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BRIEF REPORT

Variations in the H/ACA box sequence of viral telomerase RNA of isolates of CVI988 Rispens vaccine

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Abstract The use of the complete DNA sequence for the Marek's disease virus (MDV) serotype 1 vaccine strain CVI988 Rispens in comparative genomic studies with virulent strains of MDV has revealed the presence of a number of insertions, deletions and single-nucleotide polymorphisms. In this study, we investigated a SNP in the H/ACA box of the viral RNA subunit of telomerase (vTR). We sequenced vTR from four different batches of CVI988 vaccine originating from a single commercial company. The A-to-G mutation defining the SNP in the H/ACA box of CVI988 vTR was present in only some of the batches. Thus, although this mutation affects CVI988 vTR function, it is not shared by all CVI988 isolates and may be a stochastic rather than causative event in CVI988 attenuation.

Marek's disease virus (MDV; family *Herpesviridae*, genus *Mardivirus*, species *Gallid herpesvirus 2*, GaHV-2) is an oncogenic avian alphaherpesvirus which causes a highly contagious, malignant T lymphoma in chickens [30]. MDV GaHV-2 strains are divided into oncogenic strains comprising virulent pathotypes and attenuated non-oncogenic strains [29]. The non-oncogenic CVI988 (Rispens) strain, a naturally attenuated GaHV-2 strain [8, 22], has been extensively used in vaccines to control MD [2]. The MDV genome is composed of 180-kbp double-stranded DNA [14, 27]. It is structurally homologous to the genomic DNA of human simplex viruses (HSV), consisting of a long sequence (UL) and a short sequence (US), each of which is flanked by inverted terminal TRL-TRS and internal IRL-IRS repeats [4]. The repeat long (RL) region of GaHV-2 contains genes that play an important role in the pathogenesis of Marek's disease [5, 14, 27]. Among them, a GaHV-2-specific gene, *meq*, encodes a basic leucine zipper oncoprotein [13, 16], which is required for MDV-induced lymphomagenesis [1, 17]. Another viral gene of the RL region encodes the viral telomerase RNA subunit (vTR), which has 88% identity to chicken TR [9]. vTR has a major effect on the malignancy of MDV-induced lymphoma [26]. vTR promotes development of tumours, probably by enhancing telomerase activity, leading to the stabilisation of telomeres and consequently to cell immortalisation and blockade of apoptosis. Indeed, a comparative functional study has shown that vTR yields higher levels of telomerase activity than its chicken orthologue, chTR, when combined with recombinant chicken TERT protein in vitro [10]. This greater efficiency of vTR over chTR seems to be due to mutations affecting the template-pseudoknot core domain, which stabilise the interaction with chTERT. vTR has conserved vertebrate TR secondary structures comprising four main structural domains: the pseudoknot (core) domain, which contains the template sequence (the CR1 domain) for synthesis of complementary DNA and is essential for the stable assembly with TERT, the CR2 and the CR3 conserved regions; the CR4/CR5 domain, essential for functional telomerase activity by enhancing the processivity of nucleotide addition; the H/ACA boxes; and

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the CR7/CR8 domain, required for the 3′-end processing, stability, and nucleolar localisation of the RNA telomerase genes within cells [6, 7, 11, 18, 20]. Notably, the vTR gene is present in the genome of all GaHV-2 strains, regardless of their pathotype.

Comparison of RL region nucleotide sequences from 13 pathotypically distinct MDV strains recently showed a high level of nucleotide variation among strains, some of them related to pathotype [24]. Among these variants, single-nucleotide polymorphisms (SNPs) were reported in the 48 ORFs of the RL region that were examined [24]. Remarkably, 73% of the SNPs occurred in ORFs within the *meq* locus. Indeed, nucleotide changes within the *meq* locus phylogenetically characterise the Meq protein according to pathotype, with attenuated and non-attenuated strains clustering into two distinct clades [24]. Surprisingly, none of the 13 examined strains, including CVI988 isolates, had the H/ACA domain A/G SNP identified by Fragnet et al. [10] in their CVI988 isolate. This substitution results in a lack of vTR accumulation within the cell nucleus, correlates with decreased telomerase activity and might be involved in CVI988 attenuation [10]. We therefore compared the vTR sequences from different batches provided by a single commercial company to determine the possible origin of this discrepancy.

We isolated genomic DNA from four different batches (A to D) of MDV CVI988 obtained from Fort Dodge Animal Health company (Tours, France) and from peripheral blood leucocytes (PBL) of chickens infected with MDV RB-1B for cloning and sequencing. Briefly, genomic DNA was extracted from vaccine doses or PBL after incubation with 500 µg/ml proteinase K, 0.5% sodium dodecyl sulphate and 10 mM Tris (pH 8) for 2 h at 65°C. Nucleic acids were then extracted with phenol–chloroform, followed by ethanol precipitation. The resulting pellet was suspended at a final concentration of 100 µg/ml. vTR genes were PCR-amplified from the genomic DNA of MDV CVI988 batches and the RB-1B strain using the primer pairs 791 (5′-AGGCCTCGGACACGTGGCGGGTGGAA GGCTCCGC-3′) and 774 (5′-GCGTGTGGGAGCGACGC CGT-3′). These primers amplified the entire coding sequence of vTR. PCR was carried out in a final volume of 100 µl; 5% dimethyl sulphoxide (DMSO) was included in the reaction mixtures to reduce secondary structure formation. The cycling conditions used were: 35 cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 45 s, followed by a final extension at 72°C for 10 min. vTR PCR amplicons of 442 bp were purified from agarose gels (NucleoSpin Extract II) and ligated into pGEM-T Easy cloning vector (Promega) before being electroporated into *Escherichia coli*. Bacterial colonies were screened directly by PCR for the presence of insert. pGEMT-T vectors containing the insert were purified, and the vTR DNA templates were sequenced from both ends with T7 forward and SP6 reverse primers (MWG Biotech AG, Germany). We sequenced between 3 and 22 clones for each CVI988 batch and 16 clones for the RB-1B strain. The sequences of attenuated through very virulent plus GaHV-2 strains used for comparison were obtained from GenBank (Accession numbers DQ534530 through DQ534542, DQ 530348) [24].

We first checked that all 16 clones isolated from PBL genomic DNA of chickens infected with the MDV RB-1B strain had the expected A nucleotide at position 364 in the H box, shared by all the oncogenic GaHV-2 strains (Fig. 1). Most clones (12/16) contained the expected RB-1B sequence; 12 mutations located outside TR conserved domains were observed in four clones, seven of which were shared by two clones (Fig. 1).

Among the four CVI988 isolates, only batches A and D had the A-to-G mutation in the vTR H box at position 364 described by Fragnet et al. [10]. In both cases, the A-to-G mutation was shared by all the clones identified from the isolates (Fig. 1). Additionally, we identified one T-to-C mutation at position 26 upstream from the CR1 domain in 1/22 clones from CVI988 isolate A and in 3/17 clones from CVI988 isolate B, potentially defining a new SNP. In contrast, the mutations T to C or T to G at position 86 in the CR2 conserved domain and C to T or C to A at position 336 between the CR5 and CR6 domains were only found in CVI988 isolate A clones, in 7/22 and 1/22 clones, respectively.

Sequences of vTR from the 14 GaHV-2 strains obtained from GenBank compared to our RB-1B reference [9] displayed polymorphisms only in the A and C stretches. Therefore, alignments of vTR sequences from isolates of the present report and from the other 14 GaHV-2 strains did not show any nucleotide polymorphism except the SNP located at position 364 in the vTR H box, only present in our CVI988 isolates A and D (Fig. 1).

Therefore, in this study, we showed that CVI988 isolates may harbour a SNP in the vTR H box. Moreover, CVI988 isolates from batches provided by a single company may or may not harbour this SNP, but there was no variation for the SNP within a given batch. These findings thus explain the apparent discrepancy between the vTR sequence described by Fragnet et al. [10], the CVI988 BAC sequence and the data from Spatz and Silva [24]. Indeed, Spatz and Silva [24] did not find the A-to-G SNP initially identified by Fragnet et al. [10] in any MDV strains sequenced in their study. The vTR sequence of a CVI988 isolate from Intervet, another commercial company involved in the report by Spatz and Silva [24], was included in our comparative analysis as well as one CVI988-BP5 passaged in experimental chickens and the RM-1 or R2/23 attenuated strains (Fig. 1). Therefore, the A-to-G SNP shown to impair vTR nuclear accumulation and resulting telomerase

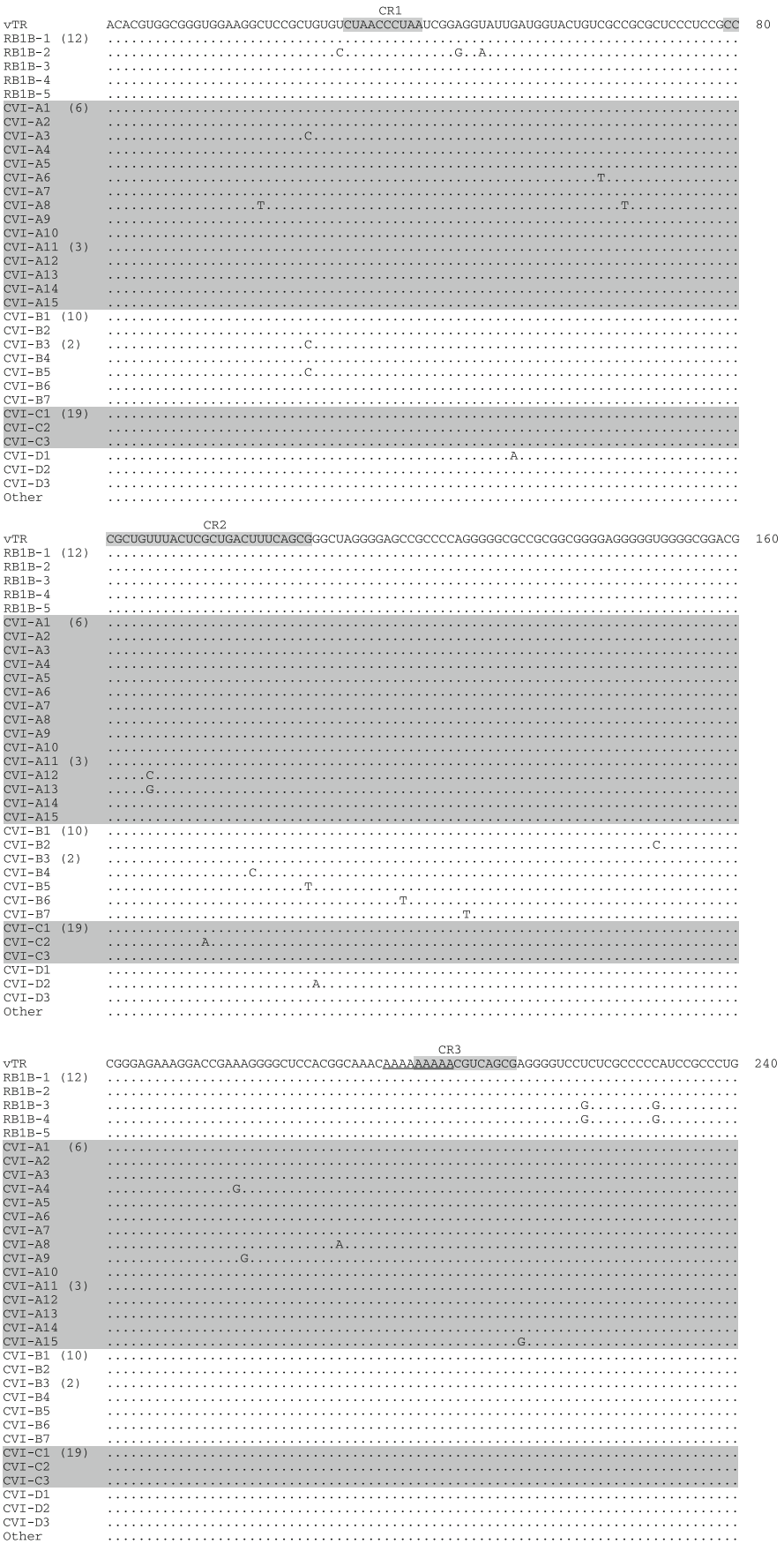
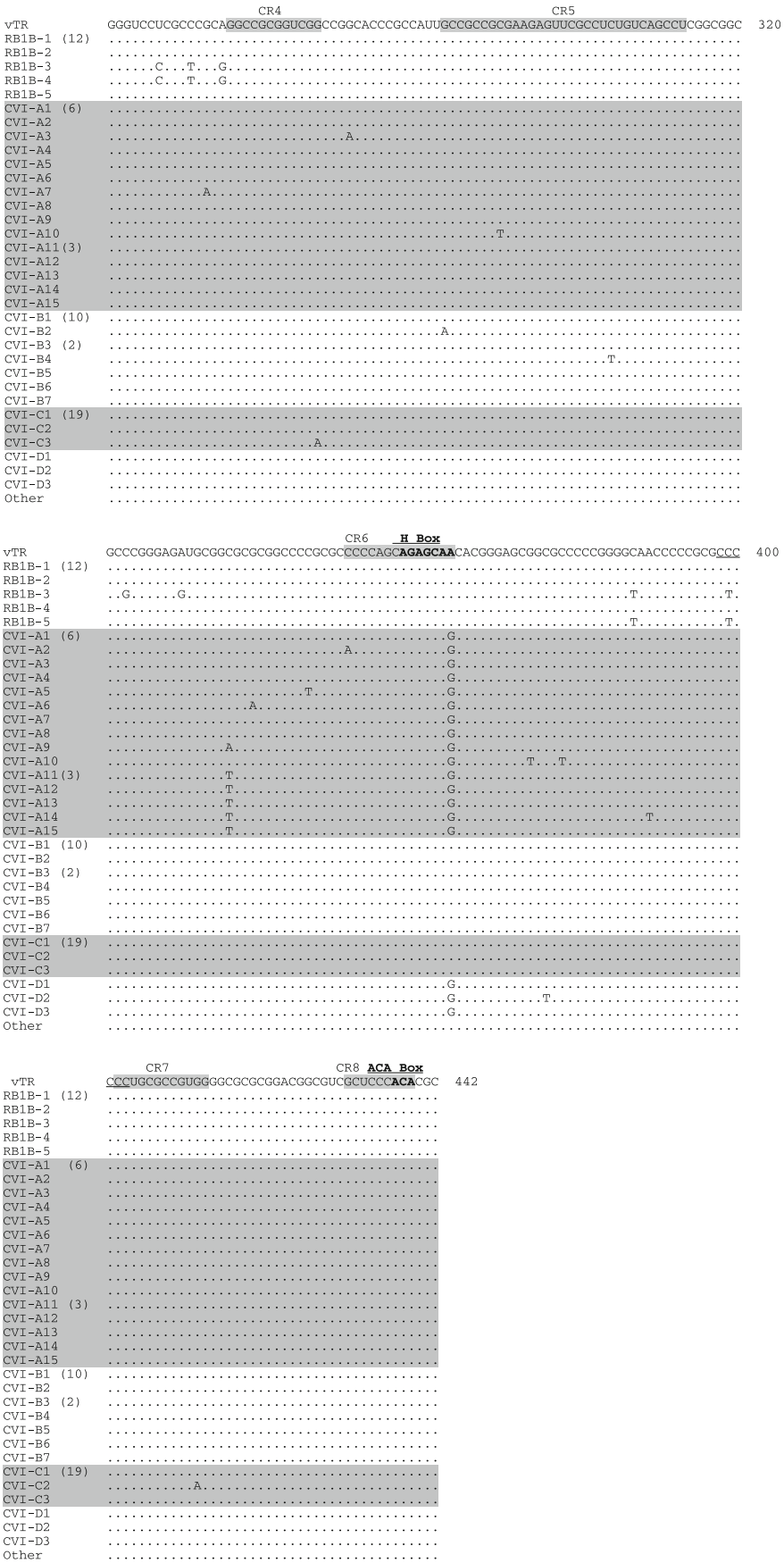


Fig. 1 Alignment of vTR RNA sequences from GaHV-2 strains. The RB-1B vTR sequence is given as reference [9]. Conserved domains *CR1*, *CR2*, *CR3*, *CR4*, *CR5*, *CR6*, *CR7*, *CR8* are shaded. The H/ACA boxes are indicated in *bold*. The stretches of A (site 196–205) and C (site 398–402) are *underlined*. RB-1B 1–5 designate sequences of different clones from DNA extracted from peripheral blood leucocytes of a chicken infected with RB-1B. CVI988 A to D are four different batches of CVI988 provided by the Fort Dodge company. Each sequence is indicated by a *number*. The number in *brackets* indicates the number of clones corresponding to the sequence. The other refers to sequences of GaHV-2 strains obtained from GenBank: CVI988-Intervet, CVI988-BP5, CVI-988 BAC, RM-1, Md11, CU-2, JM/102W, RB-1B(ADOL), 549a, 571, 595, 584a, 648a, and 686. All sequences displayed a stretch of 6 C's except RB-1B(ADOL), where 9 C's were present. The A stretch polymorphisms varied from 9 displayed in most strains to 10 in 584a, 11 in 549a, 12 in CVI988 Intervet, CVI988 BP5, CU2, and 13 for RB-1BADOL. A *dot* means same as reference

Fig. 1 continued



activity [10] does not seem to be the main causative, initial mutation responsible for GaHV-2 attenuation. This is also consistent with findings by Spatz and Silva [24] showing attenuated and non-attenuated strains clustered as a function of *meq* sequences and the identification of additional regions of variation between the CVI988 genome sequence and virulent GaHV-2 strains of MDV [25]. Previous comparative genomic studies showing major genetic changes and the occurrence of 73% of SNPs in ORFs within the *meq* locus [24], in vitro experiments demonstrating the transforming properties of MEQ oncoprotein, and the absence of lymphoma after infection with a *meq*-null GaHV-2 recombinant [15–17] collectively suggest that changes in the *meq* locus may underlie CVI988 attenuation. Genetic changes within the *meq* locus are likely to cause the inhibition of CVI988 MEQ protein transactivating activity observed in reporter assays [5, 21] and may alter CVI988 viral gene transcription observed in PBL from CVI988-infected chickens (Debba-Pavard et al., submitted). However, one of the variable regions identified by Spatz and Silva [25], the expansion of the 132-bp repeat located in the TRL and IRL regions, initially thought to be responsible for attenuation through serial passages in cell culture, has more recently been shown to occur concomitantly with attenuation but is not sufficient to cause attenuation [23]. Similarly, the mutation in the H box of CVI988 vTR may have occurred upon CVI988 amplification in cell culture, possibly participating in the loss of pathogenicity of CVI988, but is not the causative mutation of attenuation. Consistent with this, low telomerase activity observed in PBL from chickens infected with CVI988 harbouring the mutated H box may be due to a low expression level of vTR (Debba-Pavard et al., submitted) rather than failure of vTR to accumulate in the nucleus, as observed in vitro. The expansion of the 132-bp repeat in the TRL and IRL regions and the CVI988 vTR H box mutation may therefore be considered stochastic mutations occurring alongside the loss of oncogenicity of CVI988. CVI988 is a GaHV-2 virus belonging to the family *Herpesviridae*. Like other DNA viruses, it is thought to have a low mutation rate [19]. Its attenuation seems to occur as a polygenic trait, resembling the loss of fitness in cell culture or during in vivo serial passages in a natural host of foot-and-mouth disease virus replicating as viral quasispecies, with high mutation rates [3, 12].

The origin of the CVI988 vTR polymorphism is not known. We showed that it occurs among batches from a single company. The rate of mutation observed in these CVI988 isolates also differed, possibly reflecting differences in cell culture passage history of individual batches. Notably, CVI988 initially isolated from a flock of chickens as a low-pathogenic, circulating virus was used as an experimental vaccine as soon as after the fourth passage in cell culture [22]. Additional passages in cell culture resulted in an almost complete loss of residual pathogenicity at the 26th passage, whereas contact transmission and the ability to confer protection were retained [22]. In another study, contact transmission was inefficient by the 45th passage, demonstrating the continuous phenotypic and likely genotypic changes occurring in CVI988 isolates [28]. Comparative genomic studies of CVI988 isolates after different numbers of passages will be needed to determine the timing of the CVI988 vTR H box mutation and to carry out more extensive studies of stochastic and causal mutations involved in CVI988 attenuation in vivo.

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