Replication kinetics of Marek’s disease vaccine virus in feathers and lymphoid tissues using PCR and virus isolation

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CVI988 (Rispens), an avirulent strain of Marek’s disease virus, is the most widely used vaccine against Marek’s disease. The kinetics of replication of CVI988 was examined in tissues of chickens vaccinated at either 1 day or 14 days of age and sampled regularly up to 28 days post-vaccination. Age at vaccination had no significant effect on the kinetics of CVI988 virus replication. During the cytolytic phase of infection (1–7 days), virus levels peaked in the spleen, bursa and thymus with very close correlation among these organs. Virus load in peripheral blood lagged behind and did not reach high levels. Significant numbers of virus genomes were detected in the feather tips only after 7 days, but subsequently rose to levels almost 10³-fold greater than in the other tissues. This is the first accurate quantitative data for kinetics of CVI988 replication in a variety of tissues. There was good correlation between data from virus isolation and PCR, with real-time PCR being the preferred method for rapid, accurate and sensitive quantification of virus. Feathers were ideal for non-invasive sampling to detect and measure CVI988 in live chickens and, from 10 days onwards, virus load in feather tips was predictive of virus load in lymphoid tissues where immune responses will occur. The potential for real-time PCR analysis of feather samples for further investigation of the mechanism of vaccinal protection, and to assist optimization of vaccination regimes, is discussed.

INTRODUCTION

Serotype 1 strains of Marek’s disease herpesvirus (MDV-1) are highly contagious and oncogenic, and the causative agent of Marek’s disease (MD) in chickens. The virus initially replicates in a cell-associated form in the lymphoid tissues, resulting in lymphocytolysis and inflammation. A switch to latent infection occurs after about 7 days, with latently infected lymphocytes harbouring the MDV genome with limited expression of viral antigens. These lymphocytes migrate through the bloodstream to visceral organs and peripheral nerves where they become neoplastically transformed and, in susceptible chickens, proliferate to form gross lymphomas 3–4 weeks post-infection (reviewed by Calnek, 2001; Baigent & Davison, 2004). These lesions are responsible for the symptoms of and eventual mortality from MD. From 10 days post-infection onwards, MDV undergoes fully productive replication in the feather follicle epithelium. High levels of MDV antigens are expressed, and cell-free virus is shed with skin and feather debris throughout the life of an infected bird (Calnek et al., 1970). This virus is the source of infection for other chickens, via the respiratory route (Beasley et al., 1970; reviewed by Baigent & Davison, 2004).

MD is, to date, the only tumour disease for which effective vaccines are widely used. Commercial chickens are vaccinated at 1 day of age with live virus. It is likely that MD vaccine viruses establish a persistent latent infection and stimulate both cellular and humoral immune responses, reducing early viraemia and protecting against tumours and mortality after subsequent exposure to pathogenic strains (Morimura et al., 1998; Baaten et al., 2004). However, significantly, MD vaccines prevent neither superinfection nor multiplication and excretion of the challenge virus from feather tissues, and the virulent strain shed by vaccinated birds remains oncogenic to non-vaccinated birds. MDV field strains are gradually evolving towards pathotypes of greater virulence (Witter, 1997; Witter et al., 2005), a process that may be driven by vaccination itself.

CVI988 Rispens strain, an avirulent MDV-1 (Rispens et al., 1972) that has been further modified by passage in tissue culture (de Boer et al., 1986), is the most effective MD vaccine. However, vaccine breaks (suboptimal vaccinal protection of a flock) do occur and can have several causes. Inappropriate storage, handling and administration of vaccine virus can lead to the delivery of a suboptimal dose to chickens. Development of immunity can be delayed by
the presence of homologous maternal antibodies, which delay vaccine virus replication, and immune responses can be suppressed by environmental stresses or by infection with immunosuppressive pathogens. Virulent field strains can break through vaccinal protection in chickens that are infected prior to the establishment of full immunity. Finally, even in correctly vaccinated and fully immunocompetent chickens, highly virulent MDV strains can cause disease.

Maximizing the potential of CVI988 to control MD by reducing the likelihood of vaccine breaks will require optimization of the route of vaccine administration, revaccination regimes and the choice of appropriate vaccines for different breeds of chicken. This can only be achieved by a better knowledge of the kinetics of CVI988 replication at the tissue and cellular level and an understanding of how CVI988 load, at different times post-vaccination (p.v.), relates to protection against challenge. Availability of a rapid and easy sampling and detection method for measurement of CVI988 vaccine status in individual chickens would also be highly beneficial.

Existing quantitative data for kinetics of CVI988 replication are limited (Handberg et al., 2001; Davidson et al., 2002). In view of this, our aims in this study were: (i) to determine CVI988 virus load in different tissues at various times following vaccination; and (ii) to establish the optimal tissue sample and detection method to evaluate the CVI988 vaccine status of chickens. In order to establish fundamental replication kinetic data in the absence of variables seen in commercial flocks (such as infection by virulent MDV or other pathogens, and varying levels of maternal antibodies), the study was carried out in experimental birds in a controlled environment. The tissues examined were spleen, bursa, thymus, peripheral blood and feather, and the detection methods employed were virus isolation (Churchill & Biggs, 1967), conventional 132 bp repeat PCR (Silva, 1992; Becker et al., 1992) and real-time duplex PCR for absolute quantification of the MDV genome (Baigent et al., 2005).

**METHODS**

**Experimental chickens.** MD-susceptible specific-pathogen-free Rhode Island Red chickens, which lacked maternal antibodies against MDV, were hatched, reared and housed in the Experimental Animal House. Chickens were vaccinated with one commercial dose of cell-associated monovalent CVI988 vaccine (Fort Dodge Animal Health, UK) in 100 μl diluent. Experimental procedures were carried out in accordance with the guidelines of the UK Home Office.

**Virus isolation and titration.** Up to 10^7 spleen or blood lymphocytes were added to duplicate monolayers of primary chicken embryo fibroblast (CEF) cells (prepared from embryos of 10-day-old specific-pathogen-free line 0 chickens). Five days later, the monolayers were fixed using ice-cold acetone/methanol (1:1) and MDV plaques were stained using a variation of the method described by Silva et al. (1997). Briefly, cells were incubated with murine monoclonal antibody HB3 (L. J. N. Ross, personal communication), which detects MDV glycoprotein B (gB). Brown staining of plaques was developed using a secondary peroxidase-conjugated antibody (Dako), followed by the peroxidase substrate aminoethylcarbazole.

**DNA preparation and PCR.** DNA was prepared from lymphocyte pellets and feather tips using phenol/chloroform extraction (Sambrook & Russell, 2001). To confirm that DNA samples were of appropriate quality and quantity for PCR, all were subjected to 30-cycle conventional end-point PCR to detect an endogenous avian retrovirus sequence (EAV-HP1 provirus), present in all chicken cells (Sacco et al., 2004). The 40-cycle conventional PCR to detect the 132 bp repeats used previously described primers (Becker et al., 1993). Negative and positive control DNA (from non-infected or CVI988-infected CEF cells) and test sample DNA were added at 1 μg DNA per reaction. PCR products were analysed on a 1 % agarose gel containing ethidium bromide. For quantification of the MDV genome (as copies per 10^6 cells), we used our quantitative duplex PCR technique (q-PCR) (Baigent et al., 2005). Each DNA sample under test was analysed in duplicate reactions, using 50 ng sample DNA. For feather DNA samples, both conventional and real-time reactions contained 10 μg BSA (Baigent et al., 2005).

**Experimental design.** Chickens were vaccinated at 1 day of age as in the commercial environment (Experiment A) or at 14 days (Experiment B), and non-vaccinated age-matched chickens were housed separately. At each of 12 sampling times between 0 and 28 days p.v., five vaccinated chickens were chosen for sampling (by random selection of wing band numbers before going to the animal house). At the final time point, five randomly chosen non-vaccinated chickens were also sampled. Peripheral blood was taken, the birds were killed by cervical dislocation, and spleen, bursa, thymus and axillary tract pinfeathers (Experiment A) or spleen and pinfeathers (Experiment B) were collected. Because chickens were killed for sampling of tissues, a completely different group of five chickens was sampled at each time point. Lymphocytes (the primary target of MDV) were purified from organs and peripheral blood by centrifugation over Histopaque-1083 (Baigent et al., 1996, 2005) in order that virus isolation and q-PCR results could be expressed per 10^6 lymphocytes. Spleen lymphocytes and peripheral blood mononuclear cells (PBMCs) were used for titration of infectious virus. All lymphocyte and feather samples were used for DNA preparation for detection and quantification of CVI988 by PCR. In Experiment A, spleen DNA for PCR was not available at time points prior to 10 days p.v. because spleens were very small. Feather samples were not obtained prior to 10 days p.v. because feathers were not well developed before this time. In Experiment B, delaying vaccination until 14 days of age ensured that feathers were developed for sampling from time 0, and that larger spleens and larger blood samples could be taken for virus isolation and DNA preparation.

**Statistical analysis.** For q-PCR data, the arithmetic mean values for genome copy number and the 95% confidence intervals (from duplicates for individual chickens or for groups of five chickens) were determined using the log_{10} transformed copy number for each individual sample and then back transformed to obtain the actual values. It is appropriate to determine the mean values in this manner because real-time PCR read-out Ct values are proportional to log_{10} copy number. To investigate correlation between genome copies in different tissues of the same chicken, tissues were compared in pairwise combinations using regression analysis. The one-sample proportion test was used to compare different tissues and different techniques for the ability to detect CVI988. For these analyses, q-PCR MDV genome copy numbers greater than 10 (per 10^6 cells) were taken as positive.
RESULTS

Virus isolation and titration

Immunoperoxidase staining provided a specific and sensitive method for clear visualization and enumeration of MDV cytopathic plaques. Mean p.f.u. per $10^6$ lymphocytes (virus titre) was determined for spleen and PBMCs from each chicken and from each group of five chickens (summarized in Table 1). In Experiment A, problems with establishment of good cell monolayers meant that data were missing from some time points. In Experiment B, sound data were obtained for each time point (Fig. 1). Infectious virus was first detected in spleens at 2 days p.v. and in PBMCs at 3 days p.v. Following an initial peak at 5 days p.v., a transient decrease in virus titre was observed in both tissues, before rising to a second peak at 7–10 days p.v. Up to and including 10 days p.v., mean virus titre was higher in the spleen than in PBMCs, this difference being statistically significant only at 7 day p.v. ($P < 0.05$). From 10 days p.v. onwards, mean p.f.u. declined in both spleen and PBMCs and was greater in the PBMCs than in the spleen at 20 ($P < 0.0001$) and 28 days p.v. The limited data from Experiment A were supportive of a similar pattern of infectious virus titres to that in Experiment B, although at 5 and 6 days p.v., virus titres in spleen and PBMCs were approximately fivefold greater in Experiment A. This difference may reflect the different ages at which the birds were vaccinated in the two experiments.

Conventional PCR to amplify 132 bp repeats

PCR to detect the EAV-HP1 sequence confirmed that the majority of DNA samples were of appropriate quality and quantity for MDV PCR (data not shown). The few DNA samples that were of poor quality were omitted from both 132 bp repeat PCR and q-PCR analyses. The 132 bp repeat PCR products for each PBMC and feather sample from Experiment B are shown in Fig. 2. The CVI988 positive control showed many tandem repeats, giving a characteristic ‘ladder’ pattern, with the intensity of each band being similar. Test samples did not always show the same ladder pattern, with a product representing a certain number of repeats (variable between chickens, but often the same for different tissues from the same chicken) predominating in many cases. Samples were scored positive if some or all bands coinciding with the positive control pattern were observed, or negative if there were no bands or bands not coinciding with any bands in the positive control. The number of chickens that were 132 bp repeat PCR positive for each tissue at each time point is summarized in Table 1. PCR-positive spleens were first detected at 2 days p.v., positive bursa and thymus at 3 days p.v. and positive PBMCs at 4 days p.v. Numbers of positive birds subsequently increased, the majority of samples being positive between 6 and 20 days p.v. Thereafter, the proportion of positive samples decreased. No feather samples were PCR positive prior to 7 days p.v., but thereafter all were positive.

Real-time q-PCR assays

Mean standard curves, for the MDV meq gene and the chicken ovotransferrin (ovo) gene, were prepared from eight q-PCR runs for Experiment A and eight for Experiment B. Using these standard curves, MDV genome copy number per $10^6$ chicken cells was derived for DNA samples as previously described (Baigent et al., 2005). Mean values were calculated as described in Methods (Table 1, Fig. 3). In samples from non-vaccinated chickens, the calculated number of MDV genomes was, as expected, at or very close to the baseline (Table 1). In vaccinated chickens, the wide confidence intervals at each time up to 7 days p.v. reflected variation in the timing and extent of early virus replication, since Rhode Island Red chickens are not highly inbred. After 7 days p.v., there was much less variation.

In Experiment A (Fig. 3a), virus replication was very similar in bursa and thymus, the viral genome first being detected at 3 days p.v. and increasing rapidly from day 4 to a peak at day 6. Thereafter, MDV genome copies steadily declined until the termination of the experiment at 28 days p.v. The available data for spleen (10 days p.v. onwards) showed similar kinetics, but with a slightly greater virus load. In clear contrast to these lymphoid tissues, numbers of MDV genomes in feather tips peaked at 14 days p.v. and were up to $10^3$-fold greater. Thereafter, there was a gradual decline, in parallel with that observed in the lymphoid tissues, but MDV genome levels remained high in the feathers at 28 days p.v.

In Experiment B (Fig. 3b), MDV genome load and kinetics of replication in the spleen was very similar to that observed for bursa, thymus and spleen in Experiment A. In PBMCs, MDV genome was first detected at 4 days p.v., gradually increasing to a low peak at 14 days p.v. and then decreasing, parallel with genome detection in the spleen. MDV genome was first detected in significant amounts in feathers at 6 days p.v., rising rapidly to a peak at 14 days p.v. and then gradually declining, as in Experiment A.

Comparison of tissues for detecting CVI988

For each CVI988 MDV detection technique, different tissues from individual chickens were compared using the one-sample proportion test to examine the probability that a positive result in one tissue correlated with a positive result in a second tissue (Table 2). For virus isolation, there was a high probability of correlation between spleen and PBMCs. For 132 bp repeat PCR, the results from Experiment A showed low correlation of spleen with bursa or thymus, since several spleen samples were unexpectedly 132 bp repeat PCR negative, despite virus isolation and q-PCR showing that these samples contained significant levels of virus. However, correlation between bursa and thymus was high. In Experiment B, there was a high probability of correlation between spleen and PBMCs, and between feathers and PBMCs. Correlation between spleen and feathers was lower. For q-PCR data, the correlation between tissues was greater than that observed for 132 bp repeat PCR and
### Table 1. Summary of CVI988 virus detection and quantification

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Method*</th>
<th>Non-vacc.</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5†</th>
<th>6‡</th>
<th>7‡</th>
<th>10</th>
<th>14</th>
<th>20</th>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5†</td>
<td>6‡</td>
<td>7‡</td>
<td>10</td>
<td>14</td>
<td>20</td>
<td>28</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Virus isolation</td>
<td>0 (0/5)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2·4 (4/4)</td>
<td>6·5 (5/5)</td>
<td>ND</td>
<td>70·2 (5/5)</td>
<td>10·4 (5/5)</td>
<td>4·7 (4/4)</td>
<td>1·9 (5/5)</td>
</tr>
<tr>
<td>Spleen</td>
<td>132 bp PCR</td>
<td>0/5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>3/5</td>
<td>3/5</td>
<td>3/5</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>Virus isolation</td>
<td>q-PCR</td>
<td>0 (0/5)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>46·0 (4/4)</td>
<td>51·7 (1/1)</td>
<td>ND</td>
<td>ND</td>
<td>11·4 (4/5)</td>
<td>2·8 (4/4)</td>
<td>3·2 (5/5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 (0/5)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1·5 × 10⁴</td>
<td>2·3 × 10⁴</td>
<td>1·4 × 10⁴</td>
<td>1·9 × 10⁵</td>
<td></td>
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<tr>
<td>Thymus</td>
<td>132 bp PCR</td>
<td>0/5</td>
<td>0/3</td>
<td>0/5</td>
<td>0/5</td>
<td>2/5</td>
<td>3/5</td>
<td>4/4</td>
<td>5/5</td>
<td>4/4</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>4/5</td>
</tr>
<tr>
<td>q-PCR</td>
<td>6 (0/5)</td>
<td>9 (0/5)</td>
<td>6 (0/5)</td>
<td>6 (0/5)</td>
<td>40 (2/5)</td>
<td>40 (2/5)</td>
<td>1·9 × 10⁴</td>
<td>2·3 × 10⁴</td>
<td>2·7 × 10⁴</td>
<td>9·5 × 10³</td>
<td>3·6 × 10³</td>
<td>2·3 × 10³</td>
<td>350 (4/5)</td>
<td></td>
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<tr>
<td>Bursa</td>
<td>132 bp PCR</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/4</td>
<td>1/5</td>
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<td>q-PCR</td>
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<td>6 (0/5)</td>
<td>6 (0/5)</td>
<td>6 (0/5)</td>
<td>13 (1/5)</td>
<td>18 (1/5)</td>
<td>489 (2/4)</td>
<td>1·2 × 10⁵</td>
<td>1·9 × 10⁴</td>
<td>1·3 × 10⁴</td>
<td>7·3 × 10³</td>
<td>4·8 × 10³</td>
<td>473 (5/5)</td>
<td></td>
</tr>
<tr>
<td>Feather</td>
<td>132 bp PCR</td>
<td>0/5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>1·8 × 10⁷</td>
<td>2·3 × 10⁶</td>
<td></td>
</tr>
<tr>
<td>q-PCR</td>
<td>6 (0/5)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>(5/5)</td>
<td>(5/5)</td>
<td>(5/5)</td>
<td>(5/5)</td>
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</tbody>
</table>

*Results for the 132 bp repeat PCR are given as the number of positive chickens. Virus isolation results are given as mean virus titre (on CEF cells) in p.f.u. per 10⁶ lymphocytes. The number of chickens positive by virus isolation is given in parentheses. Real-time q-PCR results are given as mean mean virus genome copies per 10⁶ cells. The number of chickens positive by q-PCR is given in parentheses. ND, Not done.

†At each time point, a different group of five chickens was sampled; all tissues sampled at the same time point are from the same five chickens. At times when only four samples are given, this indicates omission of a poor DNA sample (for which EAV-HP1 PCR gave a negative result) or a poor virus isolation sample (for which the CEF monolayer laid before plaques could be fixed and stained).

‡Chickens #27 (5 days p.v.) and #38 (7 days p.v.) in Experiment A and #38 (7 days p.v.) in Experiment B were omitted from the analyses since inability to detect CVI988 genome or infectious virus in any tissue (at a time when all others in the group were CVI988 positive) indicated these chickens had missed vaccination.
was high for all pairs of tissues. For q-PCR-positive samples, we also examined whether virus genome load in one tissue was predictive of virus genome load in a second tissue, using regression analysis. In Experiment A, there was very close correlation ($r^2 = 0.9$) among the three lymphoid tissues (Fig. 4). Correlation was also good between feathers and spleen ($r^2 = 0.83$), but lower for feathers and bursa ($r^2 = 0.73$) and for feathers and thymus ($r^2 = 0.67$), these data being taken only from 10 days p.v. onwards, since feathers and spleen were not collected before this time. For Experiment B, there was no close correlation ($r^2 < 0.2$) between genome load in any pairwise combination of spleen, PBMCs and feathers over the course of the entire experiment, reflecting the different initial kinetics of virus replication in these tissues (data not shown). However, taking the data from only 10 days p.v. onwards (latent infection), there was good correlation between spleen and PBMCs ($r^2 = 0.88$) and between spleen and feathers ($r^2 = 0.69$).

Comparison of techniques for detecting CVI988

Table 1 summarizes the data obtained from the different tissues using the three techniques. While 132 bp repeat PCR gave a positive/negative result, virus isolation and q-PCR gave a continuous range of quantitative data. For each tissue, we evaluated the relative sensitivities of each technique to detect CVI988 using the one-sample proportion test to examine the probability that a positive result by one method correlated with a positive result by a second method (Table 3).

**PCR of the 132 bp repeat versus q-PCR.** There was very close agreement between the detection of MDV DNA by q-PCR and by 132 bp repeat PCR (with the exception of spleen samples from Experiment A). q-PCR was the more sensitive of the two methods: in a total of 328 samples, 24 were q-PCR positive and 132 bp repeat PCR negative, while only six were q-PCR negative and 132 bp repeat PCR positive. Generally, samples with an MDV genome load $<80$ were negative in 132 bp repeat PCR, while those with a genome load $>150$ were positive in 132 bp repeat

![Fig. 1. Isolation of infectious CVI988 virus from vaccinated chickens. Mean number of p.f.u. per 10⁶ lymphocytes (± SE) for groups of five chickens were plotted on a logarithmic scale for PBMCs (△) and spleen (■). Samples in which no plaques were observed were assigned a value of 0.01 in order that a baseline could be plotted on the logarithmic scale.](http://vir.sgmjournals.org)

![Fig. 2. PCR detection of the 132 bp repeat of CVI988 virus in vaccinated chickens. The 132 bp repeat PCR products from Experiment B for PBMC and feather samples are shown for all chickens. Days p.v. and code numbers of individual chickens (five different chickens for each time point) are given underneath. Chicken #38 (7 days p.v.) was omitted from the analyses since inability to detect CVI988 genome or infectious virus in any tissue (at a time when all others in the group were CVI988-positive), indicated that this chicken had missed vaccination. Molecular size markers (M; 1584, 1375, 947, 831 and 564 bp), negative control reactions (−) and positive control reactions (+) are shown. Since the forward primer is located 65 bp upstream of the repeat and the reverse primer 105 bp downstream of the repeat, the PCR product sizes were 302 bp (65 + 132 + 105) for a single repeat, 434 bp (302 + 132) for a double repeat, 566 bp (434 + 132) for a triple repeat, etc. The positions of the bands representing one, two and three repeats are indicated on the left-hand side of each panel.](http://vir.sgmjournals.org)
PCR, with variable 132 bp repeat PCR results for samples with a genome load between 80 and 150.

**PCR of the 132 bp repeat versus virus isolation.** Correlation between virus isolation and 132 bp repeat PCR was very good for both spleen and PBMCs in Experiment B, but lower in Experiment A. Virus isolation was more sensitive than 132 bp repeat PCR for detecting MDV in samples with low virus load: in a total of 132 spleen and PBMCs samples, 18 were negative in 132 bp repeat PCR but positive by virus isolation, while only two were negative for virus isolation and positive in 132 bp repeat PCR. In general, samples with a virus titre of $<0.2$ were negative in 132 bp repeat PCR, while those with a titre of $>0.9$ were positive, with variable PCR results for samples having a virus titre between 0.2 and 0.9.

**Virus isolation versus q-PCR.** There was very close correlation between results of virus isolation and q-PCR, and they were similarly sensitive methods for the detection of CVI988.

**DISCUSSION**

A better understanding of the kinetics of Marek’s disease vaccine virus replication, the quantification of vaccine virus load and its relationship with protection against challenge will be required as we aim towards more effective vaccines that prevent replication, or at least shedding, of challenge virus. With this in mind, we examined CVI988 virus load as a measure of kinetics of replication in experimentally vaccinated chickens. Several tissues were examined in order

![Fig. 3. Kinetics of replication of CVI988 in vaccinated chickens using real-time PCR. Mean genome copies per $10^6$ cells for groups of five chickens were plotted on a logarithmic scale, with 95% confidence limits (only the upper confidence limit is shown for clarity) for Experiment A (a) and Experiment B (b). The baseline (the minimum number of MDV genomes per $10^6$ cells detectable in each experiment) was six for Experiment A and two for Experiment B.](image)

**Table 2. Correlation between tissues for detection of CVI988 virus**

Probability is given as the percentage probability of agreement between tissues (i.e. both tissues positive or both tissues negative) using the one-sample proportion test. The 95% confidence intervals (CI) are shown.

<table>
<thead>
<tr>
<th>Method</th>
<th>Spleen versus thymus ($n=20$)†</th>
<th>Spleen versus bursa ($n=20$)†</th>
<th>Bursa versus thymus ($n=56$)</th>
<th>Spleen versus PBMCs ($n=19$)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus titre</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>132 bp PCR</td>
<td>50.0</td>
<td>28.1–71.9</td>
<td>28.1–71.9</td>
<td>94.7</td>
</tr>
<tr>
<td>q-PCR</td>
<td>100.0</td>
<td>100.0</td>
<td>85.4–100</td>
<td>84.7–100</td>
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<table>
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<tr>
<th>Experiment B</th>
<th>Spleen versus PBMCs ($n=58$)</th>
<th>Feather versus PBMCs ($n=58$)</th>
<th>Spleen versus feather ($n=58$)</th>
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<tbody>
<tr>
<td>Virus titre</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>132 bp PCR</td>
<td>81.0</td>
<td>70.9–91.1</td>
<td>50.3–75.1</td>
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<tr>
<td>q-PCR</td>
<td>87.9</td>
<td>79.5–96.3</td>
<td>65.4–87.1</td>
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</table>

*In Experiment A, feather samples were positive at all time points taken (10 days p.v. onwards), so were not included in analyses.
†In Experiment A, spleens were only sampled from 10 days p.v. onwards.
to investigate the different stages in the course of vaccine virus infection and replication.

**Kinetics of CVI988 replication in different tissues**

Different tissues showed distinct kinetics of virus replication. For the first 6 days following vaccination, CVI988 was detected predominantly in the lymphoid organs, consistent with these being the sites of primary cytolytic replication (Calnek, 2001; Baigent et al., 1998; Baigent & Davison, 1999). The rapid early increase followed by a steady decline from 6 days p.v. was consistent with replication kinetics recorded in spleens of chickens infected with virulent MDV, also using q-PCR (Yunis et al., 2004). The peak virus genome load corresponds to active cytolytic infection, while the decline marks the onset of latency. Detection of CVI988 in PBMCs and feather tips lagged behind that in lymphoid organs, consistent with the gradual circulation of lymphocytes, particularly from the spleen, into the bloodstream and thence to peripheral sites including feather tissues. Our temporal data on detection of CVI988 genome in feather tips agree with the findings of Malkinson et al. (1989) who detected virulent MDV in feather tips using dot blots. Our experiment terminated at 28 days p.v., but preliminary data from a subsequent study has shown that CVI988 genome remains readily detectable in feather tips beyond 50 days p.v. (data not shown). In a previous investigation of CVI988 replication (Handberg et al., 2001), virus isolation from PBMCs closely resembled our data, but, using PCR to

**Fig. 4.** Relationship between CVI988 genome copy numbers in different tissues. For each individual chicken, CVI988 genome copy number (per 10^6 cells) for one tissue was plotted against genome copy number in a second tissue, omitting any chickens for which virus genomes were below the detection threshold in either or both tissues. The two logarithmic plots shown are for data from Experiment A. (a) Copy number in thymus (x axis) versus copy number in bursa (□), spleen (■) and feather tips (●) (y axis). (b) Copy number in spleen (x axis) versus copy number in feather tips (●), thymus (▲) and bursa (□) (y axis). The equations and r^2 values for the regression lines are given in the plots.

**Table 3.** Comparison of techniques for detection of CVI988 virus

Probability is given as the percentage probability of agreement between tissues (i.e. both tissues positive or both tissues negative) using the one-sample proportion test. The 95% CIs are shown. Collective data from all tissues were also analysed. n, Number of samples (in Experiment A, spleens and feathers were only sampled from 10 days p.v. onwards).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>132 bp PCR versus virus isolation</th>
<th>132 bp PCR versus q-PCR</th>
<th>q-PCR versus virus isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Probability (%)</td>
<td>95% CI</td>
</tr>
<tr>
<td>Spleen</td>
<td>15</td>
<td>46-7</td>
<td>21-4–71-9</td>
</tr>
<tr>
<td>Bursa</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Thymus</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Feather</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>All</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Spleen</td>
<td>59</td>
<td>91-5</td>
<td>84-4–98-6</td>
</tr>
<tr>
<td>PBMCs</td>
<td>58</td>
<td>87-9</td>
<td>79-5–96-3</td>
</tr>
<tr>
<td>Feather</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>All</td>
<td>117</td>
<td>89-7</td>
<td>84-2–95-2</td>
</tr>
</tbody>
</table>
amplify the ICP4 gene, all spleen samples and most PBMCs samples were negative at time points between 3 and 84 days p.v., suggesting that their PCR was less sensitive than our 132 bp repeat PCR. However, consistent with our results, feather tips were PCR positive in most birds from 14 to 56 days p.v. Our ability to detect CVI988 DNA in the feather tips is likely to be associated with shedding and contact spread of the virus, although we did not measure this. The original uncloned CVI988 isolate readily spread by contact as measured by antibody levels and by virus isolation from non-vaccinated flock mates (Rispens et al., 1972). However, some later clones of the virus, notably CVI988/C (de Boer et al., 1986), showed limited transmission to contact birds (Witter et al., 1987), suggesting that the ability to spread is affected by serial passage and attenuation of CVI988 and that this characteristic may vary among different subclones of the virus.

Age at vaccination (1 day, as in the commercial environment, vs 14 days) had no significant effect on CVI988 virus load in spleen and feathers, indicating that any changes in immunocompetence over this 14 day period are probably not significant in terms of vaccinal protection.

**Mechanism of vaccinal protection**

It is likely that vaccinal protection requires the vaccine virus to be latent, since maximal protection requires 1–2 weeks between vaccination and exposure to virulent strains. Furthermore, homologous maternal antibodies, which delay vaccine virus replication and establishment of latency, also delay immunity (Bublot & Sharma, 2004). Failure of pathogenic MDV-1 strains to enter latency correlates with increased virulence (Yunis et al., 2004). Our study indicates that CVI988 enters latency in lymphoid tissues at around 7 days p.v. It is likely that greater CVI988 replication in the spleen during the cytolytic phase will better stimulate immune responses, and that maintenance of high levels of latent virus is not required. Indeed, higher protection induced by MD vaccine candidates correlated well with higher vaccine virus genome load in lymphoid organs at 6 days p.v. and with early stimulation of splenic T-cell responses (Gimeno et al., 2004). However, vaccine candidates with higher replication levels tend towards virulence (Gimeno et al., 2004) and the optimum level of vaccine virus to induce strong immune responses while avoiding pathogenic effects is likely to vary between vaccine strains and host genotypes. Genome load in lymphoid tissues is around 100-fold lower for CVI988 than for a virulent strain, RB-1B (Baigent et al., unpublished data), consistent with CVI988 being a good vaccine.

The switch from cytolytic to latent infection is associated with changes in MDV gene expression (Parcells et al., 2003), and the host’s immune response is a major factor controlling this switch (Buscaglia et al., 1988; Kaiser et al., 2003; Parcells et al., 2003). Long-term immune protection is likely to require periodic stimulation of the immune system by cells expressing MDV antigen. In feather tissues, the majority of cells probably remain lytically infected, but whether or not CVI988-infected cells at this peripheral site effectively stimulate the immune system is not known. Conceivably, CVI988 could spontaneously reactivate in other tissues to boost immune responses, but in our study there was no evidence for reactivation of latent virus (based on increasing genome copy numbers) between 14 and 28 days p.v. The effects of secondary vaccination, protective synergism and the use of adjuvants on boosting immune responses have not been fully evaluated, but could now be readily studied using real-time PCR.

**Comparison of detection methods**

It could be inferred that vaccinal protection is more closely associated with the level of infectious virus than with number of viral genomes, but these two parameters were closely related, particularly during latent phase. Conventional detection and quantification of MDV by cultivation of blood lymphocytes on permissive cells (virus isolation) measures the number of cells harbouring infectious cell-associated MDV (Churchill & Biggs, 1967). PCR techniques represent more rapid and sensitive means of measuring MDV in DNA samples from a wide variety of tissues. PCR detects all forms of MDV genome, but does not show the number of infected cells or the number of viral genomes within each infected cell, and values are an average measure of virus load per cell. A useful conventional PCR is one to amplify the 132 bp repeat region of the MDV genome, which allows distinction between virulent and attenuated MDV-1 strains. Repetition of the 132 bp sequence (Maotani et al., 1986) in the internal repeat long and terminal repeat long (IRL and TRL) genomic regions (Fukuchi et al., 1985; Silva & Witter, 1985) is the only known consistent genomic difference between attenuated and virulent strains (Silva et al., 2004). Virulent strains have two or three copies of the repeat, while CVI988 has multiple direct repeats (Silva, 1992; Becker et al., 1992, 1993). In our study, variation in number of 132 bp repeats between chickens indicated that, after several rounds of replication in vivo, subclones of CVI988 with a set number of repeats will predominate. Repeated passing of attenuated MDV in birds makes the virus more homogeneous and reduces the 132 bp region to two to three repeats (Silva et al., 2004), suggesting that, in chickens, there is strong selection to maintain two repeats. It is unknown whether there is any association between number of repeats and extent of vaccinal protection. Using 132 bp repeat PCR, Lee et al. (1999) obtained PCR-positive spleens only if chickens were vaccinated with 10 000 p.f.u. CVI988, which is 10-fold greater than the standard dose. Davidson and co-workers could detect virulent MDV-1 by 132 bp repeat PCR in feather, spleen and liver (Davidson & Borenshtain, 2003), but failed to detect CVI988 in spleen, liver and PBMCs between 3 and 37 days p.v. (Davidson et al., 2002), although some samples were positive by gB PCR. They concluded that CVI988 replicates to low levels in vaccinated chickens and that 132 bp repeat PCR is less sensitive than gB PCR because the PCR product is
distributed over multiple weaker bands. Since we were able to detect CVI988 by 132 bp repeat PCR in a variety of tissues, it is likely that we used more DNA template in the PCR. However, the relatively low sensitivity of 132 bp repeat PCR could represent a problem for analysis of samples from commercial birds, where maternal antibodies could reduce the replication of CVI988.

Unlike 132 bp repeat PCR, virus isolation and q-PCR are both quantitative and were more sensitive than 132 bp repeat PCR for detecting CVI988 in samples with low virus load. We used 20-fold less input DNA in real-time q-PCR (Baigent et al., 2005) and still obtained positive results with some samples that were 132 bp repeat PCR negative. However, 132 bp repeat PCR has the advantage of allowing distinction between virulent and vaccine strains of MDV-1 if required. Each method of virus detection has its own benefits and can provide information not available from the other two methods, but real-time q-PCR is the method of choice for measuring vaccine virus in chickens for its combination of high sensitivity and reproducibility, practicability and quantitative results.

Use of feather samples to quantify CVI988

In our work, chickens were killed to take organ samples, so the data represented mean values for five different birds at each time. Taking only those tissues that can easily and repeatedly be sampled from live birds (blood and feathers) would enable study of the time course of MDV replication in individual chickens. We found feather tips clearly to be better than blood in terms of CVI988 genome load and ease of sampling, and feathers are thus the ideal tissue for sampling for PCR confirmation of successful vaccination of commercial chickens (PCT application WO 2004/035821). Furthermore when CVI988 was measured using q-PCR, virus load in feather tips was a good predictor of virus load in spleen, the predominant site of immune responses to MDV antigens (Jeurissen, 1991; Baigent & Davison, 1999), from 10 days p.v. onwards. This indicates that virus load in feather tips should be a good predictor of vaccinal protection. Preliminary observations indicate that CVI988 replication in the feathers of vaccinated chickens in the field usually follows very similar kinetics to that in experimental chickens. However, in some flocks, the CVI988 replication curve in feathers can be skewed and the peak of virus detection delayed (R. J. W. Currie, unpublished data). While our experimental chickens are genetically homogeneous, maternal antibody-free and free of other pathogens, these factors can vary widely in commercial chickens and could influence virus replication. The altered CVI988 replication kinetics in certain flocks will not necessarily be reflected in reduced vaccinal protection and our next step is to confirm that vaccine virus load in the feather tips correlates well with protection against challenge by virulent MDV in a variety of genetic and environmental backgrounds.

In summary, we have generated a quantitative set of data for CVI988 virus load and tissue distribution for 28 days after vaccination, giving further insight into the mechanisms of protective immunity induced by this MD vaccine strain. Real-time PCR on feather samples provided the most appropriate method for detection and quantification of CVI988, and testing of commercial chickens should assist in identification of inappropriate storage, handling and administration of vaccine as causes of vaccine failure. The next step is to confirm that CVI988 load in the feather tips correlates with protection in challenge studies using virulent strains. Once this relationship is understood, the feather q-PCR test could provide data to assist optimization of existing vaccination strategies in terms of delivery route, choice of vaccine to complement host chicken genotype and identification of the optimal time for revaccination. Furthermore, by appropriate choice of q-PCR primers, we should ultimately be able to quantify both vaccine and challenge virus in the same tissue to make those advances in knowledge that are required for development of more efficient vaccine strategies.

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