Marek’s disease virus (MDV) is an oncogenic avian alphaherpesvirus. Three serotypes of MDV are distinguished on the basis of biological and serological properties. Oncogenic MDV, causing T-cell lymphoma in chickens, belongs to serotype 1. Since 1970 vaccines have been used to control the disease, and three types of vaccine are currently used, alone or in combination (Calnek & Witter, 1997). In France, herpesvirus of turkeys (HVT), a natural, avirulent serotype 3 virus, and CVI-988, a non-oncogenic strain of MDV-serotype 1, have been used since the 1970s and 1980s, respectively. These vaccines prevent tumour formation but do not prevent superinfection with circulating pathogenic MDV strains. Furthermore, new strains of MDV periodically emerge – in particular, viruses capable of causing MD in vaccinated chickens have emerged in the past 20 years (Witter, 1997). The continuous evolution of MDV strains towards greater virulence is worrying, and constitutes a serious threat to future poultry production. It should also be noted that MDV is not restricted to chickens – a number of cases of MDV infection have been reported in turkeys (Davidson et al., 2002; Voelckel et al., 1999) and quails (Pradhan et al., 1985). Recently, we were able to amplify MDV sequences from human sera (Laurent et al., 2001).

Very little is known about the molecular evolution of MDV. Herpesviruses, like other DNA viruses, have low mutation rates (estimated at $3 \times 10^{-8}$ substitutions per site per year) (McGeoch et al., 2000), and single-nucleotide polymorphism (SNP) has been shown to be a useful tool for the study of genetic polymorphism in herpesviruses (Faga et al., 2001; Franti et al., 1998).

To assess the variability of MDV over 17 years (1982–1999), we carried out a retrospective molecular epidemiological study to characterize 68 French MDV isolates obtained from field cases and compare them with reference strains from the National Center for Biotechnology Information (NCBI) database. All isolates are denoted by the year of isolation followed by their specific number. Fifty-one chicken samples corresponding to paraffin-embedded samples of retrospective MD cases (1983–98) were confirmed by histological analysis. Forty-eight of these samples originated from eight French regions located in the western part of France, with 20 originating from the region of Pays de la Loire. The remaining three samples (83-2, 90-5, 94-1) originated from the Ivory Coast (Africa), although the chickens were imported as chicks from French breeding companies. The vaccination status of all these chickens was unknown. Fourteen other samples were collected in 1999, originating exclusively from Pays de la Loire, and were blood samples. The vaccination status was known: eight had been vaccinated with CVI-988 (99-2, 99-8, 99-12, 99-15, 99-13, 99-16, 99-17, 99-18) and the remaining five had not been vaccinated. Finally, three samples were obtained from turkeys with clinical signs of MDV infection. Two of these were obtained as part of the paraffin-embedded sample collection (82-T and 91-T) and the remaining sample was a blood sample dating from 1999 (99-T). Total DNA was also

Single-nucleotide polymorphisms in two Marek’s disease virus genes (Meq and gD): application to a retrospective molecular epidemiology study (1982–1999) in France

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Marek’s disease virus (MDV) is a herpesvirus that causes a lymphoproliferative disease in chickens. Vaccines against MDV are available, but the virus is gradually becoming more virulent. A molecular epidemiology study of MDV was carried out by assessing nucleotide variation in two different genes, Meq and gD, in 68 French field isolates circulating from 1982 to 1999, compared with reference strains. Viral DNA was amplified by nested PCR and sequenced directly. Comparison of the nucleotide sequences revealed a high nucleotide sequence identity (98%). Single-nucleotide polymorphisms were identified, leading to the identification of three gene alleles for gD and six for Meq. A majority of French isolates (80.5%) clustered in the C1 type, which has been present for over 17 years. Waves of non-C1-type isolates appeared when vaccine efficacy decreased. Furthermore, specific discriminating sequences were obtained for the CVI-988 vaccine strain.
extracted from a Pouvac Marek CVI vaccine dose (Fort Dodge) and the MDV-established lymphoblastoid cell lines MSB1 (Akiyama et al., 1973) and PA5 (Fragnet et al., 2003).

DNA was extracted from cell pellets of MDV cell lines, the vaccine dose, whole blood samples (50 μl) and tissue fragments as described by Fragnet et al. (2003). Tissue fragments of paraffin-embedded blocks were previously rehydrated as described by Oud et al. (1986). Two target genes specific for MDV-serotype 1 were amplified by nested PCR. The first target corresponded to part of the glycoprotein D (gD) gene described previously (Laurent et al., 2001). The gD gene is located in the short unique region of the genome and has been identified as non-essential for the oncogenicity and horizontal transmission of MDV (Anderson et al., 1998). The second target gene selected was the Meq gene, located in the long terminal repeat region, and encodes a bZIP protein of the Fos/Jun family that is involved in MDV oncogenesis (Liu & Kung, 2000). The Meq gene fragment was amplified with the

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**Fig. 1.** Definition of gD alleles. The gD sequences from 68 French field MDV isolates were aligned with four sequences of reference strains: RB1B (AY129971), GA (AF147806), MD5 (AF243438) and Woodland (U60532). The RB1B sequence was chosen as the reference sequence. Only codons displaying mutations are indicated. Dots indicate nucleotides identical to those in RB1B. Underlining indicates the specified codons that define gD alleles. Sequences are grouped by allele types (A→D), which are indicated on the left. The re-partition in specific alleles (A→D) of the 41 gD sequences amplified from human serum in a previous study (Laurent et al., 2001) is specified in italics. The codon defining sequences of human origin, is highlighted in grey. Asterisks indicate sequences presenting specific mutations. Nucleotides in bold type indicate amino acid substitution in comparison with the RB1B strain (TCA76CCA:S→P; TAT77TG:T:Y→C; ACA79ATA:T→I; AAA100ACA:K→T; TAT101CAT:Y→H; ATG105GCT:M→V).
following primers: Meq F 5'-GAGAAGGCGGCACGGTACAGGTG-3' with Meq R 5'-AACCGGACATGTGGAGCGTTAG-3' for the first round of PCR and Meq NF 5'-GAGATGTCAGGACAGCAGCC-3' with Meq NR 5'-GGAGGTTACAGGCGGATCGTGC-3' for the second round of nested PCR. The PCR conditions were as described previously (Laurent et al., 2001). Direct sequencing of gel-purified DNA was performed twice with independent DNA templates obtained from separate PCRs.

The 68 gD and Meq sequences amplified from French field samples were aligned with sequences of MDV reference strains and cell lines (Figs 1 and 2). A high degree of conservation was observed for both genes, with a maximum
divergence between pairs of isolates of 2%. Moreover, despite their difference in function and location in the viral genome, the two genes displayed similar levels of variability, with nucleotide substitutions occurring at eight of the 203 positions (3.9%) within the gD gene and at nine of the 301 positions (3%) within the Meq gene.

We further analysed sequence polymorphisms by characterization of SNPs. In our study, as previously defined by Faga et al. (2001), we considered as SNPs the nucleotide changes shared by two viruses, because a nucleotide change found in only one virus may be a specific mutation of this virus. Under this definition, three nucleotide changes in gD and five in Meq were qualified as SNPs. The SNPs identified with MDV-gD sequences correspond to three of the four SNPs identified previously in MDV-gD sequences amplified from French human sera collected in 1990 (Laurent et al., 2001). None of the gD sequences identified was of allele B, which corresponds exclusively to MDV-gD sequences of human origin. Overall, the distribution of gD alleles differed for sequences of human and avian origin. Indeed, most of the sequences of avian origin corresponded to allele C (73%), whereas allele A accounted for the majority of the gD sequences of human origin (56%). Nevertheless, with avian MDV isolates from 1990 it appears that, except for allele B, the distribution of the avian gD sequences (72% of A; 14% of C; 14% of D) is similar to the distribution of MDV sequences amplified from human sera. However, this distribution is based on a small number of avian isolates from 1990, and our current data do not allow us to determine a precise geographical or temporal relationship between MDV in avian and human samples.

For the Meq gene, six alleles (I–VI) were defined according to the five SNPs identified (Fig. 2). The two MDV tumour cell lines PA5 and MSB1 presented the specific feature of having a second mutation in codons already affected by SNPs (codons 77 and 80, respectively), confirming that these sites are hot-spot mutation regions. Our target sequence corresponded to the region encoding part of the N-terminal region of the MEQ protein which encompassed the leucine-zipper motif and the basic region containing the major nuclear localization signals (NLS) and nucleolar functions (NoLs) (Liu & Kung, 2000). Three SNPs were identified in the basic region and may consequently affect NLS or NoLs function. The other three SNPs were in the leucine-zipper motif, but none of these were residues defining this motif. It would be of interest to assess the differences in NoLs and NLS functions of the various alleles.

The Meq sequences of French avian field samples corresponded to four of the six defined alleles (I–IV). Allele II accounted for the majority of French isolates (78%), including the three samples from turkeys. The reference strains MD5, RB1B and GA were grouped in allele III together with the sequences of 14.5% of French isolates. Allele IV, represented by the vaccine strain CVI-988, accounted for only 4.5% of the French isolates whose vaccination status was unknown. None of the eight chickens known to have been vaccinated with CVI-988 was associated with allele IV, showing that virulent MDV was detected preferentially in vaccinated chickens. Finally, 3% of French isolates clustered with the five Chinese strains in allele I. The remaining two alleles were represented by the Meq sequence amplified from quail cells (QT35, allele V; Yamaguchi et al., 2000) and MDV tumour cells (MSB1), together with the two strains of Chinese origin (st648A and st814, allele VI).

Most of the French isolates clustered in allele II, which did not include any of the reference MDV strains of foreign origin present in the database. Furthermore, strains RB1B (Schat et al., 1982), GA (Eidson & Schmittle, 1968) and MD5 (Witter et al., 1980) originating from the USA had allele III; the eight strains of Chinese origin fell into two other specific allele groups, I and VI; and the European strains, CVI988 of Dutch origin (Rispens et al., 1972) and the PA5 cell line from chickens infected with strain HPRS16 (Purchase & Biggs, 1967) of British origin, both had allele IV. It would be of interest to extend this study to MDV strains from other countries, to evaluate potential clustering as a function of geographical origin.

Theoretically, given that avian sequences displayed three and six alleles for the gD and Meq genes, respectively, 18 different combinations were possible. Only nine combinations (C1–C9) were observed, and 85% of French field samples corresponded to three of these combinations: C1, C2 and C3 (Table 1). Furthermore, 60.5% of the samples, including the three turkey samples, were grouped in a single combination (C1) where no reference MDV strain was represented. The six minor combinations (C4–C9) each corresponded to only zero to three French samples. No avian field sample was found in the C9 group, which contained the CVI-988 strain, showing that even in CVI-988-vaccinated chickens only virulent MDV were detected. PA5 was also associated with the C9 combination, but differs from CVI-988 by two other specific mutations in the Meq sequence (Fig. 2). Hence the PA5 and CVI-988 strains can be differentiated. This finding could be important for the development of a specific system for detecting the CVI-988 vaccine virus in chicks after vaccination, in order to monitor correct administration of the vaccine.

When we investigated the distribution of combinations of SNPs as a function of time, five periods (P1–P5) were distinguished that could be linked with MD epizootics and vaccination campaigns in France (Table 1). The major combination, C1, was observed throughout P1–P5. During P3, an increase in losses due to MD was observed in France and an epizootic of MD affecting turkeys was also reported. This period corresponds to the first outbreak of MD in French poultry flocks since 1984, the year in which the CVI-988 vaccine was introduced in France. During P3, the C1 combination accounted for only a minority of strains, with the proportions of the C2, C3 and C4 combinations increasing. Following this outbreak, bivalent
vaccination with HVT and CVI-988 was advocated, as had been practised for several years in the USA with SB1 and HVT. As following the introduction of the CVI-988 vaccine in France (P2), only C1 isolates were detected during the new vaccination campaign (P4), which appears to indicate that viruses of the C1 type are prevalent in France and continue to circulate regardless of poultry vaccination practices. In 1996 and subsequent years (P5) a second outbreak of MD, associated with two new forms of disease, hit poultry flocks in France and other European countries (Kross et al., 1998). During this outbreak, as during the first (P3), combinations of non-C1 types were amplified. Overall, our data suggest that the appearance of non-C1 strains could be an indicator of a decrease in vaccine efficacy; therefore it would be useful to continue surveillance of MD strains circulating in France and other countries.

### References


### Table 1. Distribution of allele combinations for French avian MDV sequences

<table>
<thead>
<tr>
<th>Combination</th>
<th>Allele</th>
<th>Field sample</th>
<th>MDV strain</th>
<th>Time period (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>C II</td>
<td>41</td>
<td>60.5%</td>
<td>NR</td>
</tr>
<tr>
<td>C2</td>
<td>A II</td>
<td>10</td>
<td>14.5%</td>
<td>NR</td>
</tr>
<tr>
<td>C3</td>
<td>C III</td>
<td>7</td>
<td>10%</td>
<td>GA</td>
</tr>
<tr>
<td>C4</td>
<td>D II</td>
<td>3</td>
<td>4.5%</td>
<td>NR</td>
</tr>
<tr>
<td>C5</td>
<td>A I</td>
<td>2</td>
<td>3%</td>
<td>NR</td>
</tr>
<tr>
<td>C6</td>
<td>A III</td>
<td>2</td>
<td>3%</td>
<td>MD5-BB1</td>
</tr>
<tr>
<td>C7</td>
<td>C IV</td>
<td>2</td>
<td>3%</td>
<td>CVI-988</td>
</tr>
<tr>
<td>C8</td>
<td>D IV</td>
<td>1</td>
<td>1.5%</td>
<td>NR</td>
</tr>
<tr>
<td>C9</td>
<td>A IV</td>
<td>0</td>
<td>0%</td>
<td>CVI-988</td>
</tr>
</tbody>
</table>

NR, Not represented.

*Year when CVI-988 vaccine was introduced in France.

†Year when practice of bivalent vaccination HVT+CVI-988 began.