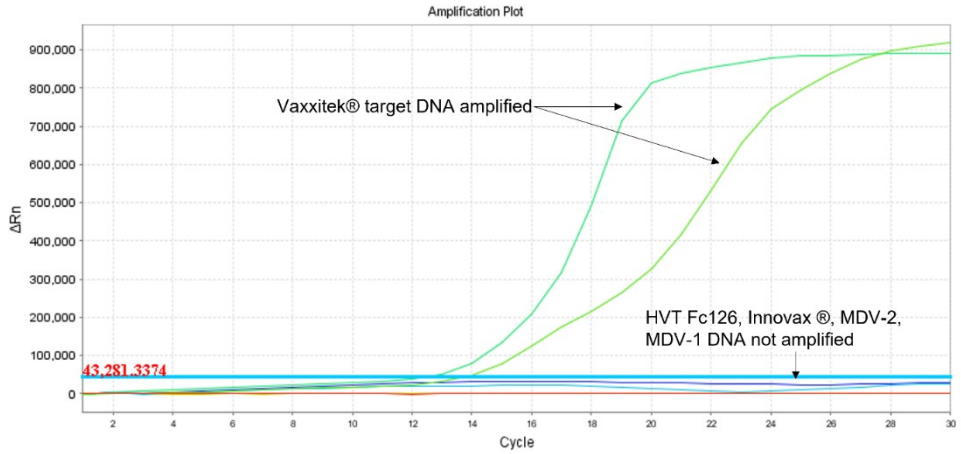

Figure 5. Specificity of Vaxxitek®-specific LAMP assay in real-time LAMP (amplification plot of fluorescence change vs cycle number).

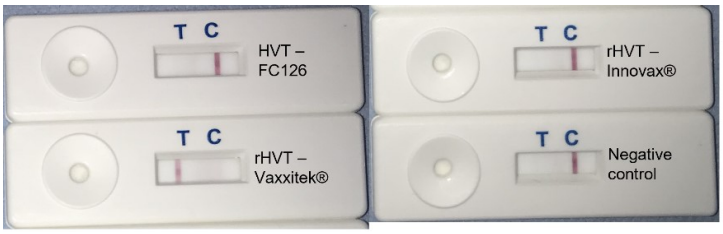

Figure 6. HVT-specific LAMP-LFD assay detects Vaxxitek® but not conventional HVT vaccine strain FC126 or rHVT vaccine Innovax®.

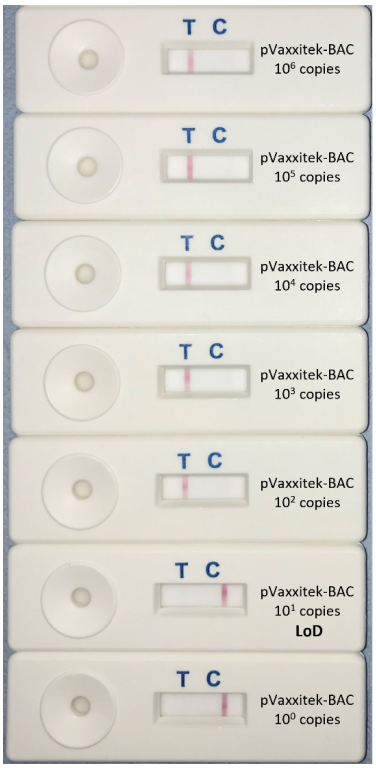

Figure 7: Limit of detection of Vaxxitek®-specific LAMP-LFD assay. Positive test line (T) shows detection of 6-FAM/Biosg-labelled LAMP amplicons. Replicates not shown.

References

Adedeji, A. J., Abdu, P. A., Luka, P. D., Owoade, A. A., & Joannis, T. M. (2017). Application of loop-mediated isothermal amplification assay in the detection of herpesvirus of turkey (FC 126 strain) from chicken samples in Nigeria. *Veterinary World*, 10(11), 1383–1388. https://doi.org/10.14202/VETWORLD.2017.1383-1388

Afonso C.L., Tulman E.R., Lu Z., Zsak L., Rock D.L., & Kutish G.F. (2001). The genome of turkey herpesvirus. *Journal of Virology*, 75(2), 971-9788. https://doi.org/10.1128/JVI.75.2.971-978.2001.

Angamuthu, R., Baskaran, S., Gopal, D. R., Devarajan, J., & Kathaperumal, K. (2012). Rapid detection of the Marek's disease viral genome in chicken feathers by loop-mediated isothermal amplification. *Journal of Clinical Microbiology*, 50(3), 961–965. https://doi.org/10.1128/JCM.05408-11

Baigent S.J., Petherbridge L.J., Smith L.P., Zhao Y., Chesters P.M., & Nair V.K. (2006). Herpesvirus of turkey reconstituted from bacterial artificial chromosome clones induces protection against Marek's disease. *Journal of General Virology*, 87(Pt 4), 769-776. https://doi.org/10.1099/vir.0.81498-0.

Baigent, S. J., Nair, V. K., & Le Galludec, H. (2016). Real-time PCR for differential quantification of CVI988 vaccine virus and virulent strains of Marek's disease virus. *Journal of Virological Methods*, 233, 23–36. https://doi.org/10.1016/j.jviromet.2016.03.002

Becherer, L., Borst, N., Bakheit, M., Frischmann, S., Zengerle, R., & Von Stetten, F. (2020). Loop-mediated isothermal amplification (LAMP)-review and classification of methods for sequence-specific detection. *Analytical Methods*, 12, 717–746. https://doi.org/10.1039/c9ay02246e

Cortes, A. L., Montiel, E. R., Lemiere, S., & Gimeno, I. M. (2011). Comparison of blood and feather pulp samples for the diagnosis of Marek's disease and for monitoring Marek's disease vaccination by real time-PCR. *Avian Diseases*, 55(2), 302–310. https://doi.org/10.1637/9578-101510-RESNOTE.1

Curtis K.A., Rudolph D.L., & Owen S.M. (2008). Rapid detection of HIV-1 by reverse-transcription, loop-mediated isothermal amplification (RT-LAMP). Journal of Virological Methods, 151(2), 264-270.

Darteil, R., Bublot, M., Laplace, E., Bouquet, J. F., Audonnet, J. C., & Riviè, M. (1995). Herpesvirus of turkey recombinant viruses expressing infectious bursal disease virus (IBDV) VP2 immunogen induce protection against an IBDV virulent challenge in chickens. *Virology*, 211(2), 481–490. https://doi.org/10.1006/VIRO.1995.1430

Dunn J.R., Dimitrov K.M., Miller P.J., Garcia M., Turner-Alston K., Brown A., & Hartman A. (2019). Evaluation of Protective Efficacy When Combining Turkey Herpesvirus-Vector Vaccines. *Avian Diseases*, 63(1), 75-83. https://doi.org/10.1637/11979-092818-Reg.1.

Francois P., Tangomo M., Hibbs J., Bonetti E.J., Boehme C.C., Notomi T., Perkins M.D., & Schrenzel J. (2011). Robustness of a loop-mediated isothermal amplification reaction for diagnostic applications. *FEMS Immunology and Medical Microbiology*, 62(1):41-48. https://doi.org/10.1111/j.1574-695X.2011.00785.x.

Fakhrul Islam, A. F. M., Walkden-Brown, S. W., Groves, P. J., & Underwood, G. J. (2008). Kinetics of Marek's disease virus (MDV) infection in broiler chickens 1: Effect of varying vaccination to challenge interval on vaccinal protection and load of MDV and herpesvirus of turkey in the spleen and feather dander over time. *Avian Pathology*, 37(3), 225–235. https://doi.org/10.1080/03079450701802230

Gatherer, D., Depledge, D. P., Hartley, C. A., Szpara, M. L., Vaz, P. K., Benkő, M., Brandt C.R., Bryant N.A., Dastjerdi A., Doszpoly A., Gompels U.A., Inoue N., Jarosinski K.W., Kaul R., Lacoste V., Norberg P., Origgi F.C., Orton R.J., Pellett P.E., Schmid D.S., Spatz S.J., Stewart J.P., Trimpert J., Waltzek T.B., & Davison A.J. (2021). ICTV virus taxonomy profile: Herpesviridae 2021. *Journal of General Virology*, 102(10), 1–2. https://doi.org/10.1099/JGV.0.001673

Gimeno, I. M. (2008). Marek's disease vaccines: A solution for today but a worry for tomorrow? *Vaccine*, 26(SUPPL. 3), C31-41. https://doi.org/10.1016/j.vaccine.2008.04.009

Gimeno I.M., Cortes A.L., Faiz N., Villalobos T., Badillo H., Barbosa T. (2016). Efficacy of Various HVT Vaccines (Conventional and Recombinant) Against Marek's Disease in Broiler Chickens: Effect of Dose and Age of Vaccination. Avian Diseases, 60(3), 662-668. https://doi.org/10.1637/11415-040116-Reg.1.

Gimeno, I. M., Cortes, A. L., Witter, R. L., & Pandiri, A. R. (2012). Optimization of the protocols for double vaccination against marek's disease by using commercially available vaccines: Evaluation of protection, vaccine replication, and activation of T cells. *Avian Diseases*, 56(2), 295–305. https://doi.org/10.1637/9930-091311-Reg.1

Handberg, K. J., Nielsen, O. L., & Jørgensen, P. H. (2001). The use of serotype 1- and serotype 3-specific polymerase chain reaction for the detection of Marek's disease virus in chickens. *Avian Pathology*, 30(3), 243-9. https://doi.org/10.1080/03079450120054659

Heckert, R. A., Riva, J., Cook, S., McMillen, J., & Schwartz, R. D. (1996). Onset of protective immunity in chicks after vaccination with a recombinant herpesvirus of turkeys vaccine expressing Newcastle disease virus fusion and hemagglutinin-neuraminidase antigens. *Avian Diseases*, 40(4), 770–777. https://doi.org/10.2307/1592296

Hein R., Koopman R., García M., Armour N., Dunn J.R., Barbosa T., & Martinez A. (2021). Review of Poultry Recombinant Vector Vaccines. *Avian Diseases*, 65(3), 438-452. https://doi.org/10.1637/0005-2086-65.3.438.

Ingrao F., Rauw F., van den Berg T., & Lambrecht B. (2017). Characterization of two recombinant HVT-IBD vaccines by VP2 insert detection and cell-mediated immunity after vaccination of specific pathogen-free chickens. *Avian Pathology*, 46(3), 289-299. https://doi.org/10.1080/03079457.2016.1265083.

Islam, A., Cheetham, B. F., Mahony, T. J., Young, P. L., & Walkden-Brown, S. W. (2006). Absolute quantitation of Marek's disease virus and Herpesvirus of turkeys in chicken

lymphocyte, feather tip and dust samples using real-time PCR. *Journal of Virological Methods*, 132(1–2), 127–134. https://doi.org/10.1016/J.JVIROMET.2005.10.009

Islam, A., & Walkden-Brown, S. W. (2007). Quantitative profiling of the shedding rate of the three Marek's disease virus (MDV) serotypes reveals that challenge with virulent MDV markedly increases shedding of vaccinal viruses. *Journal of General Virology*, 88(8), 2121–2128. https://doi.org/10.1099/vir.0.82969-0

Islam, T., Walkden-Brown, S. W., Renz, K. G., Fakhrul Islam, A. F. M., & Ralapanawe, S. (2014). Replication kinetics and shedding of very virulent Marek's disease virus and vaccinal Rispens/CVI988 virus during single and mixed infections varying in order and interval between infections. *Veterinary Microbiology*, 173(3–4), 208–223. https://doi.org/10.1016/J.VETMIC.2014.07.027

Johnson, D. I., Vagnozzi, A., Dorea, F., Riblet, S. M., Mundt, A., Zavala, G., & García, M. (2010). Protection against infectious laryngotracheitis by in ovo vaccination with commercially available viral vector recombinant vaccines. *Avian Diseases*, 54(4), 1251–1259. https://doi.org/10.1637/9401-052310-REG.1

Kawamura H., King D. J. Jr., & Anderson D.P. (1969). A herpesvirus isolated from kidney cell culture of normal turkeys. Avian Diseases, 13, 853–886.

Kiddle G., Hardinge P., Buttigieg N., Gandelman O., Pereira C., McElgunn C.J., Rizzoli M., Jackson R., Appleton N., Moore C., Tisi L.C., & Murray J.A. (2012). GMO detection using a bioluminescent real time reporter (BART) of loop mediated isothermal amplification (LAMP) suitable for field use. BMC Biotechnology 12, 15. https://doi.org/10.1186/1472-6750-12-15.

Kim, T., Volkening, J. D., & Spatz, S. J. (2020). Comparative Molecular Characterization of Three Gallid alphaherpesvirus Type 3 Strains 301B/1, HPRS24, and SB-1. *Avian Diseases*, 64(2), 174–182. https://doi.org/10.1637/0005-2086-64.2.174

López-Osorio, S., Villar, D., Piedrahita, D., Ramírez-Nieto, G., Nair, V., Baigent, S., & Chaparro-Gutiérrez, J. (2019). Molecular detection of Marek's disease virus in feather and blood samples

from young laying hens in Colombia. *Acta Virologica*, 63(4), 380–391. https://doi.org/10.4149/AV_2019_402

Mescolini G., Lupini C., Felice V., Guerrini A., Silveira F., Cecchinato M., & Catelli E. (2019). Molecular characterization of the meq gene of Marek's disease viruses detected in unvaccinated backyard chickens reveals the circulation of low- and high-virulence strains. *Poultry Science*, 98(8), 3130-3137. https://doi.org/10.3382/ps/pez095.

Morgan R.W., Gelb J.J. Schreurs C.S., Lutticken, D., Rosenberger J.K., & Sondermeijer, P.J.A. (1992). Protection of chickens from Newcastle and Marek' diseases with a recombinant herpesvirus of turkeys vaccine expressing the Newcastle disease virus fusion protein. *Avian Diseases*, 36, 858-870.

Nagamine, K., Hase, T., & Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and Cellular Probes*, 16(3), 223–229. https://doi.org/10.1006/MCPR.2002.0415

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, 28(12), E63. https://doi.org/10.1093/NAR/28.12.E63

Okazaki, W., Purchase, H. G., & Burmester, B. R. (1970). Protection against Marek's Disease by Vaccination with a Herpesvirus of Turkeys. *Avian Diseases*, 14(2), 413. https://doi.org/10.2307/1588488

Purchase H.G., Okazaki W., & Burmester B.R. (1971). Field trials with the herpes virus of turkeys (HVT) strain FC126 as a vaccine against Marek's disease. *Poultry Science*, 50, 775–783

Ralapanawe, S., Renz, K. G., Burgess, S. K., & Walkden-Brown, S. W. (2016). Field studies of the detection, persistence and spread of the Rispens CVI988 vaccine virus and the extent of co-infection with Marek's disease virus. Australian Veterinary Journal, 94(9), 329–337. https://doi.org/10.1111/avj.12479

Read, A. F., Baigent, S. J., Powers, C., Kgosana, L. B., Blackwell, L., Smith, L. P., Kennedy D.A., Walkden-Brown S.W., & Nair, V. K. (2015). Imperfect Vaccination Can Enhance the Transmission of Highly Virulent Pathogens. *PLOS Biology*, 13(7), e1002198. https://doi.org/10.1371/journal.pbio.1002198

Rémy S., Le Pape G., Gourichon D., Gardin Y., & Denesvre C. (2020). Chickens can durably clear herpesvirus vaccine infection in feathers while still carrying vaccine-induced antibodies. Veterinary Research, 51(1), 24. https://doi.org/10.1186/s13567-020-00749-1.

Renz, Katrin G., Islam, A., Cheetham, B. F., & Walkden-Brown, S. W. (2006). Absolute quantification using real-time polymerase chain reaction of Marek's disease virus serotype 2 in field dust samples, feather tips and spleens. *Journal of Virological Methods*, 135(2), 186–191. https://doi.org/10.1016/J.JVIROMET.2006.03.017

Schat, K. A. (2016). History of the First-Generation Marek's Disease Vaccines: The Science and Little-Known Facts. *Avian Diseases*, 60(4), 715–724. https://doi.org/10.1637/11429-050216-Hist

Spatz, S. J., Petherbridge, L., Zhao, Y., & Nair, V. (2007a). Comparative full-length sequence analysis of oncogenic and vaccine (Rispens) strains of Marek's disease virus. Journal of General Virology. https://doi.org/10.1099/vir.0.82600-0

Spatz S. J., Zhao Y., Petherbridge L., Smith L. P., Baigent S. J., & Nair, V. (2007b). Comparative sequence analysis of a highly oncogenic but horizontal spread-defective clone of Marek's disease virus. *Virus Genes*, 35(3), 753-766. https://doi.org/10.1007/s11262-007-0157-1

Spatz, S. J., & Schat, K. A. (2011). Comparative genomic sequence analysis of the Marek' s disease vaccine strain SB-1. *Virus Genes*, 42, 331–338. https://doi.org/10.1007/s11262-011-0573-0

Trimpert, J., Groenke, N., Jenckel, M., He, S., Kunec, D., Szpara, M. L., Spatz S.J., Osterrieder N., & McMahon, D. P. (2017). A phylogenomic analysis of Marek's disease virus reveals independent paths to virulence in Eurasia and North America. *Evolutionary Applications*, 10(10), 1091–1101. https://doi.org/10.1111/eva.12515

Walkden-Brown, S. W., Groves, P. J., Islam, A. F., Burgess, S. K., Arzey, K. E., Mascord, L., & Young, P. L. (2003). Differentiation of Marek's diesease virus serotypes using PCR: Research and field experience. *Proceedings of Australian Poultry Science Symposium*, 15, 192–196. https://rune-uat.une.edu.au/web/handle/1959.11/4327

Walkden-Brown, Stephen W., Islam, A., Groves, P. J., Rubite, A., Sharpe, S. M., & Burgess, S. K. (2013). Development, application, and results of routine monitoring of Marek's disease virus in broiler house dust using real-time quantitative PCR. Avian Diseases, 57(2 Suppl), 544–554. https://doi.org/10.1637/10380-92112-REG.1

Wei, X., Shi, X., Zhao, Y., Zhang, J., Wang, M., Liu, C., Cui H., Hu S., Quan Y., Chen H., & Wang, Y. (2012). Development of a rapid and specific loop-mediated isothermal amplification detection method that targets Marek's disease virus meq gene. *Journal of Virological Methods*, 183(2), 196–200. https://doi.org/10.1016/J.JVIROMET.2012.04.014

Witter R. L., Nazerian K., Purchase H.G., & Burgoyne G.H. (1970). Isolation from turkeys of a cell-associated herpesvirus antigenically related to Marek's disease virus. *American Journal of Veterinary Research*, 31, 525–538.

Witter, R. L. (1984). A new strategy for Marek's disease immunisation - bivalent vaccine. *Avian Pathology*, 13, 133-135. https://doi.org/10.1080/03079458408418517.

Witter, R.L. (1997). Increased virulence of Marek's disease virus field isolates. *Avian Diseases*, 41, 149-163.

Woźniakowski, G., & Niczyporuk, J. S. (2015). Detection of specific UL49 sequences of Marek's disease virus CVI988/Rispens strain using loop-mediated isothermal amplification. *Journal of Virological Methods*, 221, 22–28. https://doi.org/10.1016/J.JVIROMET.2015.04.015

Woźniakowski, G., & Samorek-Salamonowicz, E. (2014). Direct detection of Marek's disease virus in poultry dust by loop-mediated isothermal amplification. *Archives of Virology*, 159(11), 3083–3087. https://doi.org/10.1007/S00705-014-2157-5

Woźniakowski, G., Samorek-Salamonowicz, E., & Kozdruń, W. (2011). Rapid detection of Marek's disease virus in feather follicles by loop-mediated amplification. *Avian Diseases*, 55(3), 462–467. https://doi.org/10.1637/9668-012711-RESNOTE.1

Woźniakowski, G., Samorek-Salamonowicz, E., & Kozdruń, W. (2013). Comparison of loop-mediated isothermal amplification and PCR for the detection and differentiation of Marek's disease virus serotypes 1, 2, and 3. *Avian Diseases*, 57(2 Suppl), 539–543. https://doi.org/10.1637/10328-082012-RESNOTE.1

Conclusions

MD outbreaks are commonly reported in Italian commercial and backyard chicken flocks, and, occasionally, in turkeys.

Genetic diversity is key to understanding virus evolution, and the *meq* gene polymorphism is useful to preliminarily assess GaHV-2 virulence and to create epidemiological molecular linkages between various GaHV-2 strains. The performed molecular epidemiology studies revealed a significant genetic diversity of GaHV-2 strains circulating in Italian backyard poultry farms over a 3-year period and a genetic uniformity of GaHV-2 strains circulating in Italian commercial poultry farms over a 4-year period. Moreover, the molecular characteristics of a GaHV-2 strain detected from meat-type turkeys exhibiting visceral lymphomas have been reported for the first time. The detected turkey virus resembled highly virulent chicken GaHV-2 strains previously detected in Italy and, due to the presence of broiler chickens in neighbouring pens, chicken-to-turkey transmission is hypothesised.

The heterogeneous population of GaHV-2 strains currently circulating in the Italian rural sector, composed by low and high virulence strains, is causing a wide range of pathological syndromes (e.g. classical MD, acute MD, transient paralysis, sudden death). On the other hand, the homogeneous population of high virulence GaHV-2 strains currently circulating in the Italian commercial sector has been detected during acute MD outbreaks that occurred in vaccinated commercial chicken flocks. These high virulence strains are phylogenetically related to highly virulent GaHV-2 strains circulating in Italian backyard chickens and meat-type turkeys indicating that common trade routes of animals or contaminated equipment may have hypothetically served as a source of dissemination for GaHV-2 between industrial and rural compartments, where biosecurity breaches may have also occurred. The reported vaccine failures in commercial chickens, partially attributable to increased virulence of circulating GaHV-2 strains, should be carefully addressed by field veterinarians that should implement the vaccination programs in place at the time

of the outbreak. Monitoring the vaccination success in the field could be a valuable tool to investigate vaccine failure phenomena and in the second part of this doctoral project two LAMP-LFD assays for the rapid, sensitive, and species-specific detection of conventional and recombinant HVT-based vaccines, the most commonly used vaccines worldwide to prevent and control MD in commercial poultry flocks, have been developed, expanding the diagnostic capabilities, especially in resource-limited settings.

References

Abd-Ellatieff, H. A., Abou Rawash, A. A., Ellakany, H. F., Goda, W. M., Suzuki, T., & Yanai, T. (2018). Molecular characterization and phylogenetic analysis of a virulent Marek's disease virus field strain in broiler chickens in Japan. *Avian Pathology*, 47(1), 47–57. https://doi.org/10.1080/03079457.2017.1362497

Abdallah, F., Hassanin, O., Attar, E., Ali, H., Megahed, M., & Nair, V. (2018). Marek's Disease Virus in Egypt: Historical Overview and Current Research Based on the Major MDV-Encoded Oncogene Meq. *Hosts and Viruses*, 5, 35–43. http://dx.doi.org/10.17582/journal.hv/2018/5.3.35.43

Adedeji, A. J., Abdu, P. A., Luka, P. D., Owoade, A. A., & Joannis, T. M. (2017). Application of loop-mediated isothermal amplification assay in the detection of herpesvirus of turkey (FC 126 strain) from chicken samples in Nigeria. *Veterinary World*, 10(11), 1383–1388. https://doi.org/10.14202/VETWORLD.2017.1383-1388

Afonso, C. L., Tulman, E. R., Lu, Z., Zsak, L., & Rock, D. L. (2001). The Genome of Turkey Herpesvirus. *Journal of Virology*, 75(2), 971–978. https://doi.org/10.1128/JVI.75.2.971

Angamuthu, R., Baskaran, S., Gopal, D. R., Devarajan, J., & Kathaperumal, K. (2012). Rapid detection of the Marek's disease viral genome in chicken feathers by loop-mediated isothermal amplification. *Journal of Clinical Microbiology*, 50(3), 961–965. https://doi.org/10.1128/JCM.05408-11

Atkins, K. E., Read, A. F., Savill, N. J., Renz, K. G., Walkden-Brown, S. W., & Woolhouse, M. E. J. (2011). Modelling Marek's Disease Virus (MDV) infection: Parameter estimates for mortality rate and infectiousness. *BMC Veterinary Research*, 7(1), 1–12. https://doi.org/10.1186/1746-6148-7-70/FIGURES/6

Bacon, L.D., & Witter, R.L. (1992). Influence of turkey herpesvirus vaccination on the B haplotype effect on Marek's disease resistance in 15.B-congenic chickens. *Avian Diseases*, 36, 378-385.

Bacon, L. D., Witter, R. L., & Silva, R. F. (2001). Characterization and experimental reproduction of peripheral neuropathy in White Leghorn chickens. *Avian Pathology*, 30(5), 487–499. https://doi.org/10.1080/03079450120078680

Baigent, S. J., & Davison, T. F. (1999). Development and composition of lymphoid lesions in the spleens of Marek's disease virus-infected chickens: Association with virus spread and the pathogenesis of Marek's disease. *Avian Pathology*, 28, 287–300. https://doi.org/10.1080/03079459994786

Baigent, S. J., Nair, V. K., & Le Galludec, H. (2016). Real-time PCR for differential quantification of CVI988 vaccine virus and virulent strains of Marek's disease virus. *Journal of Virological Methods*, 233, 23–36. https://doi.org/10.1016/j.jviromet.2016.03.002

Baigent, S. J., Ross, J., & Davison, T. F. (1998). Differential susceptibility to Marek's disease is associated with differences in number, but not phenotype or location, of pp38+ lymphocytes. *Journal of General Virology*, 79(11), 2795–2802. https://doi.org/10.1099/0022-1317-79-11-2795

Bailey, R. I., Cheng, H. H., Chase-Topping, M., Mays, J. K., Anacleto, O., Dunn, J. R., & Doeschl-Wilson, A. (2020). Pathogen transmission from vaccinated hosts can cause dose-dependent reduction in virulence. *PLoS Biology*, 18(3), e3000619. https://doi.org/10.1371/journal.pbio.3000619

Barrow, A. D., Burgess, S. C., Baigent, S. J., Howes, K., & Nair, V. K. (2003). Infection of macrophages by a lymphotropic herpesvirus: A new tropism for Marek's disease virus. *Journal of General Virology*, 84(10), 2635–2645. https://doi.org/10.1099/vir.0.19206-0

Bertzbach, L. D., Conradie, A. M., You, Y., & Kaufer, B. B. (2020). Latest insights into Marek's disease virus pathogenesis and tumorigenesis. *Cancers*, 12(3), 647. https://doi.org/10.3390/cancers12030647

Bertzbach, L. D., Kheimar, A., Ali, F. A. Z., & Kaufer, B. B. (2018a). Viral Factors Involved in Marek's Disease Virus (MDV) Pathogenesis. *Current Clinical Microbiology Reports*, 5, 238–244. https://doi.org/10.1007/s40588-018-0104-z

Bertzbach, L. D., Laparidou, M., Härtle, S., Etches, R. J., Kaspers, B., Schusser, B., & Kaufer, B. B. (2018b). Unraveling the role of B cells in the pathogenesis of an oncogenic avian herpesvirus. *Proceedings of the National Academy of Sciences of the United States of America*, 115(45), 11603–11607. https://doi.org/10.1073/PNAS.1813964115/-/DCSUPPLEMENTAL

Bertzbach, L. D., van Haarlem, D. A., Härtle, S., Kaufer, B. B., & Jansen, C. A. (2019). Marek's disease virus infection of natural killer cells. *Microorganisms*, 7(12), 588. https://doi.org/10.3390/microorganisms7120588

Biggs P.M. (1976). Differential diagnosis of Avian Lymphoid Leukosis and Marek's Disease. In L.N. Payne Differential diagnosis of Avian Lymphoid Leukosis and Marek's Disease, Commission of the European Communities, Luxembourg. pp 67-85.

Blake-Dyke, C., & Baigent, S. (2013). Marek's disease in commercial turkey flocks. *Veterinary Record*, 173 (15), 376. https://doi.org/10.1136/vr.f6229

Blume G.R., Cardoso S.P., Oliveira M.L.B., Matiolli M.P., Gómez S.Y.M., R. J. J. L., Sant'Ana F.J.F., & Martins N.R.S. (2016). Visceral Marek's disease in white-peafowl (Pavo cristatus). *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, 68(6), 1602–1608. https://doi.org/10.1590/1678-4162-8873

Boodhoo, N., Gurung, A., Sharif, S., & Behboudi, S. (2016). Marek's disease in chickens: a review with focus on immunology. *Veterinary Research*, 47(1), 1–19. https://doi.org/10.1186/S13567-016-0404-3

Brochu, N. M., Guerin, M. T., Varga, C., Lillie, B. N., Brash, M. L., & Susta, L. (2019). A two-year prospective study of small poultry flocks in Ontario, Canada, part 1: prevalence of viral and bacterial pathogens. *Journal of Veterinary Diagnostic Investigation*, 31(3), 327–335. https://doi.org/10.1177/1040638719843577

Brown, A. C., Smith, L. P., Kgosana, L., Baigent, S. J., Nair, V., & Allday, M. J. (2009). Homodimerization of the Meq Viral Oncoprotein Is Necessary for Induction of T-Cell Lymphoma

by Marek's Disease Virus. *Journal of Virology*, 83(21), 11142–11151. https://doi.org/10.1128/JVI.01393-09

Cadmus, K. J., Mete, A., Harris, M., Anderson, D., Davison, S., Sato, Y., Helm J., Boger L., Odani J., Ficken M.D., & Pabilonia, K. L. (2019). Causes of mortality in backyard poultry in eight states in the United States. *Journal of Veterinary Diagnostic Investigation*, 31(3), 318–326. https://doi.org/10.1177/1040638719848718

Calnek BW. (1986). Marek's disease--a model for herpesvirus oncology. *Critical Reviews in Microbiology*, 12(4), 293-320. doi: 10.3109/10408418509104432.

Calnek, B.W (2001). Pathogenesis of Marek's disease virus infection. *Current Topics in Microbiology and Immunology*, 255, 25–55.

Calnek, B. W., Adldinger, H. K., & Kahn, D. E. (1970). Feather follicle epithelium: a source of enveloped and infectious cell-free herpesvirus from Marek's disease. *Avian Diseases*, 14(2), 219–233.

Chacón, R. D., Astolfi-Ferreira, C. S., Guimarães, M. B., Torres, L. N., De la Torre, D. I., de Sá, L. R. M., & Piantino Ferreira, A. J. (2019). Detection and molecular characterization of a natural coinfection of marek's disease virus and reticuloendotheliosis virus in Brazilian backyard chicken flock. *Veterinary Sciences*, 6(4), 1–14. https://doi.org/10.3390/vetsci6040092

Chakraborty, P., Vervelde, L., Dalziel, R. G., Wasson, P. S., Nair, V., Dutia, B. M., & Kaiser, P. (2017). Marek's disease virus infection of phagocytes: a de novo in vitro infection model. *The Journal of General Virology*, 98(5), 1080–1088. https://doi.org/10.1099/JGV.0.000763

Chang, K.-S., Ohashi, K., & Onuma, M. (2002). Diversity (polymorphism) of the meq gene in the attenuated Marek's disease virus (MDV) serotype 1 and MDV-transformed cell lines. *The Journal of Veterinary Medical Science / the Japanese Society of Veterinary Science*, 64(12), 1097–1101. https://doi.org/10.1292/jvms.64.1097

Chang, S., Dunn, J. R., Heidari, M., Lee, L. F., Song, J., Ernst, C. W., Ding Z., Bacon L.D., & Zhang, H. (2010). Genetics and vaccine efficacy: host genetic variation affecting Marek's disease vaccine

efficacy in White Leghorn chickens. *Poultry Science*, 89(10), 2083–2091. https://doi.org/10.3382/PS.2010-00740

Conradie, A. M., Bertzbach, L. D., Trimpert, J., Patria, J. N., Murata, S., Parcells, M. S., & Kaufer, B. B. (2020). Distinct polymorphisms in a single herpesvirus gene are capable of enhancing virulence and mediating vaccinal resistance. *PLoS Pathogens*, 16(12), e1009104. https://doi.org/10.1371/journal.ppat.1009104

Cortes, A. L., Montiel, E. R., Lemiere, S., & Gimeno, I. M. (2011). Comparison of blood and feather pulp samples for the diagnosis of Marek's disease and for monitoring Marek's disease vaccination by real time-PCR. *Avian Diseases*, 55(2), 302–310. https://doi.org/10.1637/9578-101510-RESNOTE.1

Cui Z., Qin A., Lee L.F., Wu P., & Kung H.J. (1999). Construction and characterization of a H19 epitope point mutant of MDV CVI988/Rispens strain. *Acta Virologica*, 43(2-3), 169-173.

Cui, N., Su, S., Sun, P., Zhang, Y., Han, N., & Cui, Z. (2016). Isolation and pathogenic analysis of virulent Marek's disease virus field strain in China. *Poultry Science*, 95(7), 1521–1528. https://doi.org/10.3382/ps/pew073

Darteil, R., Bublot, M., Laplace, E., Bouquet, J. F., Audonnet, J. C., & Riviè, M. (1995). Herpesvirus of turkey recombinant viruses expressing infectious bursal disease virus (IBDV) VP2 immunogen induce protection against an IBDV virulent challenge in chickens. *Virology*, 211(2), 481–490. https://doi.org/10.1006/VIRO.1995.1430

Davidson, I, Malkinson, M., & Weisman, Y. (2002). Marek's disease in turkeys. I. A seven-year survey of commercial flocks and experimental infection using two field isolates. *Avian Diseases*, 46(2), 314–321. https://doi.org/10.1637/0005-2086(2002)046[0314:MSDITI]2.0.CO;2

Davidson, Irit, Natour-Altory, A., Raibstein, I., Kin, E., Dahan, Y., Krispin, H., & Elkin, N. (2018). Monitoring the uptake of live avian vaccines by their detection in feathers. *Vaccine*, 36(5), 637–643. https://doi.org/10.1016/j.vaccine.2017.12.052

Demeke, B., Jenberie, S., Tesfaye, B., Ayelet, G., Yami, M., Lamien, C. E., & Gelaye, E. (2017). Investigation of Marek's disease virus from chickens in central Ethiopia. *Tropical Animal Health and Production*, 49(2), 403–408. https://doi.org/10.1007/s11250-016-1208-1

Denesvre, C. (2013). Marek's disease virus morphogenesis. *Avian Diseases*, 57(2 Suppl), 340–350. https://doi.org/10.1637/10375-091612-Review.1

Deng, X., Li, X., Shen, Y., Qiu, Y., Shi, Z., Shao, D., Jin Y., Chen H., Ding C., Li L., Chen P., & Ma, Z. (2010). The Meq oncoprotein of Marek's disease virus interacts with p53 and inhibits its transcriptional and apoptotic activities. *Virology Journal*, 7, 348. https://doi.org/10.1186/1743-422X-7-348

Dudnikova, E., Norkina, S., Vlasov, A., Slobodchuk, A., Lee, L. F., & Witter, R. L. (2007). Evaluation of Marek's disease field isolates by the "best fit" pathotyping assay. *Avian Pathology*, 36(2), 135–143. https://doi.org/10.1080/03079450701209857

Duffy, S., Shackelton, L. A., & Holmes, E. C. (2008). Rates of evolutionary change in viruses: Patterns and determinants. *Nature Reviews Genetics*, 9(4), 267-76. https://doi.org/10.1038/nrg2323

Dunn, J. R., Auten, K., Heidari, M., & Buscaglia, C. (2014). Correlation Between Marek' s Disease Virus Pathotype and Replication. *Avian Diseases*, 58(2), 287–292. https://doi.org/10.1637/10678-092513-Reg.1

Dunn, J. R., & Gimeno, I. M. (2013). Current status of Marek's disease in the United States and worldwide based on a questionnaire survey. *Avian Diseases*, 57(2 SUPPL. 1), 483–490. https://doi.org/10.1637/10373-091412-ResNote.1

Dunn, J. R., Pyrkosz, A. B., Steep, A., & Cheng, H. H. (2019). Identification of marek's disease virus genes associated with virulence of US strains. *Journal of General Virology*, 100(7), 1132–1139. https://doi.org/10.1099/jgv.0.001288

Fakhrul Islam, A. F. M., Walkden-Brown, S. W., Groves, P. J., & Underwood, G. J. (2008). Kinetics of Marek's disease virus (MDV) infection in broiler chickens 1: Effect of varying vaccination to challenge interval on vaccinal protection and load of MDV and herpesvirus of turkey in the spleen

and feather dander over time. *Avian Pathology*, 37(3), 225–235. https://doi.org/10.1080/03079450701802230

Firth, C., Kitchen, A., Shapiro, B., Suchard, M. A., Holmes, E. C., & Rambaut, A. (2010). Using time-structured data to estimate evolutionary rates of double-stranded DNA viruses. *Molecular Biology and Evolution*, 27(9), 2038–2051. https://doi.org/10.1093/molbev/msq088

Gall, S., Kőrösi, L., Cortes, A. L., Delvecchio, A., Prandini, F., Mitsch, P., & Gimeno, I. M. (2018). Use of real-time PCR to rule out Marek's disease in the diagnosis of peripheral neuropathy. *Avian Pathology*, 47(4), 427–433. https://doi.org/10.1080/03079457.2018.1473555

Gatherer, D., Depledge, D. P., Hartley, C. A., Szpara, M. L., Vaz, P. K., Benkő, M., Brandt C.R., Bryant N.A., Dastjerdi A., Doszpoly A., Gompels U.A., Inoue N., Jarosinski K.W., Kaul R., Lacoste V., Norberg P., Origgi F.C., Orton R.J., Pellett P.E., Schmid D.S., Spatz S.J., Stewart J.P., Trimpert J., Waltzek T.B., & Davison A.J. (2021). ICTV virus taxonomy profile: Herpesviridae 2021. *Journal of General Virology*, 102(10), 1–2. https://doi.org/10.1099/JGV.0.001673

Gennart, I., Coupeau, D., Pejaković, S., Laurent, S., Rasschaert, D., & Muylkens, B. (2015). Marek's disease: Genetic regulation of gallid herpesvirus 2 infection and latency. *Veterinary Journal*, 205(3), 339–348. https://doi.org/10.1016/j.tvjl.2015.04.038

Gimeno, I. M., Witter, R. L., Fadly, A. M., & Silva, R. F. (2005). Novel criteria for the diagnosis of Marek's disease virus-induced lymphomas. *Avian Pathology*, 34(4), 332–340. https://doi.org/10.1080/03079450500179715

Gimeno, I. M., Witter, R. L., & Reed, W. M. (1999). Four distinct neurologic syndromes in Marek's disease: effect of viral strain and pathotype. *Avian Diseases*, 43(4), 721–737. https://doi.org/10.2307/1592741

Gimeno, I., 2004. Future strategies for controlling Marek's disease. In Davison F. e Nair V., Marek's Disease, An Evolving Problem, Elsevier Academic Press, London, UK pp 62-77.

Gimeno, I. M. (2014). Marek's Disease and Differential Diagnosis with Other Tumor Viral Diseases of Poultry. In *Encyclopedia of Agriculture and Food Systems* (pp. 156–171). https://doi.org/https://doi.org/10.1016/B978-0-444-52512-3.00193-5

Gimeno, I. M. (2008). Marek's disease vaccines: A solution for today but a worry for tomorrow? *Vaccine*, 26(SUPPL. 3), C31-41. https://doi.org/10.1016/j.vaccine.2008.04.009

Gimeno, I. M., Cortes, A. L., Witter, R. L., & Pandiri, A. R. (2012a). Optimization of the protocols for double vaccination against marek's disease by using commercially available vaccines: Evaluation of protection, vaccine replication, and activation of T cells. *Avian Diseases*, 56(2), 295–305. https://doi.org/10.1637/9930-091311-Reg.1

Gimeno, I. M., Witter, R. L., Cortes, A. L., Reddy, S. M., & Pandiri, A. R. (2012b). Standardization of a model to study revaccination against Marek's disease under laboratory conditions. *Avian Pathology*, 41(1), 59–68. https://doi.org/10.1080/03079457.2011.635636

Gopal, S., Manoharan, P., Kathaperumal, K., Chidambaram, B., & Divya, K. C. (2012). Differential detection of avian oncogenic viruses in poultry layer farms and Turkeys by use of multiplex PCR. *Journal of Clinical Microbiology*, 50(8), 2668–2673. https://doi.org/10.1128/JCM.00457-12

Haesendonck R., Garmyn A., Dorrestein G.M., Hellebuyck T., Antonissen G., Pasmans F., Ducatelle R., & Martel A. (2015). Marek' s disease virus associated ocular lymphoma in Roulroul partridges (Rollulus rouloul). *Avian Pathology*, 44(5), 347–351. https://doi.org/10.1080/03079457.2015.1056088

Handberg, K. J., Nielsen, O. L., & Jørgensen, P. H. (2001). The use of serotype 1- and serotype 3-specific polymerase chain reaction for the detection of Marek's disease virus in chickens. Avian Pathology, 30(3), 243-9. https://doi.org/10.1080/03079450120054659

Hao H., Li C., Qiu Y., Wang F., Ai W., Gao J., Wei L., Li X., Sun L., Wu J., Qin G., Li R., Liu J., Lv J., Huang R., Wang H., & Chai T. (2014). Generation, transmission and infectiosity of chicken MDV aerosols under experimental conditions. *Veterinary Microbiology*, 172(3–4), 400–406. https://doi.org/10.1016/j.vetmic.2014.06.003

Hassanin, O., Abdallah, F., & El-Araby, I. E. (2013). Molecular characterization and phylogenetic analysis of Marek's disease virus from clinical cases of Marek's disease in Egypt. *Avian Diseases*, 57(2 Suppl), 555–561. https://doi.org/10.1637/10337-082912-Reg.1

Hauck R., Mays J., Dunn J.R., & Shivaprasad H.L. (2020). Two Cases of Marek' s Disease in Backyard Turkeys. *Avian Diseases*, 64(3), 347–351. https://doi.org/10.1637/aviandiseases-D-19-00177.

Heckert, R. A., Riva, J., Cook, S., McMillen, J., & Schwartz, R. D. (1996). Onset of protective immunity in chicks after vaccination with a recombinant herpesvirus of turkeys vaccine expressing Newcastle disease virus fusion and hemagglutinin-neuraminidase antigens. *Avian Diseases*, 40(4), 770–777. https://doi.org/10.2307/1592296

Hennig H., Osterrieder N., Müller-Steinhardt M., Teichert H.M., Kirchner H., & Wandinger K.P. (2003). Detection of Marek' s Disease Virus DNA in Chicken but Not in Human Plasma. *Journal of Clinical Microbiology*, 41(6), 2428–2432. https://doi.org/10.1128/JCM.41.6.2428

Huong, T. C., Murano, T., Uno, Y., Usui, T., & Yamaguchi, T. (2014). Molecular Detection of Avian Pathogens in Poultry Red Mite (Dermanyssus gallinae) Collected in Chicken Farms. *Journal of Veterinary Medical Science*, 76(12), 1583–1587. https://doi.org/10.1292/jvms.14-0253

Imai, K., Nakamura, K., Tsukamoto, K., Hihara, H., Yuasa, N., & Kobayashi, S. (1990). Isolation of marek's disease virus from japanese quail with lymphoproliferative disease. *Avian Pathology*, 19(1), 119–129. https://doi.org/10.1080/03079459008418661

Islam, A., Cheetham, B. F., Mahony, T. J., Young, P. L., & Walkden-Brown, S. W. (2006). Absolute quantitation of Marek's disease virus and Herpesvirus of turkeys in chicken lymphocyte, feather tip and dust samples using real-time PCR. *Journal of Virological Methods*, 132(1–2), 127–134. https://doi.org/10.1016/J.JVIROMET.2005.10.009

Islam, A., Harrison, B., Cheetham, B. F., Mahony, T. J., Young, P. L., & Walkden-Brown, S. W. (2004). Differential amplification and quantitation of Marek's disease viruses using real-time polymerase chain reaction. *Journal of Virological Methods*, 119(2), 103–113. https://doi.org/10.1016/J.JVIROMET.2004.03.006

Islam, A., & Walkden-Brown, S. W. (2007). Quantitative profiling of the shedding rate of the three Marek's disease virus (MDV) serotypes reveals that challenge with virulent MDV markedly increases shedding of vaccinal viruses. *Journal of General Virology*, 88(8), 2121–2128. https://doi.org/10.1099/vir.0.82969-0

Islam, T., Walkden-Brown, S. W., Renz, K. G., Fakhrul Islam, A. F. M., & Ralapanawe, S. (2014). Replication kinetics and shedding of very virulent Marek's disease virus and vaccinal Rispens/CVI988 virus during single and mixed infections varying in order and interval between infections. *Veterinary Microbiology*, 173(3–4), 208–223. https://doi.org/10.1016/J.VETMIC.2014.07.027

Johnson, D. I., Vagnozzi, A., Dorea, F., Riblet, S. M., Mundt, A., Zavala, G., & García, M. (2010). Protection against infectious laryngotracheitis by in ovo vaccination with commercially available viral vector recombinant vaccines. *Avian Diseases*, 54(4), 1251–1259. https://doi.org/10.1637/9401-052310-REG.1

Jurajda V., & Klimes B. (1970). Presence and survival of Marek's disease agent in dust. *Avian Diseases*, 14(1), 188–190.

Kennedy, D. A., Cairns, C., Jones, M. J., Bell, A. S., Salathé, R. M., Baigent, S. J., Nair V.K., Dunn P.A., & Read, A. F. (2017). Industry-Wide Surveillance of Marek's Disease Virus on Commercial Poultry Farms. *Avian Diseases*, 61(2), 153-164. https://doi.org/10.1637/11525-110216-Reg.1

Kheimar, A., Previdelli, R. L., Wight, D. J., & Kaufer, B. B. (2017). Telomeres and telomerase: Role in Marek's disease virus pathogenesis, integration and tumorigenesis. *Viruses*, 9(7), 173. https://doi.org/10.3390/v9070173

Kim, T., Volkening, J. D., & Spatz, S. J. (2020). Comparative Molecular Characterization of Three Gallid alphaherpesvirus Type 3 Strains 301B/1, HPRS24, and SB-1. *Avian Diseases*, 64(2), 174–182. https://doi.org/10.1637/0005-2086-64.2.174

Kumar K.S., Suresh P., Sukumar K., Saravanan S., Raja A., & Thangavelu A. (2022). Quantitative profiling of Marek's Disease Virus in vaccinated layer chicken. *Veterinary Microbiology*, 264, 109305. https://doi.org/10.1016/J.VETMIC.2021.109305

Lee, L. F., Liu X., & Witter R. L. (1983). Monoclonal antibodies with specifficity for three different serotypes of Marek's disease viruses in chickens. *The Journal of Immunology*, 130, 1003–1006.

Lee, S.-I., Takagi, M., Ohashi, K., Sugimoto, C., & Onuma, M. (2000). Difference in the meq Gene between Oncogenic and Attenuated Strains of Marek's Disease Virus Serotype 1. *Journal of Veterinary Medical Science*, 62(3), 287–292. https://doi.org/10.1292/jvms.62.287

Lian, X., Ming, X., Xu, J., Cheng, W., Zhang, X., Chen, H., & Ding, C. (2018). First molecular detection and characterization of Marek' s disease virus in red-crowned cranes (Grus japonensis): a case report. *BMC Veterinary Research*, 14(122), 1–11. https://doi.org/https://doi.org/10.1186/s12917-018-1437-9

Liu, J. L., Lin, S. F., Xia, L., Brunovskis, P., Li, D., Davidson, I., Lee LF, & Kung, H. J. (1999). Meq and V-IL8: Cellular genes in disguise? *Acta Virologica*, 43(2–3), 94–101.

López-Osorio, S., Villar, D., Piedrahita, D., Ramírez-Nieto, G., Nair, V., Baigent, S., & Chaparro-Gutiérrez, J. (2019). Molecular detection of Marek's disease virus in feather and blood samples from young laying hens in Colombia. *Acta Virologica*, 63(4), 380–391. https://doi.org/10.4149/AV_2019_402

López-Osorio, Sara, Piedrahita, D., Espinal-Restrepo, M. A., Ramírez-Nieto, G. C., Nair, V., Williams, S. M., Baigent S., Ventura-Polite C., Aranzazu-Taborda D.A., & Chaparro-Gutiérrez, J. J. (2017). Molecular characterization of Marek's disease virus in a poultry layer farm from Colombia. *Poultry Science*, 96(6), 1598–1608. https://doi.org/https://doi.org/10.3382/ps/pew464

Lounas, A., Besbaci, M., Akkou, M., & Tali, O. (2021). Occurrence of Marek's disease in vaccinated Algerian broiler breeder flocks: A histopathological survey. *Veterinary World*, 14(11), 3021. https://doi.org/10.14202/VETWORLD.2021.3021-3027

Lupiani, B., Lee, L. F., Kreager, K. S., Witter, R. L., & Reddy, S. M. (2013). Insertion of reticuloendotheliosis virus long terminal repeat into the genome of CVI988 strain of Marek's disease virus results in enhanced growth and protection. *Avian Diseases*, 57(2 SUPPL. 1), 427–431. https://doi.org/10.1637/10445-110412-ResNote.1

Machida, Y., Murata, S., Matsuyama-Kato, A., Isezaki, M., Taneno, A., Sakai, E., Konnai S., Ohashi, K. (2017). Isolation and purification of Gallid herpesvirus 2 strains currently distributed in Japan. *The Journal of Veterinary Medical Science*, 79(1), 115–122. https://doi.org/10.1292/jvms.16-0329

Mete, A., Gharpure, R., Pitesky, M. E., Famini, D., Sverlow, K., & Dunn, J. (2016). Marek's Disease in Backyard Chickens, A Study of Pathologic Findings and Viral Loads in Tumorous and Nontumorous Birds. *Avian Diseases*, 60(4), 826–836. https://doi.org/10.1637/11458-062216-Reg

Molouki, A., Ghalyanchilangeroudi, A., Abdoshah, M., Shoushtari, A., Abtin, A., Eshtartabadi, F., Mahmoudzadeh Akhijahani M., Ziafatikafi Z., Babaeimarzango S.S., Allahyari E., Ahmadzadeh L., Fallah Mehrabadi M.H., Lim S.H.E., Rouhani K., Hosseini H., & Nair, V. (2021). Report of a new meq gene size: The first study on genetic characterisation of Marek's disease viruses circulating in Iranian commercial layer and backyard chicken. *British Poultry Science*, 1–8. https://doi.org/10.1080/00071668.2021.1963677

Morgan R.W., Gelb J.J. Schreurs C.S., Lutticken, D., Rosenberger J.K., & Sondermeijer, P.J.A. (1992). Protection of chickens from Newcastle and Marek' diseases with a recombinant herpesvirus of turkeys vaccine expressing the Newcastle disease virus fusion protein. *Avian Diseases*, 36, 858-870.

Morrow, C. & Fehler, F. (2004). Marek's disease: a worldwide problem. In F. Davison & V. Nair (Eds.), Marek's Disease. An Evolving Problem (pp. 49–61). London: Elsevier Academic Press.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., & Erlich, H. (1986). Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harbor Symposia on Quantitative Biology*, 51 Pt 1(1), 263–273. https://doi.org/10.1101/SQB.1986.051.01.032

Murata, S., Hayashi, Y., Kato, A., Isezaki, M., Takasaki, S., Onuma, M., Osa Y., Asakawa M., Konnai S., & Ohashi, K. (2012). Surveillance of Marek's disease virus in migratory and sedentary birds in Hokkaido, Japan. *Veterinary Journal*, 192(3), 538–540. https://doi.org/10.1016/j.tvjl.2011.07.006

Nagamine, K., Hase, T., & Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and Cellular Probes*, 16(3), 223–229. https://doi.org/10.1006/MCPR.2002.0415

Nair, V. (2018). Spotlight on avian pathology: Marek's disease. *Avian Pathology*, 47(5), 440–442. https://doi.org/10.1080/03079457.2018.1484073

Neumann, U., & Witter, R. L. (1979). Differential Diagnosis of Lymphoid Leukosis and Marek's Disease by Tumor-Associated Criteria I. Studies on Experimentally Infected Chickens. *Avian Diseases*, 23(2), 417. https://doi.org/10.2307/1589572

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, 28(12), E63. https://doi.org/10.1093/NAR/28.12.E63

Okazaki, W., Purchase, H. G., & Burmester, B. R. (1970). Protection against Marek's Disease by Vaccination with a Herpesvirus of Turkeys. *Avian Diseases*, 14(2), 413. https://doi.org/10.2307/1588488

Osterrieder, N., Kamil, J. P., Schumacher, D., & Tischer, B. K. (2006). Marek' s disease virus: from miasma to model. *Nature Reviews Microbiology*, 4(4), 283–294. https://doi.org/10.1038/nrmicro1382

Padhi, A., & Parcells, M. S. (2016). Positive selection drives rapid evolution of the meq oncogene of Marek's disease virus. *PLoS ONE*, 11(9). https://doi.org/10.1371/journal.pone.0162180

Pandey, U., Bell, A. S., Renner, D. W., Kennedy, D. A., Shreve, J. T., Cairns, C. L., Jones M.J., Dunn P.A., Read A.F., & Szpara, M. L. (2016). DNA from Dust: Comparative Genomics of Large DNA Viruses in Field Surveillance Samples. MSphere, 1(5), e00132-16. https://doi.org/10.1128/mSphere.00132-16

Pandiri, A. K. R., Cortes, A. L., Lee, L. F., & Gimeno, I. M. (2008). Marek's disease virus infection in the eye: Chronological study of the lesions, virus replication, and vaccine-induced protection. *Avian Diseases*, 52(4), 572–580. https://doi.org/10.1637/8284-031308-Reg.1

Payne L.N. (2004). Pathological responses to infection. In F. Davison e V.Nair, Marek's Disease, An Evolving Problem, Elsevier Academic Press, London, UK, 62-77.

Payne, L.N., Biggs,P.M. (1967). Studies on Marek's disease. II. Pathogenesis. *Journal of the National Cancer Institute* 39, 281–302.

Pennycott, T. W., Duncan, G., & Venugopal, K. (2003). Marek's disease, candidiasis and megabacteriosis in a flock of chickens (Gallus gallus domesticus) and Japanese quail (Coturnix japonica). *The Veterinary Record*, 153(10), 293–297. https://doi.org/10.1136/VR.153.10.293

Pennycott, T. W., & Venugopal, K. (2002). Outbreak of Marek's disease in a flock of turkeys in Scotland. *Veterinary Record*. https://doi.org/10.1136/vr.150.9.277

Pohjola, L., Rossow, L., Huovilainen, A., Soveri, T., Hänninen, M. L., & Fredriksson-Ahomaa, M. (2015). Questionnaire study and postmortem findings in backyard chicken flocks in Finland. *Acta Veterinaria Scandinavica*, 57(1). https://doi.org/10.1186/s13028-015-0095-1

Qian, Z., Brunovskis, P., Rauscher 3rd, F., Lee, L., & Kung, H. J. (1995). Transactivation activity of Meq, a Marek's disease herpesvirus bZIP protein persistently expressed in latently infected transformed T cells. *Journal of Virology*, 69(7), 4037–4044. http://www.ncbi.nlm.nih.gov/pubmed/7769661

Ralapanawe, S., Renz, K. G., Burgess, S. K., & Walkden-Brown, S. W. (2016a). Field studies of the detection, persistence and spread of the Rispens CVI988 vaccine virus and the extent of co-infection with Marek's disease virus. *Australian Veterinary Journal*, 94(9), 329–337. https://doi.org/10.1111/avj.12479

Ralapanawe, Sithara, Walkden-Brown, S. W., Islam, A. F. M. F., & Renz, K. G. (2016b). Effects of Rispens CVI988 vaccination followed by challenge with Marek's disease viruses of differing

virulence on the replication kinetics and shedding of the vaccine and challenge viruses. *Veterinary Microbiology*, 183, 21–29. https://doi.org/10.1016/J.VETMIC.2015.11.025

Read, A. F., Baigent, S. J., Powers, C., Kgosana, L. B., Blackwell, L., Smith, L. P., Kennedy D.A., Walkden-Brown S.W., & Nair, V. K. (2015). Imperfect Vaccination Can Enhance the Transmission of Highly Virulent Pathogens. *PLOS Biology*, 13(7), e1002198. https://doi.org/10.1371/journal.pbio.1002198

Renz, K. G., Cheetham, B. F., & Walkden-Brown, S. W. (2013). Differentiation between pathogenic serotype 1 isolates of Marek's disease virus and the Rispens CVI988 vaccine in Australia using real-time PCR and high resolution melt curve analysis. *Journal of Virological Methods*, 187(1), 144–152. https://doi.org/10.1016/J.JVIROMET.2012.09.018

Renz, Katrin G., Cooke, J., Clarke, N., Cheetham, B. F., Hussain, Z., Fakhrul Islam, A. F. M., Tannock G.A., & Walkden-Brown, S. W. (2012). Pathotyping of Australian isolates of Marek's disease virus and association of pathogenicity with meq gene polymorphism. *Avian Pathology*, 41(2), 161–176. https://doi.org/10.1080/03079457.2012.656077

Renz, Katrin G., Islam, A., Cheetham, B. F., & Walkden-Brown, S. W. (2006). Absolute quantification using real-time polymerase chain reaction of Marek's disease virus serotype 2 in field dust samples, feather tips and spleens. *Journal of Virological Methods*, 135(2), 186–191. https://doi.org/10.1016/J.JVIROMET.2006.03.017

Rispens, B.H., van Vloten, H., Mastenbroek, N., Maas, H.J., & Schat, K.A. (1972a). Control of Marek's disease in the Netherlands. I. Isolation of an avirulent Marek's disease virus (strain CVI 988) and its use in laboratory vaccination trials. *Avian Diseases* 16, 108-125.

Rispens, B.H., van Vloten, H., Mastenbroek, N., Maas, J.L., & Schat, K.A. (1972b). Control of Marek's disease in the Netherlands. II. Field trials on vaccination with an avirulent strain (CVI 988) of Marek's disease virus. *Avian Diseases*, 16, 126-138.

Ross, N. L. J. (1999). T-cell transformation by Marek's disease virus. *Trends in Microbiology*, 7, 22–29. https://doi.org/10.1016/S0966-842X(98)01427-9

Schat, K. A. (2005). Isolation of Marek's disease virus: revisited. *Avian Pathology*, 34(2), 91–95. https://doi.org/10.1080/03079450500059289

Schat, K. A. (2016). History of the First-Generation Marek's Disease Vaccines: The Science and Little-Known Facts. *Avian Diseases*, 60(4), 715–724. https://doi.org/10.1637/11429-050216-Hist

Schat, K.A., Calnek, B.W., & Fabricant, J. (1982). Characterisation of two highly oncogenic strains of Marek's disease virus. *Avian Pathology,* 11, 593-605.

Schat, K.A., Chen, C.-L. L., Calnek, B. W., & Char, D. (1991). Transformation of T-lymphocyte subsets by Marek's disease herpesvirus. *Journal of Virology*, 65(3), 1408–1413. https://doi.org/10.1128/jvi.65.3.1408-1413.1991

Schock A., Garcia-Rueda C., Byas R., Nuñez A., & Baigent S. (2016). Severe outbreak of Marek' s disease in crested partridges. *Veterinary Record*, 179(17), 443–444. https://doi.org/10.1136/vr.i5768

Schat K.A., & Nair,V. (2008). Marek's disease. In Fadly A.M,Glisson,J.R., McDougald L.R., Nolan L.K., Swayne D.E. Disease of Poultry, 12th ed. Iowa State University Press, Ames, IA, 452-514.

Shamblin, C. E., Greene, N., Arumugaswami, V., Dienglewicz, R. L., & Parcells, M. S. (2004). Comparative analysis of Marek's disease virus (MDV) glycoprotein-, lytic antigen pp38- and transformation antigen Meq-encoding genes: Association of meq mutations with MDVs of high virulence. *Veterinary Microbiology*, 102(3–4), 147–167. https://doi.org/10.1016/j.vetmic.2004.06.007

Solomon, J.J., Witter, R.L., Stone, H.A., & Champion, L.R. (1970). Evidence against embryo transmission of Marek's disease virus. *Avian Diseases*, 14, 752–762.

Spatz, S. J., Petherbridge, L., Zhao, Y., & Nair, V. (2007). Comparative full-length sequence analysis of oncogenic and vaccine (Rispens) strains of Marek's disease virus. *Journal of General Virology*. https://doi.org/10.1099/vir.0.82600-0

Spatz, S. J., & Schat, K. A. (2011). Comparative genomic sequence analysis of the Marek' s disease vaccine strain SB-1. *Virus Genes*, 42, 331–338. https://doi.org/10.1007/s11262-011-0573-0

Spatz, S. J., Smith, L. P., Baigent, S. J., Petherbridge, L., & Nair, V. (2011). Genotypic characterization of two bacterial artificial chromosome clones derived from a single DNA source of the very virulent gallid herpesvirus-2 strain C12/130. *Journal of General Virology*, 92(7), 1500–1507. https://doi.org/10.1099/vir.0.027706-0

Suchodolski, P. F., Izumiya, Y., Lupiani, B., Ajithdoss, D. K., Lee, L. F., Kung, H. J., & Reddy, S. M. (2010). Both homo and heterodimers of Marek's disease virus encoded Meq protein contribute to transformation of lymphocytes in chickens. *Virology*, 399(2), 312–321. https://doi.org/10.1016/j.virol.2010.01.006

Sun, G. R., Zhang, Y. P., Lv, H. C., Zhou, L. Y., Cui, H. Y., Gao, Y. L., Qi X.L., Wang Y.Q., Li K., Gao L., Pan Q., Wang X.M., & Liu, C. J. (2017). A Chinese variant Marek's disease virus strain with divergence between virulence and vaccine resistance. *Viruses*, 9(4), 71. https://doi.org/10.3390/v9040071

Swayne, D. E., Fletcher, O. J., & Schierman, L. W. (1989). Avian Pathology Marek's disease virus-induced transient paralysis in chickens. 1. Time course association between clinical signs and histological brain lesions. *Avian Pathology*, 18, 385–396. https://doi.org/10.1080/03079458908418613

Tai, S. H. S., Hearn, C., Umthong, S., Agafitei, O., Cheng, H. H., Dunn, J. R., & Niikura, M. (2017). Expression of Marek's Disease Virus Oncoprotein Meq During Infection in the Natural Host. *Virology*, 503, 103–113. https://doi.org/10.1016/j.virol.2017.01.011

Trimpert, J., Groenke, N., Jenckel, M., He, S., Kunec, D., Szpara, M. L., Spatz S.J., Osterrieder N., & McMahon, D. P. (2017). A phylogenomic analysis of Marek's disease virus reveals independent paths to virulence in Eurasia and North America. *Evolutionary Applications*, 10(10), 1091–1101. https://doi.org/10.1111/eva.12515

Tulman, E. R., Afonso, C. L., Lu, Z., Zsak, L., Rock, D. L., & Kutish, G. F. (2000). The Genome of a Very Virulent Marek's Disease Virus. *Journal of Virology*, 74(17), 7980–7988. https://doi.org/10.1128/jvi.74.17.7980-7988.2000

Walkden-Brown, Stephen W., Islam, A., Groves, P. J., Rubite, A., Sharpe, S. M., & Burgess, S. K. (2013). Development, application, and results of routine monitoring of Marek's disease virus in broiler house dust using real-time quantitative PCR. *Avian Diseases*, 57(2 Suppl), 544–554. https://doi.org/10.1637/10380-92112-REG.1

Walkden-Brown, S. W., Groves, P. J., Islam, A. F., Burgess, S. K., Arzey, K. E., Mascord, L., & Young, P. L. (2003). Differentiation of Marek's diesease virus serotypes using PCR: Research and field experience. *Proceedings of Australian Poultry Science Symposium*, 15, 192–196. https://rune-uat.une.edu.au/web/handle/1959.11/4327

Wei, X., Shi, X., Zhao, Y., Zhang, J., Wang, M., Liu, C., Cui H., Hu S., Quan Y., Chen H., & Wang, Y. (2012). Development of a rapid and specific loop-mediated isothermal amplification detection method that targets Marek's disease virus meq gene. *Journal of Virological Methods*, 183(2), 196–200. https://doi.org/10.1016/J.JVIROMET.2012.04.014

Witter, R.L. (1982). Protection by attenuated and polyvalent vaccines against highly virulent strains of Marek's disease virus. *Avian Pathology,* 11, 49-62.

Witter, R.L., 1987. New serotype-2 and attenuated serotype-1 Marek's disease vaccine viruses: comparative efficacy. *Avian Diseases* 31, 752-765.

Witter, R.L. (1997). Increased virulence of Marek's disease virus field isolates. *Avian Diseases*, 41, 149-163.

Witter, R.L., & Burmester, B.R., 1979. Differential effect of maternal antibodies on efficacy of cellular and cell-free Marek's disease vaccines. *Avian Pathology*, 8, 145-156.

Witter, R. L., Calnek, B. W., Buscaglia, C., Gimeno, I. M., & Schat, K. A. (2005). Classification of Marek's disease viruses according to pathotype: Philosophy and methodology. *Avian Pathology*, 34(2), 75-90. https://doi.org/10.1080/03079450500059255

Witter R.L., Sharma J.M., & Fadly A.M.. (1980). Pathogenicity of variant Marek's disease virus isolants in vaccinated and unvaccinated chickens. *Avian Diseases*, 24, 210–232.

Witter, R.L., Solomon, J.J., & Burgoyne, G.H. (1969). Cell culture techniques for primary isolation of Marek's disease-associated herpesvirus. *Avian Diseases* 13, 101-118.

Woźniakowski, G., & Niczyporuk, J. S. (2015). Detection of specific UL49 sequences of Marek's disease virus CVI988/Rispens strain using loop-mediated isothermal amplification. *Journal of Virological Methods*, 221, 22–28. https://doi.org/10.1016/J.JVIROMET.2015.04.015

Woźniakowski, G., & Samorek-Salamonowicz, E. (2014). Direct detection of Marek's disease virus in poultry dust by loop-mediated isothermal amplification. *Archives of Virology*, 159(11), 3083–3087. https://doi.org/10.1007/S00705-014-2157-5

Woźniakowski, G., Samorek-Salamonowicz, E., & Kozdruń, W. (2011). Rapid detection of Marek's disease virus in feather follicles by loop-mediated amplification. *Avian Diseases*, 55(3), 462–467. https://doi.org/10.1637/9668-012711-RESNOTE.1

Woźniakowski, G., Samorek-Salamonowicz, E., & Kozdruń, W. (2013). Comparison of loop-mediated isothermal amplification and PCR for the detection and differentiation of Marek's disease virus serotypes 1, 2, and 3. *Avian Diseases*, 57(2 Suppl), 539–543. https://doi.org/10.1637/10328-082012-RESNOTE.1

Yehia, N., El-Sayed, H. S., Omar, S. E., Erfan, A., & Amer, F. (2021). Genetic evolution of Marek's disease virus in vaccinated poultry farms. *Veterinary World*, 14(5), 1342-1353. https://doi.org/10.14202/VETWORLD.2021.1342-1353