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Real-time PCR for differential quantification of CVI988 vaccine virus and virulent strains of Marek's disease virus

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CVI988/Rispens vaccine, the 'gold standard' vaccine against Marek's disease in poultry, is not easily distinguishable from virulent strains of Marek's disease herpesvirus (MDV). Accurate differential measurement of CVI988 and virulent MDV is commercially important to confirm successful vaccination, to diagnose Marek's disease, and to investigate causes of vaccine failure. A real-time quantitative PCR assay to distinguish CVI988 and virulent MDV based on a consistent single nucleotide polymorphism in the pp38 gene, was developed, optimised and validated using common primers to amplify both viruses, but differential detection of PCR products using two short probes specific for either CVI988 or virulent MDV. Both probes showed perfect specificity for three commercial preparations of CVI988 and 12 virulent MDV strains. Validation against BAC-sequence-specific and U_S2-sequence-specific q-PCR, on spleen samples from experimental chickens co-infected with BAC-cloned pCVI988 and wild-type virulent MDV, demonstrated that CVI988 and virulent MDV could be quantified very accurately. The assay was then used to follow kinetics of replication of commercial CVI988 and virulent MDV in feather tips and blood of vaccinated and challenged experimental chickens. The assay is a great improvement in enabling accurate differential quantification of CVI988 and virulent MDV over a biologically relevant range of virus levels.

1. Introduction

Marek's disease (MD) is a highly infectious lymphoid neoplasm of chickens caused by oncogenic serotype-1 strains (MDV-1) of Marek's disease virus (family *Herpesviridae,* subfamily *Alphaherpesvirinae,* genus *Mardivirus*). Susceptible breeds of chicken develop visceral and neural lymphomatous lesions (Baigent and Davison, 2004; Calnek, 2001), resulting in death or carcass condemnation. This economically important disease has been successfully controlled since the 1970s by vaccination using cell-associated live avirulent and nononcogenic vaccine strains of all three *Mardivirus* serotypes. Serotype 2 (MDV-2) or serotype 3 (herpesvirus of turkeys, HVT) vaccine viruses are naturally avirulent in chickens (Okazaki et al., 1970; Schat and Calnek, 1978), while CVI988/Rispens vaccine (de Boer et al., 1986; Rispens et al., 1972) is a naturally-attenuated MDV-1 strain. MD vaccine viruses establish persistent infection and lifelong immunity, effectively protecting against tumours and mortality. However, vaccination does not prevent superinfection, replication and shedding of virulent challenge viruses (Davison and Nair, 2005; Gimeno, 2008), so chickens can potentially be infected simultaneously with both vaccine and virulent MDV strains.

Accurate differential measurement of vaccine and virulent virus in the same individual chicken is both commercially important and experimentally useful. In the field, it could confirm successful vaccination and assist in identifying causes of vaccine failure, such as administration of a sub-optimal vaccine dose (Landman and Verschuren, 2003), interference with vaccine virus replication by maternal antibodies (King et al., 1981; de Boer et al., 1986), and infection with virulent MDV field strains (Witter et al., 2005). In the laboratory, it could be used to study mechanisms of vaccinal protection and differences in efficacy between vaccines.

Abbreviations: 40-Ct, cycle threshold value in real-time PCR subtracted from 40; BAC, bacterial-artificial-chromosome; dpc, days post challenge; dpv, days post vaccination; FAM-BHQ1, probe with carboxyfluorescein (FAM) reporter fluorochrome and Black Hole Quencher; LOD, limit of detection by q-PCR; ovo, chicken ovotransferrin gene; PBL, peripheral blood lymphocytes; pp38-CVI, PCR probe specific for CVI988 pp38 gene sequence; pp38-Vir, PCR probe specific for virulent MDV pp38 gene sequence; q-PCR, real-time quantitative polymerase chain reaction; SNP, single nucleotide polymorphism; YY-TAMRA, probe with Yakima Yellow reporter fluorochrome and tetramethylrhodamine quencher.

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Therefore, it is important to have a reliable test for differentiation of MDV-infected and vaccinated animals (DIVA test). Real-time quantitative polymerase chain reaction (q-PCR) is a sensitive and specific molecular diagnostic method. Serotype-specific q-PCR can be used to distinguish and differentially quantify MD vaccine and challenge virus, where vaccine is of serotype 2 (Islam et al., 2004; Renz et al., 2006) or serotype 3 (Islam et al., 2004, 2006a,b), and thus of a different serotype to challenge virus (always serotype 1). CVI988 is the 'gold standard' vaccine against MD because it is antigenically and genetically 98% identical to virulent strains (Spatz et al., 2007a), and therefore elicits a very effective immune response. However, the downside of this is that CVI988 is not easily distinguishable from virulent MDV-1, restricting the application of differential molecular diagnostics (Baigent et al., 2006b). To date, development of a q-PCR assay which is both accurately quantitative and able to universally distinguish all virulent MDV strains from CVI988, has not been possible.

Using bacterial-artificial-chromosome (BAC) cloned CVI988 virus (pCVI988), which carries the BAC vector sequences in place of the MDV-1 U_S2 gene, q-PCR assays for differential quantification of pCVI988 from virulent MDV (by targeting the BAC vector sequence and the U_S2 gene respectively) were developed previously (Baigent et al., 2011). Although these assays allowed investigation of replication of both pCVI988 vaccine virus and virulent MDV-1 in experimental infections (Baigent et al., 2011), they cannot be used in the field as commercial CVI988 does not contain a BAC sequence. However, the BAC-U_S2 q-PCR system, and the DNA samples generated in that study, represents an ideal system against which to validate any prospective CVI988-specific and virulent MDV-1-specific q-PCR assays.

Spatz and Silva (2007) identified several types of sequence difference in the repeat long (RL) genomic regions of 13 MDV-1 strains of varying virulence: polynucleotide sequence expansions, short tandem repeat polymorphisms, sequence deletions, frameshift mutations and single nucleotide polymorphisms (SNPs). The efforts of other researchers to develop DIVA PCR assays for CVI988 and virulent MDV have focused on the polynucleotide sequence expansion of the 132-bp repeat region, the 177-bp insertion of duplicated sequence in the CVI988 *meq* gene, and SNPs in the pp38 gene. Conventional PCR to amplify the 132-bp repeat region (Becker et al., 1992; Silva, 1992) could distinguish CVI988 from virulent MDVs based on a difference in the number of 132-bp repeats, but had relatively low sensitivity (Davidson et al., 2002). A highly sensitive quantitative *meq* gene q-PCR, that detects both CVI988 and virulent MDV (Baigent et al., 2005a), can be used to quantify either CVI988 or virulent MDV if only one of those viruses is present (Baigent et al., 2005a,b, 2006a,b), or can be paired with the 132-bp repeat PCR to assist with distinction between CVI988 and virulent MDV in mixed infections. However, 132-bp repeat PCR is not very sensitive for CVI988 (since the multiple bands of the CVI988 PCR products have much lower intensity than the single/double bands of the virulent MDV PCR products), not absolutely conclusive (Baigent et al., 2005b; van Iddekinge et al., 1999; Niikura et al., 2006; Petherbridge et al., 2003; Silva and Gimeno, 2007), laborious, and the repetitive nature of the 132-bp repeat region precludes its use as a quantitative assay.

The *meq* gene is polymorphic, with variation in the number of sequence repeats in the proline-rich region (Chang et al., 2002). Murata et al. (2007) developed a non-quantitative nested PCR to distinguish between standard *meq* and large (L-)*meq* (with a 177-bp insertion). However, while virulent MDV strains have standard *meq*, CVI988 may have either L-*meq* or standard *meq* (Lee et al., 2000), so the 177-bp insertion is not a reliable differential marker for CVI988 and virulent MDV. Based on a stable polymorphism in the *meq* gene, Renz et al. (2013) developed two sensitive and specific real-time PCRs to differentiate and accurately quantify 20 Australian virulent MDV isolates and three commercial CVI988 vaccines used in Australia. However, virulent isolates from USA, Asia and Europe do not differ from CVI988 at this polymorphism so, while this assay could be very successfully used in Australia, it could not be used globally (Renz et al., 2013).

The MDV-1 pp38 gene has a SNP at base #320, which is consistent between CVI988 (which has 'G' at this position) and all sequenced virulent strains (which have 'A') (Cui et al., 1991; Endoh et al., 1994; Spatz et al., 2007a,b). The current study therefore investigated the possibility of using SNP #320 as a reliable biomarker for differentiating and quantifying CVI988 and virulent strains by real-time PCR (pp38 SNP q-PCR assay). Subsequent to development of this assay, Gimeno et al. (2014) published a real-time PCR assay also based on SNP #320. However, their assay differs from the assay reported in the current paper in terms of the molecular methods used to amplify and detect the polymorphism, their ability to fully validate the assay, and its quantitative accuracy.

The aims of the current work were (1) to design and optimise a q-PCR assay based on SNP #320 in the pp38 gene to specifically distinguish CVI988 from virulent MDV-1 strains; (2) to investigate the feasibility of making this assay accurately quantitative over a biologically relevant range of virus levels; (3) to validate the assay against the BAC/U_S2 q-PCR assay; and (4) to use the assay to investigate replication of CVI988 and virulent MDV in tissues of vaccinated, challenged experimental chickens.

2. Materials and methods

2.1. Design of q-PCR primers and probes

Primers and probes were designed by AlleLogic Biosciences Corp (Hayward, CA). Primer and TaqMan® probe sequences, and their location within the pp38 gene, are shown in Fig. 1. The forward and reverse primers (pp38-FP and pp38-RP) are common to both the CVI988 and virulent MDV-1 pp38 sequences, and the 99-bp amplicon covers the polymorphic region within which the probes were designed. One 15-mer probe (pp38-Generic) was designed to target a region common to all MDV-1 strains. Two 15-mer probes were designed to incorporate the consistent G/A SNP #320, one specific for the CVI988 sequence (pp38-CVI), and one specific for the virulent MDV-1 sequence (pp38-Vir(1)). Subsequently, an additional probe (pp38-Vir(3)) was designed for detection of virulent MDV-1, to have a melting temperature similar to that of the pp38-CVI probe and the pp38-Generic probe. Probe pp38-Vir(3) also incorporates the 'non-consistent' SNP #326, so it is possible that affinity for some field strains may be reduced. However, in this study, the specificity of pp38-Vir(1) and pp38-Vir(3) was identical, but pp38-Vir(3) was chosen as the probe for routine detection of virulent MDV-1 because it was more sensitive. Each probe was labelled with 5′FAM reporter, and 3′BHQ1 quencher. All probes were used in combination with the same primer pair. All primers and probes were manufactured by Sigma Genosys (UK).

2.2. DNA samples from virus stocks and chicken tissues

HVT strain FC126, MDV-1 strain HPRS-B14, and USA MDV-1 strains 675A, 584A, 648A, 660A, 595, Md5, 549, 571, JM102/W (Witter, 1997; Witter et al., 2005) were obtained from Dr. A.M. Fadly (Avian Disease and Oncology Laboratory, USA) all as 7th duck embryo fibroblast passage stocks. MDV-1 strain RB-1B was prepared as previously described (Baigent et al., 2007). European MDV-1 isolate C12/130 (Barrow and Venugopal, 1999) was prepared as previously described (Smith et al., 2011). Three commercial CVI988 vaccine stocks were used: Pfizer Poulvac Marek CVI, Merial BioMarekR, and Intervet Nobilis Rismavac. MDV-2 strain