An Overview on Marek’s Disease Virus Evolution and Evidence for Increased Virulence in Brazil

ABSTRACT

Marek’s disease virus (MDV) has been shown to be evolving to higher virulence. One of the genetic sites involved in virulence which enables such characterization is the 339-amino acid Meq protein encoding gene (meq). The reemergence of clinical Marek’s disease (MD) in vaccinated flocks can be associated to changes in meq. Our studies have shown the presence of very virulent MDV strains in the Brazilian industrial and free-range poultry. We present an overview of MD increasing severity and indicate the necessity of using phylogenetic tools for best accompanying MDV evolution.

INTRODUCTION

During the last fifty years, the increased intensification of poultry has provided high numbers of chickens concentrated in industrial farms and specific geographical areas. The proximity of large flocks of chickens, of varied immune and health status, has enabled the circulation of infections, such as Marek’s disease (MD), infectious bronchitis, infectious bursal disease, with the emergence of a large number and diversity of pathogens, as described for MDV. The extensive vaccination of flocks against MDV has additionally provided selective pressure and possibly genetic diversity for the evolutive advantage of immunity-evading strains. After a few decades of MD vaccination, MDV strains have emerged with ever increasing virulence (Eidson et al., 1978; Eidson et al., 1981; Imai et al., 1992; McKimm-Breschkin et al., 1990; Powell & Lombardini, 1986; Sung, 2002; Venugopal, 1996; Witter, 1997; Witter et al., 2005). The estimated economic burden of MD may reach US $ 1 to 2 billion annually (Atkins, 2013).

The breakthrough description of a herpesvirus in MD tumors enabled the differentiation between Marek’s disease and Lymphoid Leukosis (Churchill & Biggs, 1967; Nazerian et al., 1968; Solomon et al., 1968), formerly considered as part of the avian leukosis complex. Consequently, research rapidly provided the tools for the prevention of MD. The understanding of the transmission mechanism and risk of infection was achieved when experimental infection with cell-free MDV of feather follicle desquamation epithelium was demonstrated (Beasley et al., 1970; Calnek et al., 1969; Calnek et al., 1970a). The virus was serially passaged in primary kidney cell monolayers and successfully attenuated (Churchill et al., 1969), and given to one-day-old chicks induced protection against the challenge (Calnek et al., 1970b), subsequently also acquired by naturally a virulent strains isolated from turkeys (Witter et al., 1970b) and chickens (Biggs & Milne, 1972; Cho & Kenzy, 1972). Among the isolated a virulent low virulence MDV strains, the CVI 988 vaccines became popular or of (Rispens et al., 1972) and SB-1 (Schat & Calnek, 1978).
Marek’s disease virus

MDV is classified as *Gallid Herpesvirus 2*, genus *Mardivirus*, family *Herpesviridae*, subfamily *Alphaherpesvirinae*, and divided into three serotypes MDV-1 (RB-1B, MD5 and CVI988), MDV-2 (SB-1 e HPRS24), and the antigenically related *Meleagrid Herpesvirus* -1 (known as serotype three; herpes virus of turkeys- strain FC126) (ICTV, 2018; Dunn *et al.*, 2014). Only strains of serotype 1 are capable of causing disease, while MDV-2 e MDV-3 strains are a virulent (Calnek, 2001). The classification of MDV according to pathotype was reviewed, including the philosophical and methodological aspects (Witter *et al.*, 2005). The correlation between MDV replication and virulence was shown for vMDV and vvMDV strains, although a non-significant difference was found between very virulent (vv) and vv+MDV isolates (Dunn *et al.*, 2014). MDV genome is large and encodes for more than 200 genes, including genes that are involved in pathogenicity, such as *meq* (Jones *et al.*, 1992; Lupiani *et al.*, 2004; Nair, 2013). MDV genomic integration was demonstrated in host cells (Nikura *et al.*, 2006). MDV encodes a basic-leucine zipper protein (MDV EcoRl-Q), similar to the fos/jun oncogenes products, that is highly expressed in tumors (Jones *et al.*, 1992). Meq is involved in the transformation of T-lymphocytes but not needed for replication (Lupiani *et al.*, 2004). Meq protein is a 339-phosphoprotein expressed abundantly by *meq* during the lytic and latent phases of cellular interaction (Gennart *et al.*, 2015), activating transcription and involved in the transformation of T lymphocytes (Gennart *et al.*, 2015; Brown *et al.*, 2009). Although *meq* is consistently associated to pathogenicity, other genes were shown to be involved (Wozniakowski *et al.*, 2010; Jarosinski *et al.*, 2006), such as *vTR* (Fragnet *et al.*, 2005; Trapp *et al.*, 2006) and *vil-8* e *pp38*. However, the oncogenicity was retained by a MDV mutant (RB1BD4.5lac) lacking unique short region genes (Parcells *et al.*, 1995).

The *meq* encoded oncogenic protein Meq is detected in all MD tumors (Ross, 1999). Meq interferes negatively with the expression (down-regulates) of cellular apoptosis genes, and up-regulates viral genes involved in cellular transformation (Liu & Kung, 1999), as well as its own expression. MDV lacking *meq* is not oncogenic, as for serotypes 2 and 3, and its deletion of pathogenic strains will result in loss of oncogenicity (Silva *et al.*, 2010; McPherson & Delany, 2016). The equilibrium of cell-virus interaction during persistence is genetically determined in Herpes simplex virus (HSV) persistently infected cells, and gradual increase in virulence as opposed to cellular resistance would result in tumorigenesis (Cummings *et al.*, 1989).

Latency starts approximately within one week of infection, mainly in T lymphocytes CD4+, although the transition from cytolytic to latent infection is not entirely understood (Nair, 2013). Latently infected T CD4+ cells in genetically susceptible unvaccinated chickens are transformed and originate tumors (McPherson & Delany, 2016), and may systemically disseminate MDV through the feather follicle epithelium, where the productive replication may resume, disseminating MDV to the environment and housing in desquamating epithelial cells (Baigent & Davison, 2004; Baigent *et al.*, 2013). During latency in T CD4+, the productive (lytic) infection is suppressed and apoptosis is blocked (Baigent *et al.*, 1998). MDV reactivation in latently infected lymphocytes will result in elevated genetic expression (McPherson & Delany, 2016). The mechanisms of latency and transformation are not well understood (Nair, 2013), although both are associated to genomic integration (NAIR, 2005), and a few T CD4+ lymphocytes will undergo transformation and give rise of T-cell tumor lineages (Calnek, 2001). Latency is mediated by the Meq protein by blocking apoptosis and gene expression is transactivated and reactivation is dependeble on *meq* repression and expression of phosphoprotein 38 (pp38), Hep and Mys encoding open reading frames (Parcells *et al.*, 2003), and the susceptibility is determined by higher numbers of pp38+ lymphocytes (Baigent *et al.*, 1998).

**Figure 1** – Marek’s disease chronological increase in severity and acuteness. (Adapted from Osterrieder *et al.*, 2006).

**MD in Brazil**

Although MD has been studied in the poultry producing countries all over the world, studies in Brazil are scarce, especially regarding the characterization of MDV strains virulence. Research in our laboratory...
(in press) has revealed the widespread occurrence of pathogenic and very pathogenic MDV in free-range and industrial chickens, with also the detection of vv+MDV (Fig. 2). Natural outbreaks, for instance in free-range chickens and involving the classical MD, include enlarged peripheral nerves, such as vagus at the proventricular/ventricular region (Fig. 3A) and at the cervical region (Fig. 3B). Preliminary findings suggest that the eventual future reemergence of MD in Brazil could be principally associated to genetic changes in meq and resulting in insufficient protection through single HVT vaccination, as described elsewhere (Wozniakowski & Samorek-Salamonowicz, 2014).

Figure 2 – Evolutionary relationships of Brazilian Marek’s disease virus strains

Brazilian industrial or free-range chickens MDV strains were evaluated as based on meq gene sequences. The strain 157 (Accession number KY322682) was grouped with GXY2 (EF546430.1), a very virulent MDV strain from China which caused acute tumors in CVI988 (Rispens) or HVT vaccinated chickens. Strains 500 (KY322683), 573 (KY322684), 578 (KY322685), 590 (KY322689), 754b (KY322689) and 755 (KT768121.1) had identity with virulent MDV (vMDV). Strains 500 (KY322683) (Fig. 2), 754b (KY322689) and 755 (KT768121.1) caused severe peripheral nerve inflammatory disease in free-range chickens. Strains 1042 (KY352470), 924-3 (KY322691) and 155 (KY322681) had substitutions in the meq oncogene compatible with highly virulent MDV strains (vvMDV), and grouped separately. Herpesvirus of turkeys (HVT) vaccine strain was added as a heterologous herpesvirus. The evolutionary history was inferred using the Neighbor-Joining method (Saitou N. and Nei M., 1987). The optimal tree with the sum of branch length = 5.66821085 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei and Kumar, 2004) and are in the units of the number of base substitutions per site. The analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 353 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar, Stecher & Tamura, 2016).

Figure 3 – Natural Marek’s disease case by strain 500. (A) Note the enlarged left vagus (black arrow) below the proventriculus (thin arrow). (B) Enlarged left vagus (cervical region) along with the jugular vein (white arrow), with enlarged branches.
An additional preoccupation has come to light in Brazil, with the description of MD in peafowl (Pavo cristatus) at a Zoological Park, characterized by visceral lymphomatosis and the detection of a virulent MDV (serotype 1) strain, through PCR and partial sequencing of the Meq protein encoding gene (Blume et al., 2016). Tumoral disease in the liver, spleen, kidneys and skin, although with mild clinical expression, was characterized by histopathology as MD in free-range chickens in Rio de Janeiro (Abreu et al., 2016). Thickened feather follicles and focal whitish tumors suggestive of MD in liver, heart, kidneys, spleen, ovary, proventriculus and peripheral nerves of broilers and layers of the poultry industry were described during 1999 to 2003 (Sousa, 2010) in Minas Gerais, Brazil.

**MD vaccination**

MD control through vaccination was paramount for the growth of the poultry industry. However, the increasing virulence of MDV strains may however compromise the success of control through vaccination (Witter, 1997) and reports of vvMDV are spread worldwide (López-Osório et al., 2017). The pioneering experiments with vaccines were developed almost immediately after the description of MD etiology (Churchill & Biggs, 1967), with its attenuation by the end of the 1960’s (Churchill et al., 1969) and the discovery of a virulent strains in turkeys (Witter et al., 1970) and chickens (Biggs & Milne, 1972; Cho & Kenzy, 1972).

The vaccination of chickens against MD is mandatory in the Brazilian poultry industry and must be given at the 18th day of incubation or at the day of hatching (Brasil, 2007). MD vaccines in use in Brazil should contain 1,500 plaque forming units per dose and are prepared with live *Meleagrid Herpesvirus* 1 (FC-126) and/or CVI988/Rispens (*Gallid Herpesvirus* 2) or SB1 (*Gallid Herpesvirus* 3) and may be monovalent or polyvalent (OIE, 2018). However, imperfect vaccination would enhance transmission of highly virulent MDV strains and other pathogens (Read et al., 2015).

**Increased virulence**

A comprehensive review was previously published, indicating the advancement of knowledge regarding MDV interaction with host cells and virulence (Osterrieder et al., 2006) (Fig. 1).

One of the driving forces involved in MDV selection to higher virulence is the immune response, possibly more relevant if vaccine derived (Davison & Nair, 2005). The pioneering description of potential evolution to evading vaccination protection was proposed very early after adoption of HVT vaccine (Okazaki et al., 1973). Virulence may evolve partially due to a compromise loss between damage and infection, and although pathogenicity might evolve from competition with the host, bacterial virulence would evolve from within the host pathogen competition (Smith et al., 2011).

Since the first description of MD in 1907, up to 1915, the principal form of clinical presentation was chronic polyneuritis. Starting from 1915 to approximately 1925, increasing number of cases of immunosuppression were registered. From 1925 up to 1975, visceral lymphoma, immuno-suppression and chronic polyneuritis were the clinical forms described in ever increasing incidence. From 1975, transient paralysis began to be encountered in the field. In 1985, cases of acute cerebral edema and acute eczema were additionally described. The classical form of MD was characterized as a paralytic syndrome of relatively low occurrence involving peripheral nerve inflammation, involving more commonly the sciatic, brachial, trigeminal, and vagus nerves, but rarely exceeding 10-15% mortality (OIE, 2018). By the end of the 1950s, with the intensification of the poultry production, more virulent forms of MD were described (Benton & Cover, 1957), characterized by up to 40% mortality in layers and the occurrence of up to 10% of broilers with visceral lymphomatosis.

During the 1960s the more aggressive forms of MD were also described in the United Kingdom (Biggs, 1965), and United States, characterized as more acute and precocious disease with early onset of visceral tumors (Biggs, 1966).

After the generalized use of day-old chicks vaccination against MD, in the beginning of the 1970s, the clinical disease was nearly eradicated, with punctual problems associated to the administration or titer of vaccines, or too early exposure to field MDV (Buscaglia & Crosetti, 1993). However, by the end of the 1970’s, visceral and early disease outbreaks were described, and associated to variant MDV strains, including in HVT (*Herpesvirus of turkeys*) vaccinated flocks, in the US and elsewhere (Eidson et al., 1978; Eidson et al., 1981; Schat et al., 1981; Powell & Lombardini, 1986; McKimm-Breschkin et al., 1990; Imai et al.; 1992; Sung, 2002).

Several very virulent strains of MDV (vvMDV) have been described since the 1980s, with the pathogenicity determined by the capacity of oncogenesis in HVT vaccinated chickens (Eidson et al., 1981). According to the capacity of evading the specific immunity derived
from HVT vaccination, the more virulent MDV strains have been classified as mildly virulent (mMDV), virulent MDV (vMDV), very virulent MDV (vvMDV), and very virulent plus MDV (v+vMDV) (Witter, 1997), being all strains above virulent capable of breaking the vaccination protection (Wozniakowski & Samorek-Salomonowicz, 2014).

The increased occurrence of vMDV strains has also been reported in vaccinated flocks in Germany, France, the Mediterranean countries, Japan and the UK (Boer et al., 1985). The description of naturally occurring vvMDV and v+vMDV has also been reported in Argentina in the early 1990s, but initially associated to vaccination error (Buscaglia & Crosetti, 1993). The outbreaks in Argentina were associated to four varying prototypes (Buscaglia et al., 1995), the first vvMDV strains of disease in vaccinated flocks in South America. In Colombia (López-Osório et al., 2017) the strain UDEACO-2013, isolated from an outbreak in chickens, was genetically related to hypervirulent strains of the United States, with the oligonucleotide position substitutions 176 (P/A), 217(P/A) and P233 (P/L) considered as indicative of vvMDV, although not related to other strains around the world, although the amino acid substitution at residue 77 (E/K) was suggestive of mMDV.

The emergence of higher virulence of MDV is associated to the selective pressure induced by the immunity derived from vaccination (Witter, 1997), in addition to the increased genetic resistance by chickens, and has resulted in the widespread description of higher virulence strains, such as in Argentina, India and China (Buscaglia et al., 2004; Zhang et al., 2011). In addition, the interaction of MDV with chicken anemia virus might result in evolving advantage (Zanella et al., 2001).

Within the last 15 years, v+vMDV strains have been described principally in vaccinated flocks (Zhang et al., 2011), indicating a lack of protective spectrum for the HVT strain. The molecular analysis of 1020 nucleotides which encode 339 amino acids of the Meq protein of Chinese MDV strains obtained from 2006 to 2008 has shown that all isolates possessed two substitutions of amino acids at residues 139 (T/A) and 176 (P/R), similar to sequences of the atenuated strain CVI988. However, six isolates have shown substitutions at positions 176 or 177 (P/T). Results have suggested a specific clade for the Chinese strains.

Very virulent (vv) MDV strains were first reported in vaccinated chickens in Europe (Powell & Lombardini, 1986) and subsequently in Australia (McKimm-Breschkin et al., 1990), and Japan (IMAI et al., 1992). In Korea (Sung, 2002), five strains of vMDV were described in layers and broilers with tumours, with one strain resulting in severe immunosuppression and high incidence of tumors (93.3%) in inoculated SPF chickens. Strains obtained of broiler flocks with visceral tumors in China were not adequately protected with the CVI988 vaccine strain (Zhang et al., 2015), protecting only 66% after the challenge with the LTS strain. MDV phosphoprotein pp38 and meq transformation protein encoding genes were evaluated, and meq mutations were associated to higher virulence (Shamblin et al., 2004).

The meq oncogene sequences of MDV strains of 2006-2008 were analyzed in China (Zhang et al., 2011), revealing 19 strains of broilers with sanitary problems. In Guangxi, the vMDV strains in vaccinated flocks were genetically distinct of the CV1988/Rispens vaccine strain, in use for 14 years (Teng et al., 2011). The characterization of MDV strains (2007-2010) of vaccinated flocks in Poland (Wozniakowski et al., 2011), revealed the recombination of MDV and REV (reticuloendotheliosis) viruses. Twelve out of 24 isolates had 68 bp insertions in the meq gene, and 0.78, 0.8, 0.82, 1.6 kb and other random LTR-REV insertions in 28 of 29 evaluated strains, although the insertions could influence MDV replication, were not associated to virulence. MDV field strains (n=85) isolated in Poland within the years 1974-2012 were compared, evaluating 85 sequences of MDV076 (RLORF7) region of meq, 60 sequences of MDV077 encoding a 23 kDa protein which binds alpha-enolase and 58 sequences of MDV077.5 (RLORF6) genes. Although the 23 kDa and LORF6 sequences were related to low pathogenic MDV, the RLORF7 sequences were similar to vMDV and vvMDV strains. However, specific motifs within the three genes could be associated to virulence, indicated an increased virulence since 2006 and strains obtained in 2012 were similar to vMDV+ strains (Wozniakowski et al., 2014).

In Egypt, vaccinated chickens showing neurological and tumoral lesions were investigated. Lesions were mostly observed in the liver, spleen and gonads, as localized or diffuse tumors, although the meq oncogene was detected in five out of the 30 chickens, with substitutions in positions 77(E/K), 80(Y/D), 88(T/A), 112(F/S), 139(A/T) and 176(R/P), although with deducted amino acid sequences showing five strains with identity (≥ 98%) with the vMDV strains ATE (Hungary), C12/130 (UK) and Chinese LMS, YA, W503 and GX070060 (Hassanin et al., 2013).
MDV meq sequences of strains were evaluated in Japan (Murata et al., 2013) and China (Yu et al., 2013), revealing point mutations and diversity potentially associated to higher oncogenicity. In Iraq (Wajid et al., 2013), MDV was detected in 49.5% of provinces, and based on meq sequences, with similar occurrence and identity of vaccinated and non-vaccinated broiler flock’s strains.

The genetic diversity of MDV in Saudi Arabia (Mohamed et al., 2016), as based on the meq gene, has shown that the strains of chickens with visceral tumors were similar to strains described in Poland, and indicated that the international trade or migratory birds might have a role in the transportation of virus. Although vaccination was implemented for commercial chickens in Colombia (López-Osorio et al., 2017), with CVI988/Rispens + HVT vaccine strains in the first day, sporadic cases of MD continue to occur, with mortality reaching up to 30% by 50 weeks of age, and no visible lymphomas were observed. Although MD outbreaks in vaccinated flocks in Argentina (Buscaglia & Crosetti, 1993) during the early 1990s were associated to vaccination failure, MDV strains of higher virulence were detected in later outbreaks (Buscaglia et al., 1995).

The emergence of vvMDV strains in Brazil strains might result of similar mechanisms as described in Germany, France, Mediterranean countries, Japan and the UK (Boer et al., 1985), Argentina (Buscaglia et al., 1995) and Colombia (López-Osorio et al., 2017). Here, the selective pressure induced by vaccination of chicks with HVT strain FC126, might have similarly enabled varying strains with evolving advantage, as detected by the end of the 1970’s in vaccinated flocks, in the US and elsewhere (Eidson et al., 1978; Eidson et al., 1981; Schat et al., 1981; Powell & Lombardini, 1986; McKimm-Breschkin et al., 1990; Imai et al., 1992; Baigent et al., 1998; Sung, 2002). However, differently to other countries, the emergence of clinical disease is still negligible. Different environmental condition for build up and challenge, as observed for infected cells in dust (Baigent et al., 2013) might play a role and may provide additional information regarding risk. In Brazil, most commonly, new chick flocks are housed in carefully cleaned and disinfected houses, which might have being provided reduced challenge, at least with clinically significant doses of field MDV. The occurrence of vvMDV in free-range flocks might arise from the eventual proximity of industrial and free-range flocks, common in certain regions, and might also result in continuous spill-over and spill-back mechanisms, although strains could also have emerged independently.

MDV strains detected in Brazil were evaluated for meq gene sequences (Fig. 2). Strain 157 (Accession number KY322682) was grouped with GXY2 (EF546430.1), a very virulent MDV strain which caused acute tumors in CVI988 (Rispens) or HVT vaccinated chickens in China. Strains 500 (KY322683), 573 (KY322684), 578 (KY322685), 590 (KY322689), 754b (KY322689) and 755 (KT768121.1) were considered virulent MDV (vMDV). Strains 500 (KY322683) (Fig. 3), 754b (KY322689) and 755 (KT768121.1) were detected in chickens with severe peripheral nerve inflammatory disease. Strains 1042 (KY352470), 924-3 (KY322691) and 155 (KY322681) had identity with highly virulent MDV strains (vvMDV) (Fig. 2).

CONCLUSION

The increasing virulence of MDV may pose as a threat to the standard MD prevention strategy, progressively reducing the success of vaccine protection, especially for programs based on HVT strain vaccines. Research and continuing surveys may provide answers regarding the epidemiology of MD, the evolving virulence of circulating MDV strains, and might enable determining the best fit vaccination protocols and strategy.

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