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Relationship Between Levels of Very Virulent MDV in Poultry Dust and in Feather Tips from Vaccinated Chickens

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SUMMARY. To assess the effect of various vaccine strains on replication and shedding of virulent Marek's disease virus from experimentally infected chickens, quantitative PCR (q-PCR) methods were developed to accurately quantify viral DNA in infected chickens and in the environment in which they were housed. Four groups of 10 chickens, kept in poultry isolators, were vaccinated at 1 day old with one of four vaccines covering each of the three vaccine serotypes, then challenged with very virulent MDV strain Md5 at 8 days of age. At regular time-points, feather tips were collected from each chicken and poultry dust was collected from the air-extract prefilter of each isolator. DNA was extracted from feather and dust samples and subjected to real-time q-PCR, targeting the U_S2 gene of MDV-1, in order to measure Md5 level per 10^4 feather tip cells or per microgram of dust. Accuracy of DNA extraction from dust and real-time q-PCR were validated by comparing either q-PCR cycle threshold values or the calculated MDV genome level; for use in q-PCR, DNA was extracted from serial dilutions of MDV-infected dust diluted with noninfected dust, or DNA from MDV-infected dust was diluted with DNA from noninfected dust. The results confirmed the accuracy and sensitivity of dust DNA extraction and subsequent q-PCR and showed that differences in virus levels between dust samples truly reflect differences in shedding. Vaccination delayed both replication of Md5 in feather tips and shedding of Md5. First detection of Md5 in feather tips always preceded or coincided with first detection in dust in each group. pCVI988 and HVT+SB-1 were the most efficient vaccines in reducing both replication and shedding of Md5. There was close correlation between mean virus level in feathers of each group and mean virus level in the dust shed by that group. This relationship was similar in each of the vaccinated groups, demonstrating that measurement of the virus in dust can be used to monitor accurately both the infection status of the chickens and environmental contamination by MDV.

RESUMEN. Relación entre los niveles del virus de Marek muy virulento en el polvo de instalaciones avícolas y en cañones de las plumas en pollos vacunados.

Para evaluar el efecto de diferentes cepas de vacunas sobre la replicación y diseminación del virus muy virulento de la enfermedad de Marek en pollos infectados experimentalmente, se desarrollaron métodos cuantitativos de PCR (q-PCR) para determinar con precisión el ADN viral en los pollos infectados y en el entorno en el que se alojaron. Se alojaron cuatro grupos de 10 pollos por unidad de aislamiento, estas aves fueron vacunadas al primer día de edad con una de cuatro vacunas que incluyen los tres serotipos vacunales. Posteriormente fueron desafiados a los ocho días de edad con la cepa muy virulenta del virus de la enfermedad de Marek, Md5. En intervalos de tiempo regulares, se recolectaron cañones de plumas de cada pollo y también se recolectó el polvo del prefiltro del extractor de cada unidad de aislamiento. Se extrajo el ADN de los cañones de las plumas y de las muestras de polvo y se analizaron por PCR en tiempo real, para el gene U_S2 del virus de Marek 1, con el fin de medir el nivel de virus Md5 por 10^4 células de plumas o por microgramo de polvo. Se validó la precisión de la extracción de ADN del polvo y el método de PCR en tiempo real mediante la comparación de los ciclos umbrales del método de PCR cuantitativo o por los valores del genoma del virus de Marek. Para su uso en método de PCR cuantitativo, el ADN fue extraído de diluciones seriadas de polvo contaminado con el virus de Marek, diluido con polvo no contaminado, o también, el ADN extraído de polvo contaminado con el virus de Marek se diluyó con ADN de polvo no contaminado. Los resultados confirmaron la precisión y la sensibilidad de la extracción de ADN del polvo y el método de PCR cuantitativo subsecuente, y mostró que las diferencias en los niveles virales entre las muestras de polvo verdaderamente reflejan diferencias en la eliminación del virus. La vacunación retrasó la replicación de la cepa Md5 en los cañones de las plumas y la eliminación de esta cepa Md5. La primera detección de la cepa Md5 en los cañones de las plumas siempre precedió o coincidió con la primera detección de polvo en cada grupo. Las vacunas pCVI988 y HVT + SB-1 fueron las más eficaces en la reducción de la replicación y de la diseminación de la cepa Md5. Hubo una estrecha correlación entre el nivel promedio de virus en las plumas en cada grupo y el nivel promedio de virus en el polvo eliminado por ese grupo. Esta relación fue similar en cada uno de los grupos vacunados, lo que demuestra que la medición del virus en el polvo se puede utilizar para muestrear con precisión tanto el estado de infección de los pollos y la contaminación ambiental por el virus de Marek.

Key words: Marek's disease virus, vaccination, poultry dust, cell-free infectious MDV, feathers, real-time PCR

Abbreviations: BAC = bacterial artificial chromosome; CEF = chick embryo fibroblast cells; Ct = cycle threshold; dpc = days postchallenge; dpi = days postinfection; GLM = general linear model; HVT = herpes virus of turkeys; MD = Marek's disease; MDV = Marek's disease virus; MDV-1 = Marek's disease virus serotype 1; MDV genomes/μg dust = MDV genomes per microgram of dust; *ovo* = chicken *ovotransferrin* gene; pfu = plaque-forming units; q-PCR = quantitative PCR; RIR = Rhode Island red chickens; SPF = specific-pathogen-free; U_S = unique short region of MDV genome; vv = very virulent

Serotype 1 strains of Marek's disease herpesvirus (MDV-1) are highly contagious and oncogenic and are the causative agent of Marek's disease (MD) in chickens. The Marek's disease virus (MDV) naturally infects the host via the respiratory route. Initial

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cytolytic replication in the lymphoid tissues is followed by a switch to latent infection at about 7 days. Latently infected lymphocytes carry the virus through the bloodstream to peripheral nerves and visceral organs, where they may proliferate to form gross lymphomas in susceptible breeds of chicken (3,9), and to the skin. Here, MDV undergoes fully productive replication in the feather follicle epithelium. Characteristic lesions are observed in the feather pulp (13) and MDV DNA can be detected at this site (5). Cell-free MDV is shed into the environment with skin and feather debris from about 7 days postinfection (dpi) onward (20), and shedding continues throughout the life of an infected bird (10). MDV-infected feather materials and poultry dust can remain infectious for at least 1 yr (11,18), and cell-free virus is the source of infection for other chickens via the respiratory route (8). MDV DNA for use in PCR can be extracted from both feather pulp (4,5) and poultry dust (20,21,32).

MD is effectively controlled by live attenuated-avirulent vaccine strains, which establish a persistent latent infection and stimulate the immune response to reduce early viremia and, thus, protect against tumors and mortality after subsequent exposure to virulent MDV (2,23). However, significantly, MD vaccines can be considered ''imperfect'' (16) because they prevent neither super-infection nor multiplication and shedding of the challenge virus from the skin (20,21,32), and virulent MDV shed by vaccinated chickens remains pathogenic to nonvaccinated chickens. Furthermore, the ability of vaccines to confer protection has decreased due to the emergence of increasingly virulent field strains classified as very virulent (vv) and very virulent plus [vv+] pathotypes (33,34), an evolutionary process which may be driven by vaccination itself (24,33).

The first generation commercial vaccine against MD, herpesvirus of turkeys (HVT) (26), was introduced in Europe and the United States in the early 1970s. In the United States but not in Europe, HVT was superseded in the 1980s by the more-protective second generation bivalent vaccine, serotype 2 MDV SB-1 strain (12,30) combined with HVT. The third generation vaccine, CVI988/Rispens strain, is an avirulent MDV-1 (29) which has been further attenuated by passage in tissue culture (15). The most effective ''gold standard'' MD vaccine, CVI988, is now widely used in the United States, Europe, South America, and Australasia. However, vaccine failures may occur due to inappropriate vaccine storage and handling, delivery of a suboptimal vaccine dose to chickens, impairment of vaccine virus replication by homologous, maternally derived antibodies, infection prior to development of full vaccinal immunity, or to breakthrough of protection by highly virulent field strains (6). Thus, these ''classical'' MD vaccines are approaching their limits of protective efficacy (17,24). Novel strategies may be required in the development of improved MD vaccines that will prevent replication or shedding (or both) of virulent MDV by the vaccinated host.

To assess the efficiency of vaccine viruses in reducing replication and shedding of virulent MDV, it is essential to have methods for accurate quantification of MDV in chickens and in the environment in which those chickens are housed. It is also of importance to know whether the levels of MDV genome in the chicken reflect levels of environmental contamination.

The aims of this work were to 1) validate a method for extraction of DNA from poultry dust samples for reproducibility and sensitivity, 2) validate subsequent real-time quantitative PCR (q-PCR) on dust DNA for reproducibility and sensitivity, and 3) examine the correlation between mean MDV genome level in feather tips and mean MDV genome level in dust shed by vaccinated chickens.

Four vaccines, representing each of the three generations of commercial MD vaccines and an experimental vaccine virus, were used. In order to establish fundamental data in the absence of variables seen in commercial flocks (such as varying levels of maternal antibodies or co-infection with other pathogens), this study was carried out in experimental specific-pathogen-free (SPF) birds in a controlled environment.

MATERIALS AND METHODS

Experimental chickens. Fifty SPF, maternal–antibody-free Rhode Island red (RIR) chickens, hatched in the Poultry Production Unit at the Pirbright Institute (Compton Laboratory, Berkshire, U. K.), were randomly divided between five positive pressure avian isolators (Controlled Isolation Systems Inc., San Diego, CA). Each isolator contained one experimental group of 10 chickens. At 18 dpi, three randomly selected chickens in each of the vaccinated groups were culled to reduce crowding in the isolators as the chickens grew, leaving seven chickens per vaccinated group from 19 days onward. All procedures were performed according to the guidelines of the United Kingdom Home Office.

Vaccination. All vaccines were administered subcutaneously at 1 day of age in a volume of 100 µl. Group 1 received HVT strain FC126, second chick embryo fibroblast (CEF) passage stock from commercial HVT vaccine (Poulvac, Fort Dodge Animal Health). Group 2 received pCVI988-10 (28), a bacterial artificial chromosome (BAC)-cloned derivative of Poulvac CVI988 (Fort Dodge Animal Health); this was used because it can be readily distinguished from wild-type virulent MDV-1 by real-time q-PCR (7). pCVI988-10 and Poulvac CVI988 both give 100% protection against mortality following infection with a vvMDV strain (28). Group 3 received pRB-1B-2, a BAC-cloned experimental virus which is derived from vvMDV strain RB-1B (31) but has genomic deletions that result in a loss of pathogenicity and oncogenicity (27). Group 4 received a bivalent vaccine, HVT+SB-1 (Marexine, Intervet). Group 5 was not vaccinated. The pCVI988, pRB-1B-2, and HVT were administered at a target dose of 1000 plaque-forming units (pfu) per chicken, consistent with the approximate commercial dose of Poulvac CVI988. In previous experiments, we had shown that this dose of either pCVI988 or HVT provided 100% protection (at 30 days postchallenge [dpc]) against mortality following infection with a vvMDV strain. HVT+SB-1 was administered at one commercial dose per chicken because the pfu titer of this vaccine was not provided. Titration of the prepared vaccine viruses onto CEF confirmed that the dose administered (per chicken) was: pCVI988-10, 1800 pfu; pRB-1B-2, 725 pfu; HVT, 975 pfu; bivalent vaccine HVT component, 8100 pfu and SB-1 component, 595 pfu.

Virus challenge. The challenge stock of vvMDV strain Md5 (35) was prepared from monolayers of CEF that were cocultured with splenocytes collected from experimental chickens 7 days postinoculation with Md5 of seventh duck embryo fibroblast passage (a gift from Dr. A. M. Fadly, Avian Disease and Oncology Laboratory, East Lansing, MI). The culture was subject to one further pass in CEF to produce the stock used for challenge. Each group of chickens was challenged intra-abdominally at 8 days of age, at a target dose of 500 pfu, in a volume of 100 µl. Titration of the prepared Md5 challenge virus onto CEF confirmed the dose administered was 490 pfu/chicken.

Sample collection and DNA preparation. Feather and dust samples were collected twice weekly at intervals of either 3 or 4 days (3, 6, 10, 13, 18, 21, 24, 27 and 31 dpc). Five feathers were collected from the axillary feather tracts of each chicken. The barb-free proximal end of each feather contained the fleshy feather pulp (hereafter referred to as the feather tip) and was sliced into smaller pieces and stored at −20 C. Dust samples were collected by changing and replacing the air-extract prefilters in the isolators. The prefilters were shaken inside a plastic bag, the dust collected into tubes, and three 5-mg aliquots of each sample were weighed and stored at −20 C. DNA was prepared from feather tips and from 5-mg dust samples using a DNeasy-96 kit (Qiagen) according to the manufacturer's instructions for extraction of DNA from tissues. DNA was eluted in a volume of 200 µl and stored at −20 C prior to PCR.

Table 1. Triplicate q-PCR measures for 40-Ct value and MDV genome load for each replicate of MDV-infected poultry dust serially diluted with noninfected dust.

MDV-infected dust sample	Dilution factor	40-Ct value			MDV genomes per microgram dust		
		Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3
A[A]	1	16.38	16.21	16.41	7045.03	6281.55	7189.09
	0.1	10.84	11.04	10.38	167.67	191.90	122.93
	0.01	8.76	8.95	8.70	41.20	46.84	39.57
	0.001	4.38	4.39	3.28	2.14	2.16	1.02
	0.0001	1.87	0.98	0.18	0.39	0.23	0.13
B[B]	1[C]	11.34	—	—	234.96	—	—
	0.1	6.49	5.67	5.80	8.91	5.12	5.59
	0.01	2.29	2.20	0[D]	0.52	0.49	0[D]
C[E]	1	5.41	5.49	5.92	4.29	4.53	6.06
	0.1	2.83	0.64	1.81	0.75	0.17	0.38

[A]All replicates at 0.00001 dilution were negative.
[B]All replicates at 0.001, 0.0001, and 0.00001 dilutions were negative.
[C]Only one value for undiluted sample of dust B because of limited amount of dust available.
[D]This sample gave an outlying value and was not included in the analyses.
[E]All replicates at 0.01, 0.001, 0.0001, and 0.00001 dilutions were negative.

Real-time q-PCR to measure Md5 MDV. The chosen q-PCR target sequence for Md5 was a region of the MDV serotype 1 unique short region (U_S) 2 gene (7). This enabled specific detection of Md5 without detection of the vaccine viruses because the U_S2 gene is absent from HVT (serotype 3), SB-1 (serotype 2), and the BAC-cloned viruses pCVI988-10 and pRB-1B-2 (in which the U_S2 gene is replaced by the BAC F plasmid sequence). Duplex real-time q-PCR assays were performed to quantify both the MDV-1 U_S2 gene and the chicken reference gene *ovotransferrin* (*ovo*) in the same reaction using 40 cycles on an ABI 7500 instrument (Applied Biosystems, Foster City, CA), essentially as previously described (4,5). All reactions contained 10 μg bovine serum albumin to overcome the inhibitory effect of melanin pigment (4). For feather tip samples, standard curves were used to quantify Md5 as genomes per 10^4 feather tip cell equivalents (4); standard curves were prepared using 10-fold serial dilutions of DNA from MDV-1-infected CEF (for U_S2 reaction) and noninfected CEF (for *ovo* reaction) and accurately calibrated against plasmid constructs of known target gene copy number. Because dust is a complex mixture of substances, DNA extracted from dust will not contain exclusively chicken DNA and MDV DNA; thus, it would not be appropriate to quantify MDV per 10^4 cell equivalents (19). Instead, for dust samples the standard curve for the U_S2 reaction was used to quantify Md5 as genomes per microgram of dust (MDV genomes/μg dust; based on the mass of dust used to prepare DNA and the volume of dust DNA used per reaction).

Validation of DNA extraction and real-time q-PCR from dust. In measuring MDV DNA content, the accuracy of DNA extraction from dust and of real-time q-PCR was validated by comparing either 40-cycle threshold (Ct) values (q-PCR cycle threshold value subtracted from 40) or the calculated MDV genome copy number; data were generated from two different sets of serial dilutions, and each set was performed in triplicate. The first set of dilutions was prepared using MDV-infected dust diluted with noninfected dust (which more accurately simulates quantification of MDV in dust samples containing different amounts of cell-free virus). The second set was prepared using DNA extracted from MDV-infected dust diluted with DNA extracted from noninfected dust. Noninfected dust had been previously collected from the prefilter of an isolator housing 20 noninfected chickens and was confirmed by q-PCR to be negative for MDV DNA. Three independent samples of MDV-infected dust (hereafter referred to as samples A, B, and C), which had previously been used to prepare DNA and shown by q-PCR to encompass a 1000-fold range of MDV content, were selected. Tenfold serial dilutions (10^{-1}–10^{-5}) of each of the samples A, B, and C were prepared by adding 3 mg MDV-infected dust to 27 mg noninfected dust. Thorough mixing of dust was achieved using a vortex mixer at each dilution step. A clean, disposable spatula was used to dispense dust at each dilution step. Three 5-mg aliquots were taken from each dilution of each of the three dust samples A, B, and C and DNA was prepared. Additionally, 10-fold serial dilutions (10^{-1}–10^{-5}) of DNA prepared from infected dust samples A, B, and C were made by adding 5 μl MDV-infected dust DNA into 45 μl of noninfected dust DNA. Each dilution series was prepared in triplicate. Mixing of DNA was achieved using a vortex mixer at each dilution step. A clean pipette tip was used for each dilution. DNA samples from all dilution series were subject to q-PCR to quantify the MDV genomes. Mean values for triplicate samples were determined using the $\log_{10}$ transformed copy number for each individual sample and then back-transformed to obtain the actual values.

Statistical analyses. Using Minitab statistical software (v15), all sets of serial dilutions of dust and dust DNA were compared using regression. Both q-PCR 40-Ct values, and the corresponding $\log_{10}$ MDV genome copies, were regressed against the $\log_{10}$ dilution of dust or DNA, as appropriate. Analysis of variance (general linear model [GLM]) was used to compare the slopes and intercepts for each serial dilution. Mean levels of Md5 in feather tips or dust were compared between groups using an analysis of variance GLM. To investigate the correlation between MDV genome level in feather tips and dust for groups of chickens, regression analysis was performed. For each group of chickens and each time-point, the mean level of Md5 MDV in the feather tips of 10 chickens (Md5 genomes per 10^4 feather tip cells) was plotted against the level of Md5 in the dust collected from that group at that time-point (Md5/μg dust).

RESULTS

Validation of extraction of DNA from dust, and validation of real-time q-PCR. Table 1 summarizes real-time q-PCR data for dilutions prepared using MDV-infected dust diluted with noninfected dust. Table 2 summarizes real-time q-PCR data for dilutions prepared using DNA extracted from MDV-infected dust diluted into DNA extracted from noninfected dust. Both tables show 40-Ct values and calculated number of MDV genomes/μg dust for each triplicate sample of each 10-fold dilution and for each of the three dust samples. Dust sample A was highly infected with MDV, having an approximately 30-fold higher number of MDV genomes/μg dust than did sample B and 1500-fold higher than sample C. A negative q-PCR result was obtained only after a 10^5-fold dilution for sample A dust or DNA, after a 10^3-fold dilution for sample B dust or DNA, after a 10^2-fold dilution for sample C dust, and after a 10^1 dilution for sample C DNA.

Table 2. Triplicate q-PCR measures for 40-Ct value and MDV genome load for each replicate of DNA from MDV-infected poultry dust serially diluted with DNA from noninfected dust.

MDV-infected dust sample	Dilution factor	40-Ct value			MDV genomes per microgram dust		
		Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3
A[A]	1	16.24	15.95	16.10	6410.00	5270.82	5832.21
	0.1	12.88	13.08	12.92	664.15	760.10	682.32
	0.01	9.60	9.70	9.78	72.63	77.70	82.00
	0.001	5.98	6.35	5.70	6.31	8.11	5.23
	0.0001	4.15	3.83	2.89	1.84	1.48	0.78
B[B]	1	11.41	11.24	11.13	246.32	219.63	203.92
	0.1	8.02	8.38	8.10	25.01	31.88	26.39
	0.01	4.99	4.99	4.97	3.24	3.24	3.19
C[C]	1	5.13	5.02	4.89	3.56	3.30	3.03

[A]All replicates at 0.00001 dilution were negative.
[B]All replicates at 0.001, 0.0001, and 0.00001 dilutions were negative.
[C]All replicates at 0.1, 0.01, 0.001, 0.0001, and 0.00001 dilutions were negative.

For dilutions of dust, and also for dilutions of dust DNA, there was very good repeatability between 40-Ct values and, hence, the number of MDV genomes, for triplicate samples; this confirmed the accuracy and reproducibility of MDV q-PCR. Tenfold dilutions of dust DNA were very accurate as shown by the fact that MDV genomes/μg dust decreased by approximately 10-fold at each dilution (Table 2). Tenfold dilutions of dust were somewhat less precise (Table 1), presumably reflecting the fact that accurate measurement, dispensing, and mixing was considerably more difficult for samples of dust than for solutions of DNA.

Figure 1 shows validation based on calculated number of MDV genomes/μg dust for dust samples (Fig. 1a) and for samples of DNA extracted from dust (Fig. 1b). Both the number of MDV genomes (Fig. 1) and the 40-Ct value (not shown) were highly correlated with sample dilution factor for each dust and DNA sample ($R^2 \geq 0.98$ and $P < 0.0001$ in every case), confirming accurate quantification. There were slight differences in the slope of the relationship between dilution series for independent samples of dust ($F_{4,44} = 4.99$, $P = 0.002$), but this accounted for less than 0.6% of the variance in the statistical model (adjusted $R^2 = 98.39\%$). The slope of the common line was 1.07 ± 0.04, not significantly different from one. The slopes between the dilution series of dust and dilution series of DNA did not differ significantly. The data give an accurate demonstration of the sensitivity and reproducibility of both the DNA extraction and the PCR.

The standard curve for the U_S2-specific q-PCR reaction (not shown) showed that the limit of detection was 26 MDV genomes. The volume of DNA used in each reaction equated to that extracted from 100 μg dust. Thus, the limit of detection for calculated Md5 load was 0.26 genomes/μg dust.

Efficiency of vaccination in reducing replication and shedding of Md5. All chickens in every vaccinated group (groups 1–4) were protected against mortality during the experimental period to 31 dpc. All chickens in the nonvaccinated group (group 5) reached humane end-point between 5 and 10 dpc, consistent with the fact that maternal–antibody-free RIR chicks are highly susceptible to early mortality following intra-abdominal infection with Md5. Thus, feather and dust samples were only available from the latter group at 3 and 6 dpc. At these two time-points, the level of Md5 in the feather tips was significantly lower in the four vaccinated groups than in the nonvaccinated group ($P < 0.0001$ in all cases). The level of Md5 in the dust was significantly lower in the pCVI988, HVT, and HVT+SB-1 vaccinated groups than in the nonvaccinated group ($P < 0.05$), but there was no significant difference between the pRB-1B-2 vaccinated group and the nonvaccinated group.

Table 3 shows the proportion of feather and dust samples positive for Md5 by q-PCR in each group at each time-point. Vaccination delayed both the replication of detectable levels of Md5 in the feather tips and the shedding of detectable levels of Md5 into the dust. The first time-point for detection of Md5 in the feather tips always preceded or coincided with the first time-point for detection in the dust in each group. Over the time-course of 3–31 dpc, vaccine type had a significant effect on level of Md5 in feather tips ($P < 0.001$), and dust ($P < 0.05$; Fig. 2). For each sample type, the level of Md5 was significantly lower in the pCVI988 and HVT+SB-1-vaccinated groups than in the pRB-1B-2-vaccinated group, while the HVT-vaccinated group did not significantly differ from any of the other three vaccinated groups.

Relationship between Md5 level in feather tip and dust samples. For the four vaccinated groups, the correlation between the level of Md5 in feather tips and the level of Md5 in dust, measured by q-PCR, is shown in Figure 3. Each point on the plot represents the mean virus level in dust and the mean virus level in feathers for a particular virus at a particular time-point. There was a close correlation ($R^2 = 0.87$) between the mean virus level in feathers in each group and the virus level in dust shed by that group. However, the slope of the line was 0.82 (with 95% confidence intervals of 0.70–0.93). As the slope is <1, this indicates that a high virus genome load in feathers is less-effectively translated into a virus genome load in dust.

DISCUSSION

In this study we utilized published methods for extraction and q-PCR measurement of MDV DNA in poultry dust samples (20,21) to determine the association between MDV genome load in feather tips and that in poultry dust. We initially performed further validations on the DNA extraction and q-PCR methodologies to confirm their accuracy and sensitivity. We then directly demonstrated a very close correlation between mean MDV genome levels in feather tips and mean MDV genome levels in dust shed by vaccinated chickens from 3–31 dpc.

The ''poultry dust'' in the environment of a poultry house is a heterogenous and variable mixture containing particles of dried feces, feed, litter, dander from the feathers and skin of chickens, and pathogens associated with any of the above. These particles may be in the form of powder, lumps, or fibers. The amount of dander shed by individual chickens will depend upon factors such as their age and size (20) and also their health status. MDV has been detected in the

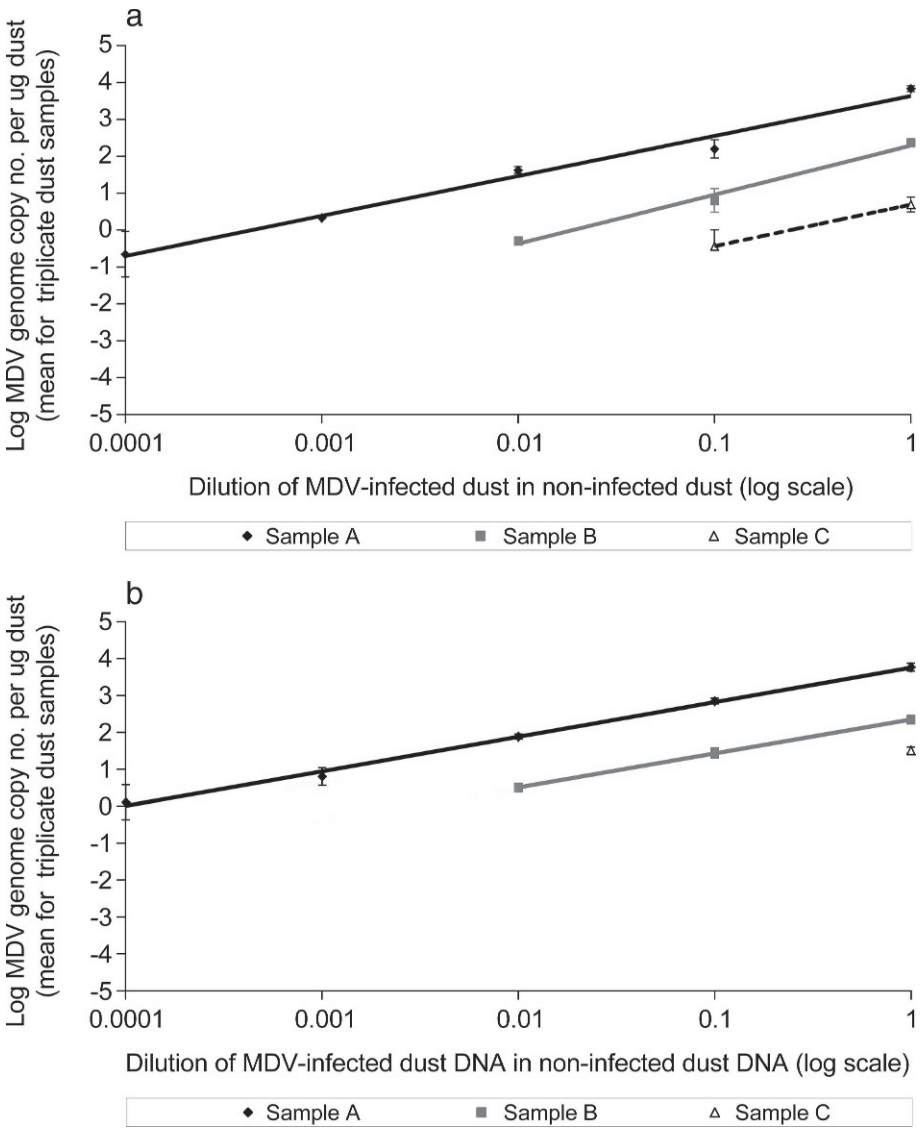

Fig. 1. Validation of dust DNA extraction and q-PCR based on calculated MDV genomes per microgram dust. Three independent samples of MDV-infected dust (samples A, B, and C) were selected. Validation was carried out by comparing calculated number of MDV genomes for two different sets of serial dilutions, performing triplicates for each set. (a) Dilution series 1: MDV-infected dust diluted with noninfected dust. (b) Dilution series 2: DNA prepared from MDV-infected dust diluted with DNA prepared from noninfected dust. DNA samples from all dilution series were subject to q-PCR to quantify MDV genomes. Mean values for triplicate samples were determined using the $\log_{10}$ transformed copy number for each individual sample, plotted against dilution factor, and then regression analysis was performed.

feather tips (5) and poultry dust (20) as early as 7 dpi, increasing rapidly to 21 dpi (21). In fact, mathematical models predict that shedding of MDV may begin as early as 6 dpi (1). MDV can persist long-term within the poultry dust which is, thus, an ideal sample to collect for monitoring levels of MDV because it can be obtained noninvasively, is easy to store and transport, and gives an overall picture of the level of MDV contamination of the poultry house (19).

In order to compare dust samples with respect to MDV level, we used a standard mass of poultry dust for preparation of DNA (5 mg), a standard volume for elution of DNA (200 µl), and a standard volume of DNA solution in q-PCR (4 µl). We demonstrated the accuracy, sensitivity, and reproducibility of MDV DNA extraction from dust, as well as the accuracy, sensitivity, and reproducibility of MDV q-PCR on DNA prepared from dust, thereby confirming that differences in virus levels measured in dust reflect differences in shedding and not inaccuracies in weighing of dust or q-PCR errors. Thus, these methods can reliably be used to monitor environmental contamination by MDV.

The association between presence, level, and timing of MDV detection in the feather follicle, and its transmission to contact chickens, has long been known (22,25). However, the relationship between the level of MDV in the feather tissues and the environment has never previously been reported, and the current study is the first to demonstrate a close correlation between these levels. We examined whether measurement of MDV in poultry dust can be used to accurately monitor infection status of the flock; that is, whether the level of MDV in poultry dust reflects the level of infection in the chickens. Samples of feather tips and poultry dust were collected from groups of vaccinated experimental chickens which were infected with vvMDV strain Md5 and housed in poultry isolators. There was close correlation between mean virus level in feathers (10 birds) and mean virus level in dust (three aliquots) for each vaccinated group at multiple time-points between 3–31 dpc.

Table 3. Proportion of feather and dust samples positive for Md5 by q-PCR.

Vaccine group	Sample	Proportion of samples positive by q-PCR at given time (dpc)[A]								
		3	6	10	13	18	21	24	27	31
Nonvaccinated	Feather	4/10	10/10	—[B]	—	—	—	—	—	—
	Dust	0/3	3/3	—	—	—	—	—	—	—
pCVI988-10 vaccinated	Feather	0/10	2/10	10/10	6/10	10/10	6/7	7/7	7/7	7/7
	Dust	0/3	0/3	1/3	0/3	3/3	3/3	3/3	3/3	3/3
pRB-1B-2 vaccinated	Feather	0/10	2/10	10/10	10/10	10/10	7/7	7/7	7/7	7/7
	Dust	0/3	2/3	2/3	3/3	3/3	3/3	3/3	3/3	3/3
HVT vaccinated	Feather	1/10	0/10	3/10	6/10	10/10	7/7	7/7	7/7	7/7
	Dust	0/3	0/3	1/3	3/3	3/3	3/3	3/3	3/3	3/3
HVT+SB-1 vaccinated	Feather	0/10	2/10	9/10	10/10	10/10	7/7	7/7	7/7	7/7
	Dust	0/3	1/3	1/3	1/3	3/3	3/3	3/3	3/3	3/3

[A]Feather samples were collected from every chicken in each group: from 3–18 dpc, $n = 10$ chickens per vaccinated group; from 21–31 dpc, $n = 7$ chickens per vaccinated group.
[B]No samples available at these time-points because all chickens had succumbed to mortality by 10 dpc.

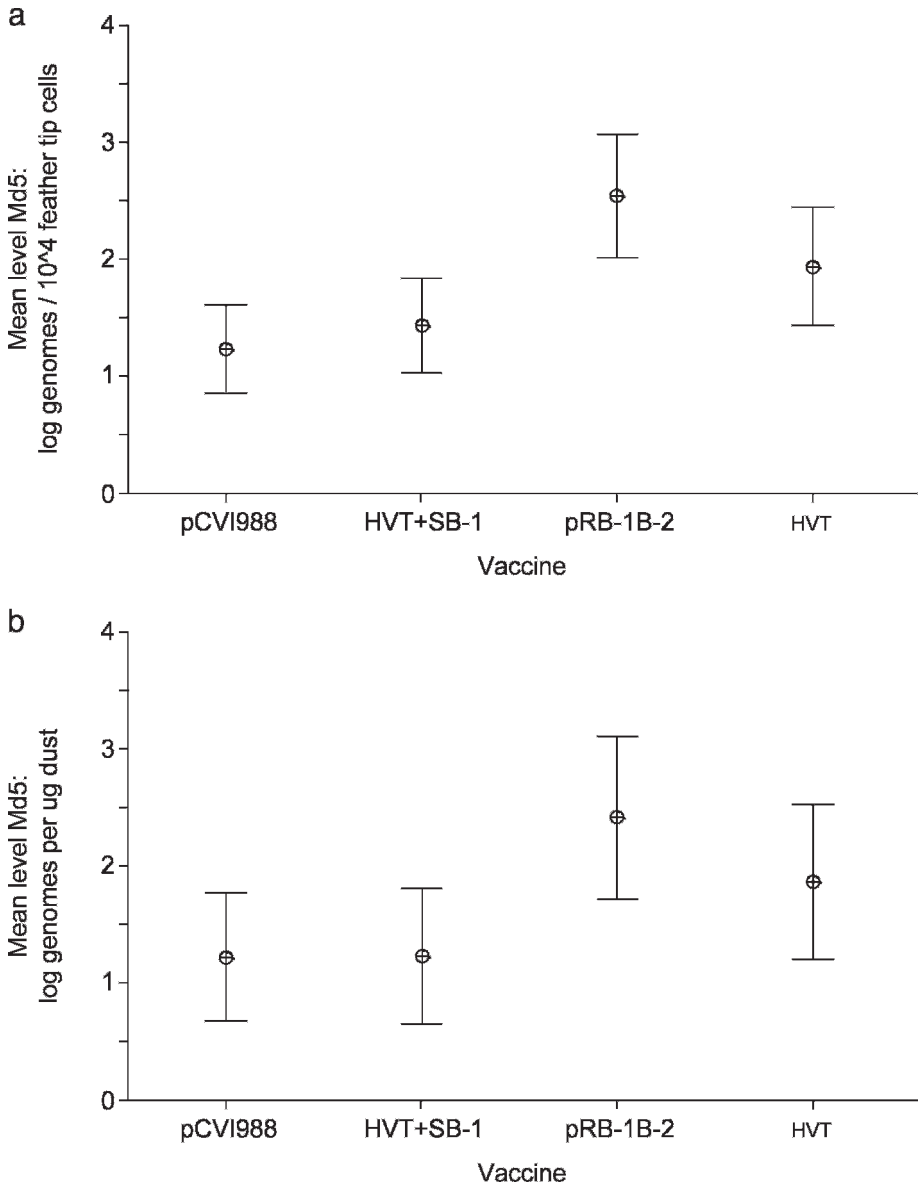

Fig. 2. Mean level of Md5 in feather tips and dust from 3–31 dpc. The mean level of Md5, across all time-points from 3–31 dpc, is shown for each vaccinated group with 95% confidence intervals in (a) feather tips (Md5 genomes per 10^4 feather tip cells) and (b) dust (Md5 genomes/μg dust).

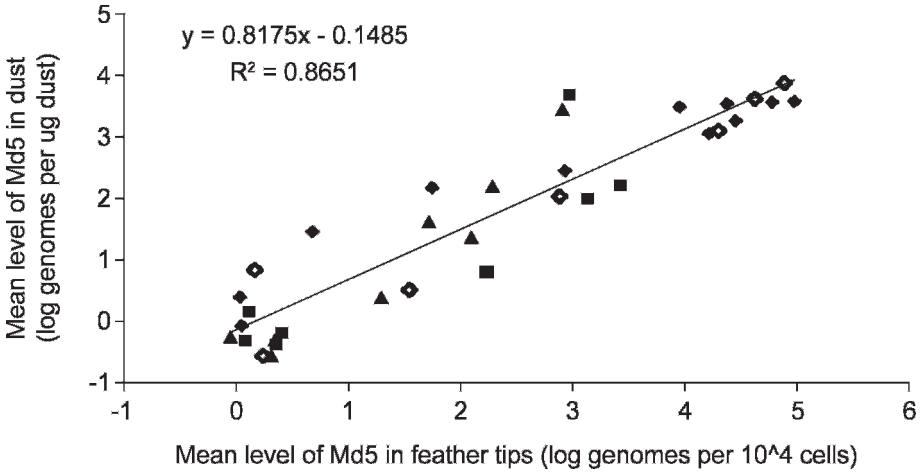

Fig. 3. Correlation between Md5 genome level in feather tips and dust. Virus levels in feather tips and in dust were measured by q-PCR. Each point on the plot represents the correlation of mean virus level in feathers (seven or 10 birds) and mean virus level in dust (three aliquots) at a particular time-point for a particular group: birds vaccinated with HVT (black diamonds), pCVI988 (black triangles), HVT+SB-1 (black squares), or pRB-1B-2 (white diamonds).

Furthermore, this relationship was similar in groups vaccinated with viruses representing each of the three MD vaccine serotypes, confirming published data on demonstrations that measurement of MDV in dust can be used to accurately monitor infection status of vaccinated chickens (20).

A large proportion of commercial flocks may be infected with virulent MDV. However, infection status is not necessarily correlated with protection because vaccinated chickens can still become infected and shed infectious virus but be protected from disease. Thus, a high level of MDV in poultry dust may not be associated with MDV-induced mortality in that flock. Even so, there are practical implications for the finding of a high level of MDV contamination. A high MDV load in the dust is an indicator of the pressure imposed on a vaccinated flock by virulent MDV, which may select for increasingly virulent strains. It should also reinforce the biosecurity and disinfection precautions that must be considered with a heavily contaminated flock and poultry house, particularly if young or unvaccinated chicks are scheduled to enter the house after a contaminated flock.

It should be noted that the level of MDV in feather tips reflects the mean level of MDV at this site on the day on which those feathers were collected, whereas the samples of dust reflect the shedding of MDV during the time since the prefilter was last replaced during the previous dust collection. Furthermore, presence of MDV in the feather tips is considered a prerequisite for shedding of MDV. Thus, observed changes in level of MDV in the dust will tend to lag behind observed changes in the feather tips. This could explain why the slope of the relationship between feather and dust titers is <1. Because MDV load was normalized to a given mass of dust, and expressed as MDV genomes/μg dust, we do not consider that the difference between a 3-day sampling interval and a 4-day sampling interval would have a significant effect on the calculated MDV load because the additional shedding of MDV genomes would approximately correlate with the additional mass of dust shed on one additional day.

The maximum level of MDV measured in the dust samples from experimental chickens in this study was almost 10^3-fold greater than that measured in the dust of a commercial poultry house on day 35 after placement of chickens (19), but was similar to the peak level measured in isolators housing vaccinated experimental chickens infected with Australian MDV isolates (20). (Note that data from (19) and (20) are presented as MDV genomes per milligram of dust while our data are presented as MDV genomes per microgram of dust). This finding reflects the fact that the experimental chickens were housed in a confined space and were simultaneously injected with a high dose of MDV, while commercial birds are housed in a much greater airspace and gradually become infected with a variable dose of cell-free MDV; thus, the single time-point at which dust was collected may not represent the peak of shedding. Those researchers also estimated the daily shedding rate of MDV per chicken based on daily dander production and MDV shedding (20) and on total MDV content in the air of the poultry housing by using the estimated particulate content per m^3 of air (21). The latter measurement is much more difficult to determine but has distinct advantages in that it measures total environmental contamination by MDV by factoring in the total amount of dust as well as the level of virus per milligram of dust. Using such data, a mathematical model was developed to estimate viral shedding rates for MDV (1).

It is important to recognize that q-PCR measures the level of MDV DNA, which does not necessarily represent the level of infectious MDV, and also to recognize that detection of high levels of MDV in the feathers or dust does not necessarily indicate disease, as properly vaccinated chickens are likely to be protected against clinical signs. As demonstrated in this study, despite conferring protection against mortality following Md5-infection, none of the four vaccines prevented replication and shedding of Md5 consistent with previous observations using other highly virulent challenge strains (14,17,20,21,32). However, the data presented in Figure 2 indicate that the second generation vaccine (HVT+SB-1) and the cloned third generation vaccine (pCVI988) were more effective at reducing MDV titers in feathers and dust than were the first generation vaccine (HVT) and the experimental vaccine (pRB-1B-2). The three monovalent vaccines were all administered at a similar dose (range 725–1800 pfu), so the data are directly comparable. The dose of HVT+SB-1 was notably greater (total 8700 pfu), a factor which may contribute to its greater efficiency compared with the pRB-1B-2 and monovalent HVT in this study.

This study was carried out in experimental birds in a controlled environment in order to establish fundamental data in the absence of variables present in commercial poultry flocks. There are some caveats with respect to correlation of MDV levels in poultry dust and feather samples in commercial flocks. Infection will be via the

respiratory route and will not be synchronous or uniform (in terms of either dose or time of infection) in all chickens of the flock. Thus, the level of MDV measured in the feathers of individual chickens may be considerably higher or lower than that measured as a ''flock average'' in the poultry dust.

Nevertheless, we have confirmed the findings of previously published work (19,20,21) that poultry dust can be used as an easy and noninvasive sample for q-PCR measurement of MDV DNA, and we have applied this method to assess the efficiency of vaccination in reducing replication and shedding of virulent MDV in an experimental model.

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