

A Appendix for Industry-wide surveillance of Marek's disease virus on commercial poultry farms

A.1 Vaccine virus correlations with wild type virus

4 Although vaccination does not preclude infection with and transmission of wild type Marek's disease virus, several studies have shown that vaccines can alter wild type virus infection and shedding rates (11, 14, 15). We explored correlations between vaccine virus concentration and wild type virus concentration in dust. To do this, we measured HVT and SB-1 virus
8 concentration in 396 qPCR reactions, using dust samples from the cross-sectional data. We also measured Rispens vaccine virus concentration using all of the samples in the cross-sectional data. Note that Rispens virus was inferred to be absent in some samples based on a missing artifact in wild type virus qPCR (Appendix A.16). We then tested whether there
12 was a correlation between these vaccine virus concentrations and wild type virus dynamics using linear mixed effects models. We performed this analysis on the presence-absence version of the wild type virus data, and the wild type virus concentrations themselves. Due to the large number of samples with no detectable Rispens virus, we repeated the analysis
16 of a Rispens vaccine effect excluding any samples determined to be negative for Rispens virus (leaving 534 qPCR reactions). In all cases, we compared a model including a fixed effect of vaccine virus concentration to a model lacking that effect using a likelihood ratio test. Models were fit in R using the function 'glmer' in the package 'lme4' with REML set
20 to FALSE. Likelihood ratio tests were performed using the function 'lrtest' in the package

‘lmtest’. Models took the form:

$$M \sim V + (1|F), \tag{1}$$

where M is the data on wild type Marek’s disease virus, V is the data on the vaccine virus of interest, and $(1|F)$ denotes the random effect of farm. The random effect was necessary
24 to account for clustering of the data. In fig. S1, we show that wild type virus concentration was not correlated with HVT ($\chi^2 = 0.3$, $d.f. = 1$, $p = 0.58$) or SB-1 ($\chi^2 = 0.4$, $d.f. = 1$, $p = 0.54$) virus concentration, but that there was a negative correlation between wild type virus concentration and Rispens virus concentration, both when Rispens negative samples
28 were included ($\chi^2 = 37.4$, $d.f. = 1$, $p < 0.001$) and when they were excluded ($\chi^2 = 18.1$, $d.f. = 1$, $p < 0.001$). Reanalysis treating the wild type virus in dust as presence-absence data yielded very similar patterns, showing no correlation with HVT ($\chi^2 = 1.1$, $d.f. = 1$, $p = 0.30$) or SB-1 ($\chi^2 = 0.0$, $d.f. = 1$, $p = 0.94$), but a negative correlation with Rispens
32 virus both when Rispens negative samples were included ($\chi^2 = 18.6$, $d.f. = 1$, $p < 0.001$) and excluded ($\chi^2 = 12.8$, $d.f. = 1$, $p < 0.001$).

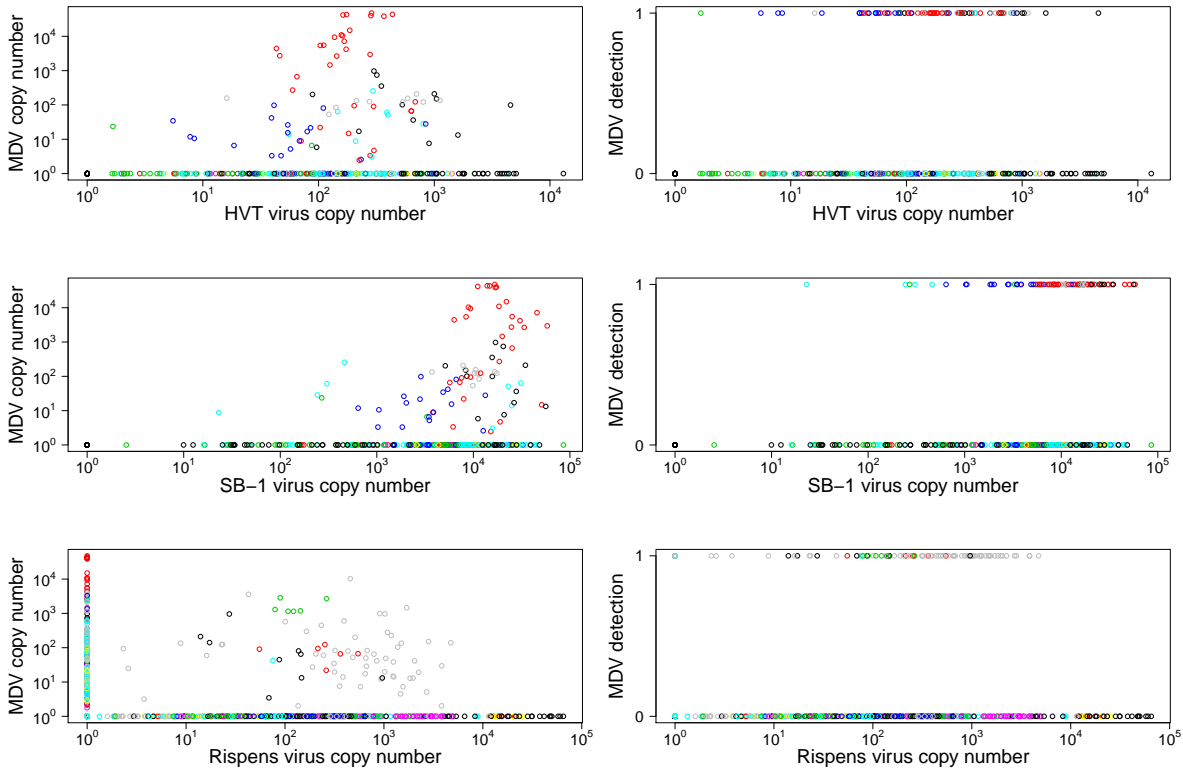


Figure S1: Correlation between wild type virus and each of the three vaccine virus types. Different colors denote data collected from different farms. Copy numbers are based on virus genome copies detected per qPCR template. Note that the value one was added to each copy number measure. MDV detection is plotted as zero if virus was undetected by qPCR, or one if virus was detected.

A.2 Assessing spatial heterogeneity

A typical commercial broiler chicken house in Pennsylvania is 400 ft × 50 ft. One might
 36 therefore wonder whether virus concentrations are spatially variable. We explored this possibility through an observational study. We divided houses into six arbitrary sections as shown in fig. S2. In each section, 5 dust samples were collected from wooden ledges located along

the wall of the house, and in sections that also contained fans, we collected an additional
40 five samples from the louvers that cover the fans. This collection method was performed in
2 houses, for 2 flocks of birds. In each case, three sections contained wooden ledges in addi-
tion to fans, and three sections contained wooden ledges but no fans. Virus quantities were
measured using the qPCR method described in the main text. Here we used cycle threshold
44 scores (CT scores) as our estimate of virus concentration. CT scores are inversely correlated
with the log of virus concentration, such that small CT scores represented high concentra-
tions of virus. Values of 40 were assigned to samples that never crossed the fluorescence
threshold, because our qPCR assay was run for a total of 40 cycles.

48 We tested for heteroscedasticity, that is a change in variance with the mean, in the CT
scores by examining the mean squared difference in CT score between our replicate samples
against the mean in CT score for the two samples. A likelihood ratio test found a significant
effect of mean CT score on the mean squared difference ($\chi^2 = 5.18, d.f. = 1, p = 0.023,$
52 fig. S3). Nonetheless the effect was small relative to the variation in our data ($R^2 = 0.03$). We
therefore ignored this heteroscedasticity when testing for effects of “location” and “substrate”
in our data.

The data were divided into three balanced datasets for analysis. The first dataset con-
56 tained all samples collected from wooden ledges, and no fan samples, hereafter referred to as
“wood data”. The second dataset contained all samples collected from fans, and no wooden
ledge samples, hereafter referred to as “fan data”. The third dataset contained all samples
collected from fans, and the samples that were collected from wooden ledges in sections that

60 also contained fans, hereafter referred to as “fan and wood data”.

We next constructed linear mixed effects models, and fit them in “R” using the function “lmer” in the package “lme4” (2). These models contained fixed effects of “House”, “Flock”, and the interaction between them. For the fan and wood data, we additionally included a
64 fixed effect of “Substrate” to allow for differences in CT score between samples collected from wooden ledges and those collected from fan louvers. All models also contained a random effect of “Sample”. For the wood data and fan data, we were interested in determining the significance of “Section”, and so we also constructed models that also included or excluded
68 this factor. Section was nested in house and flock. These simpler models were compared to their respective more complex models using likelihood ratio tests. We found that “Section” was highly significant in the wood data ($\chi^2 = 104.16, d.f. = 1, p < 0.001$), but it was not significant in the fan data ($\chi^2 = 1.76, d.f. = 1, p = 0.18$). This suggests that fan louver
72 dust samples were more well mixed than wooden ledge dust samples, presumably, because the fans were designed to pull air from throughout the house. For the wood and fan data, we compared two models that contained all factors above, where one also included an effect of “Substrate”. This analysis showed a significant effect of substrate ($\chi^2 = 43.2, d.f. =$
76 $1, p < 0.001$), where virus concentration was higher on samples collected from wood samples than from fan samples. These analyses suggest that dust samples collected from fan louvers would show less spatial variation than samples collected from wooden surfaces, and that virus concentrations would differ when collected from wooden ledges or fan louvers. We
80 therefore used only samples collected from fan louvers during our surveillance. Despite this

preliminary data suggesting that fans do not show spatial variation, we were conservative in our collection methods by sampling from the louvers of multiple fans during each collection trip.

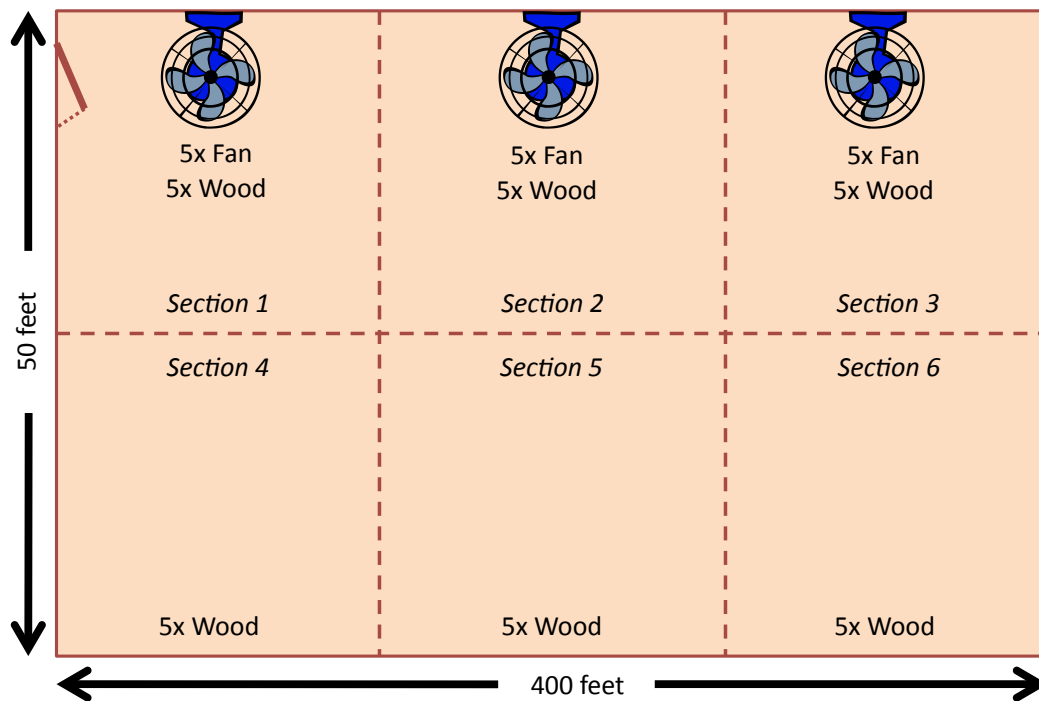


Figure S2: Schematic of a typical commercial broiler chicken house. Dotted lines illustrate partitioning used in the above sampling experiment, and do not represent physical separators within the houses.

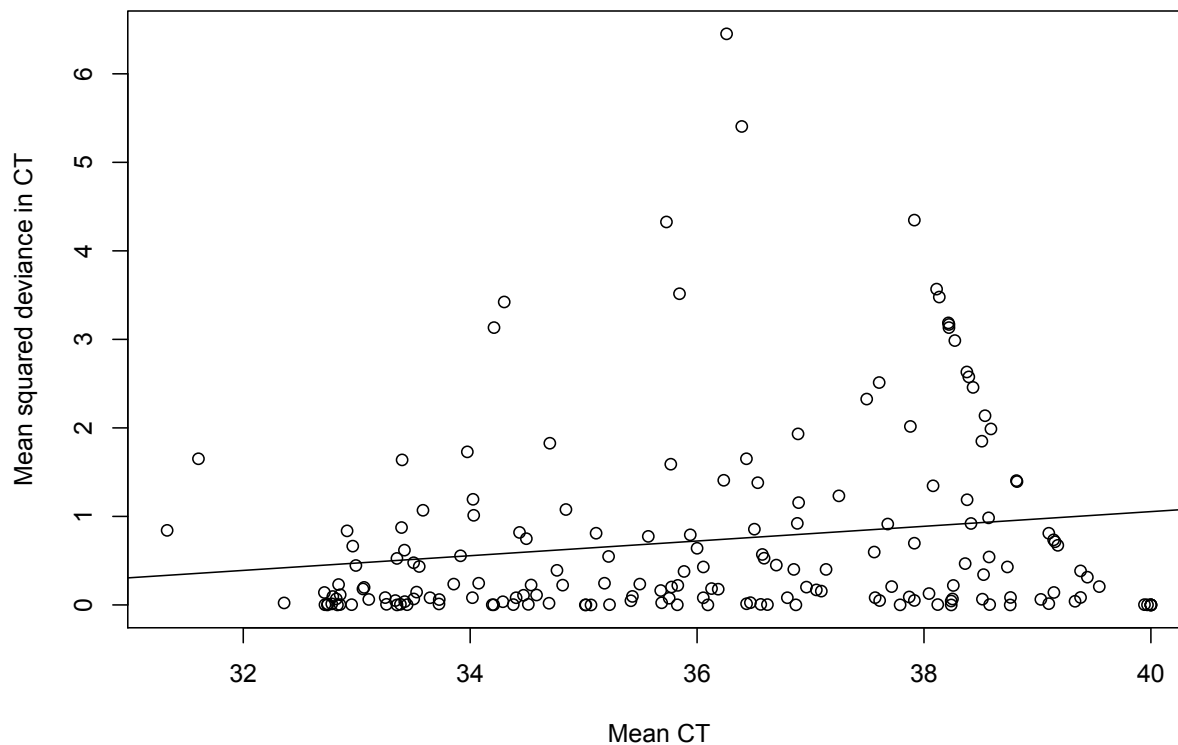


Figure S3: Mean square deviance of biological replicates as a function of CT score. The solid line is the best fit regression line.

84 **A.3 qPCR methods**

For fan dust samples, at the earliest convenience, typically within one week, we weighed out duplicate 2 mg samples of dust on a Mettler Toledo balance (Cat # 97035-620). Virus DNA was extracted from dust samples using the Qiagen DNeasy Blood & Tissue kit (Cat
 88 # 69506). The following changes were made to the standard “Animal Tissue” protocol. In step 1, we used 2 ± 0.2 mg of chicken dust, and we increased the amount of Buffer ATL

to 380 μ l. In step 2, we incubated our samples overnight at 56 °C with constant shaking at 1,100 RPM on a VWR symphony Incubating Microplate Shaker (Cat # 12620-930). In
92 step 3, we doubled the volume of ethanol and Buffer AL to 400 μ l each. Between steps 3 and 4, we centrifuged samples at 17,000 RCF to pellet undigested dust to avoid clogging the spin column in step 4. Lastly, in step 4, we bound the DNA to the spin column twice using half of our supernatant each time. This last step was necessary because of the increased
96 sample volume that was generated from our modifications to steps 1 and 3. Air tube and feather tip samples were processed identically standard “Animal Tissue” protocol, with the exception of the added centrifugation between steps 3 and 4. Unlike the fan dust samples, the entire template was used in initial DNA extraction, and so these samples were processed
100 in singlicate.

We used the primer-probe combination of Baigent et al. (1) to quantify wild-type virus in the presence of vaccine virus. This assay targets a portion of the *pp38* gene, which is present only in Marek’s disease virus serotype 1. Two of these vaccine strains (HVT and SB-1) are
104 different serotypes; their genomes do not contain a *pp38* gene, and therefore they do not amplify in the qPCR assay. The third vaccine strain (Rispens) is a serotype 1 virus, as are wild-type strains, and all have a *pp38* gene. The qPCR was designed to target a region of the *pp38* gene that contains a single nucleotide polymorphism that differs between Rispens
108 virus and all sequenced wild-type strains. The Rispens virus did amplify minimally in the qPCR, resulting in minor fluorescence. We were nonetheless able to accurately determine the presence and quantity of wild-type virus when it was present at a ratio of as low as 1

copy per 10 Rispens virus copies (Appendix A.16). Below this ratio, the wild-type virus was
112 often still detected, but the estimate of its quantity became biased. In Appendix A.17 we
show that this interference was unlikely to affect our conclusions.

All qPCR assays were run on a 7500 Fast Real-Time PCR System machine (Cat #4351107).
The pp38-FP and pp38-RP primers and the pp38-Vir(1) probe from Baigent et al. (1) were
116 used at concentrations of 300 nM, 300 nM, and 100 nM respectively. We used the PerfeCTa
qPCR FastMix, UNG, Low ROX reaction mix (Cat #95078), and we followed the standard
protocol with the exception of adding bovine serum albumin (BSA) from Sigma Aldrich (Cat
#B4287) to a final concentration of 1 $\mu\text{g}/\mu\text{l}$. The final reaction volume contained 21 μl of
120 master mix and 4 μl of DNA template. Our cycling conditions included an initial denatu-
ration at 95 °C for 20 seconds, followed by 40 two-step cycles of denaturation at 95 °C for 3
seconds and annealing/extension at 60 °C for 30 seconds. All assays were carried out using
'ABI Sequence Detection Software' version 1.4. We used the prMd5pp38-1 plasmid provided
124 by John Dunn at the Avian Disease and Oncology Lab as a standard. For all qPCR runs,
a quantification curve was generated using five serial tenfold dilutions of the standard in
duplicate, and C_T numbers were translated into DNA copy numbers using this quantifica-
tion curve. As a positive control, each run contained a well of Marek's disease virus DNA
128 extracted from an *in vivo* infection. As negative controls, each run contained a well with
distilled water, and a well with a high concentration of Rispens virus DNA. As previously
mentioned, Rispens virus DNA can result in minor fluorescence, and so this Rispens-virus-
positive well was used to set the critical threshold of our qPCR. In practice, this meant

132 that the critical threshold was set to just above the fluorescence value of this well at cycle
40. The standard deviation between technical replicates of a dust sample was estimated
to be $0.319 \pm 0.006 \log_{10}$ units of virus per mg dust (Appendix A.9). This error estimate
included multiple sources of variation, including heterogeneity within a dust sample, in the
136 DNA extraction efficiency, and in the qPCR amplification and quantification. The standard
deviation between biological replicates was $0.556 \pm 0.022 \log_{10}$ units (Appendix A.9). Our
tests to confirm the accuracy of our qPCR approach are summarized in Appendix A.4.

A.4 qPCR accuracy

140 The accuracy of our qPCR was assessed using a combination of dilutions and mixtures.
First, we wanted to determine whether our qPCR was able to accurately quantify virus copy
number over a wide range of virus densities. We did this by creating a set of samples from
serial ten-fold dilutions of our DNA standards. Our highest concentration was 2.41×10^7
144 virus genome copies per $4 \mu\text{l}$, and our lowest dilution was 2.41×10^0 virus genome copies per
 $4 \mu\text{l}$. With the exception of the highest density, in which target DNA is substantially higher
than any of our field samples, and lowest density, which was below our limit of detection,
these dilutions followed an observed linear decline in Ct of 3.39 per 10-fold dilution. The
148 decline in Ct per 10-fold dilution can be used to calculate the amplification efficiency of a
qPCR assay with a 100% efficiency relating to a decline of 3.32. Our observed curve related
to an amplification efficiency of 97.1% with an R^2 of 0.999. In practice, each plate of qPCR
reactions contained a similar dilution series used as a standard for quantification. From these

152 dilution series, we found a median linear decline in Ct of 3.34 per 10-fold dilution, with 95%
of assays between 3.23 and 3.44. This related to an amplification efficiency of 99.4%, ranging
between 95.3% and 104.0%. In these assays, our median limit of detection was 55.0 virus
copies per mg of dust, with 95% of assays between 21.4 and 168.6.

156 We next wanted to test whether nontarget contaminating DNA would interfere with
the accuracy of the assay. In unpublished data, we saw that in DNA extracted from dust,
nontarget DNA was more prevalent than wild-type virus DNA at a ratio of approximately
5000 to 1 by mass. We therefore mixed plasmid DNA of the virus used as our standard with
160 plasmid DNA of the chicken ovotransferrin (*ovo*) gene to create samples with 500:1, 5000:1,
and 50000:1 ratios of chicken DNA to wild-type DNA by mass. In practice, we mixed a
concentration of 1000 copies of the virus plasmid per $4\mu\text{l}$ with the necessary quantity of the
plasmid having chicken *ovo* gene insert, and this was performed three times for each target
164 concentration. These samples then underwent serial 10-fold dilutions to generate samples
with 100 and 10 copies of the virus plasmid per $4\mu\text{l}$. Our typical standards were run with
these samples to ensure that the assay remained accurate across these conditions. We found
that contamination from nontarget DNA added very little bias to our estimates (fig. S4).

168 We next attempted to validate our quantification of virus from dust by mixing virus
positive dust with virus negative dust and comparing the resulting virus concentrations
measured by qPCR to the values expected. We used 8 different samples of virus positive
dust that spanned a range of virus concentrations, and we mixed this with a dust sample
172 that came from a farm in which virus was never detected in any of our samples. Positive

dust and negative dust were mixed to generate ratios of positive to negative 1:0, 10:1, 3:1, 1:1, 1:3, 1:10, 1:30, and 1:100. These mixtures were then processed in an identical manner to our field collected samples. We analyzed the results using linear models. These models

176 took the form:

$$\text{lm}(V \sim 0 + F + F : S), \quad (2)$$

$$\text{lm}(V \sim 0 + F : S). \quad (3)$$

Here we use the “R” syntax for linear models. V is the measured concentration of virus per mg of mixed dust, F is the fraction of dust that came from the virus positive sample, and S is a factor denoting the sample of virus positive dust used. “ $F : S$ ” is used to express an
180 interaction term between F and S , and “0” is used to express that the intercept of the model is forced through 0. In eq. (1), “F” is treated as a factor when it is on its own. If a likelihood ratio test were unable to reject the model described by eq. (2), that would suggest that the data followed the expected dilution pattern. This was exactly what we found (fig. S5,
184 likelihood ratio test, $\chi^2 = 10.5, d.f. = 7, p = 0.161$), confirming the validity of our DNA extraction protocol.

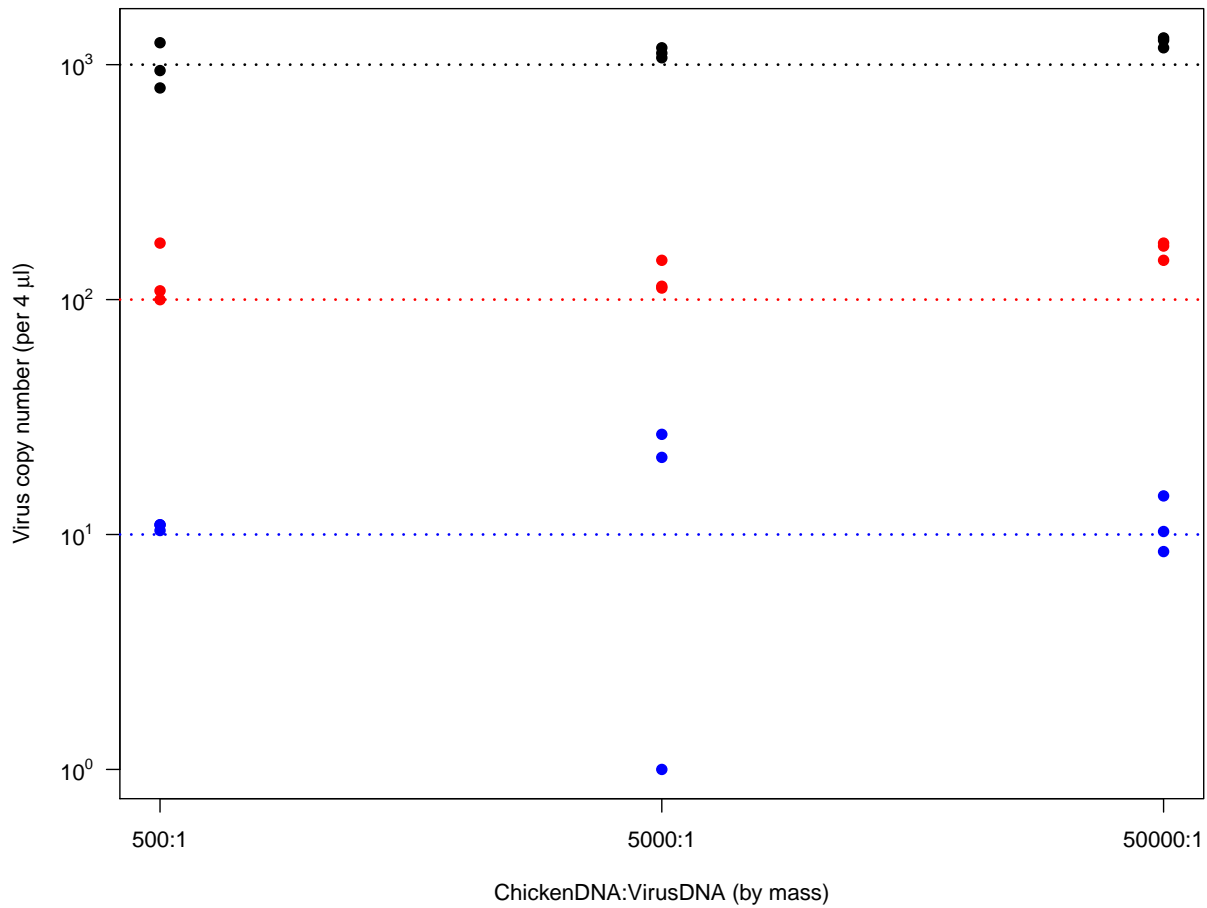


Figure S4: The effect of nontarget DNA (chicken plasmid DNA) presence on virus quantification. Each point shows the virus copy number measured by qPCR. Expected virus quantities for each point are shown by the color-matched dotted lines. These data show that over a wide range of non-target DNA ratios, quantification of virus DNA was unaffected by contaminating DNA.

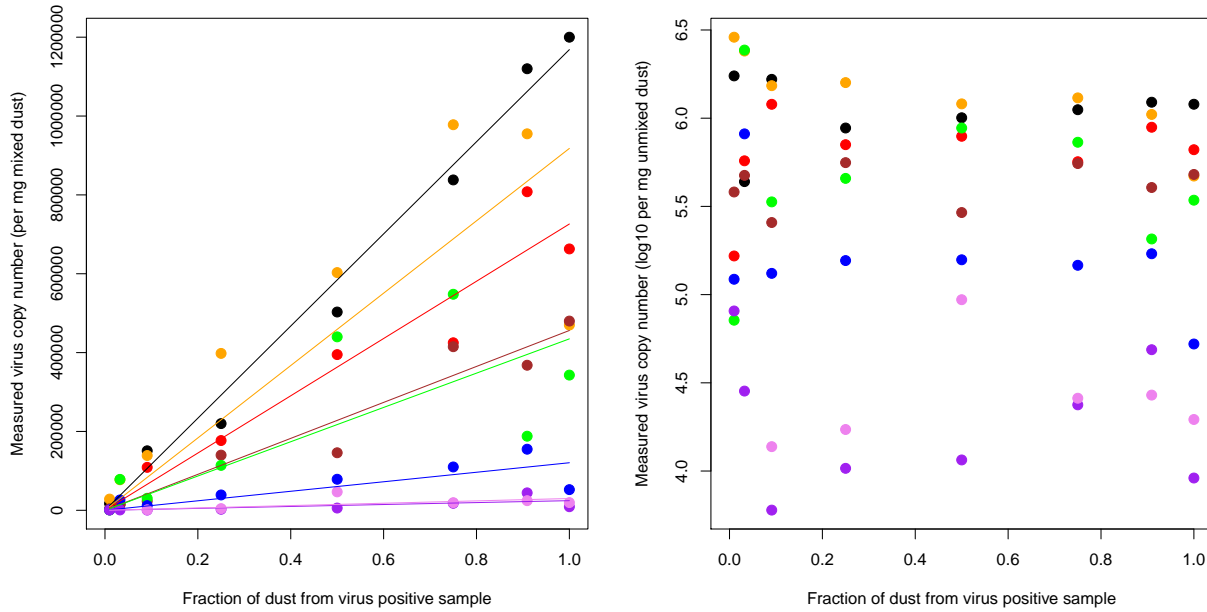


Figure S5: The effects of dust mixing on virus quantification. Panel A shows the measured virus copy number that resulted from the pre-DNA-extraction mixing of virus positive and virus negative dust. Colored points show data that resulted from using different samples of virus positive dust. Color-matched lines are the regression lines that resulted from fitting eq. (2). Panel B shows back calculated estimates of virus copy number per mg of dust in the unmixed virus positive dust. This value was calculated by dividing virus copy number in the mixed dust by the fraction of dust that was from the virus positive sample. The general flatness of color-matched points across the range of mixtures validated our claim that virus copy number can be assessed from dust samples.

A.5 Virus detectability

We used virus shed rates from the published literature and data from our surveillance to
 188 estimate how many chickens would need to be infected before the virus concentration in
 the house would be sufficiently high for our assay to detect its presence. The concentration
 of virus infected dust in a house at the end of a cohort can be described by the following

equation.

$$C = \frac{(T - \tau)(1 - \phi)VI}{T N}. \quad (4)$$

192 Above, C is the concentration of virus per mg of dust, T is the duration of a cohort, τ is the incubation period between infection and maximum virus shedding, ϕ is the fraction of house dust that is produced by non-chicken sources such as litter or feed, V is the amount of virus produced per mg of dust from an infected chicken, I is the number of birds infected, and N is
196 the total number of birds in the chicken house. This is of course only a rough approximation. The true equation would depend on a multitude of unknown variables including the breed specific virus shed rate of the chickens, and the virulence rank of the virus. Nevertheless, this calculation is a useful starting point for interpreting our limit of detection.

200 In the above equation, we made several assumptions. First, we assumed that virus was well mixed. Our analysis has already shown that the virus was not perfectly mixed within dust samples, but the samples were only moderately variable (see Appendix A.9) suggesting that it would have little impact on our detection limit. Second, we assumed that virus
204 infection happened immediately after placement. This assumption seems reasonable given that the virus is highly infectious and resistance increases with bird age (17, 19). Third, we assumed that dust samples were collected at the end of a cohort. In fig. S6, we showed how cohort duration affected our ability to detect virus. This figure could also have been
208 interpreted as our sensitivity to detect virus if collected at the time on the x-axis, although note that this would underestimate our ability to detect virus, making it overly conservative.

Fourth, we assumed that the cohort to cohort infection level was at an equilibrium value. This assumption was necessary to account for dust that persisted between flocks. In our
 212 experience, however, for most farms, very little dust was held over between flocks, and so this assumption was probably unnecessary. Fifth, we assumed that ventilation rates were negligible. If this assumption were violated, it would make our analysis more conservative, because dust produced early would be more likely to be removed by ventilation than dust
 216 produced late, and early dust is likely to have less virus than late dust.

Given these caveats, we solved the above equation for I to determine how many infected chickens would be needed to detect virus in a chicken house.

$$I = \frac{CNT}{V(1 - \phi)(T - \tau)}. \quad (5)$$

We can then substituted in values for C , V , N , ϕ , T , and τ to calculate I . In practice we
 220 used a conservative estimate of the limit of detection $C = 10^2$ (Appendix A.4), two different estimates of V from bivalent vaccinated birds (10), a higher end estimate of the number of chickens in the broiler houses we visited $N = 30,000$, and the incubation period before maximum shedding $\tau = 21$ from Islam and Walkden-Brown (10). For the fraction of dust
 224 that came from non-chicken sources ϕ , we multiplied the fraction of dust not attributable to feed for caged layers, 0.15, by the fraction of dust not attributable to litter for layer birds raised on bedding material, 0.385 (4), and subtracted this product from the number one. This gave us the estimate of $\phi = 0.94$. We used a range of values for T to show how the
 228 duration of a cohort would affect these results. Using these numbers, our limit of detection

was estimated to be between 18.1 and 53.6 infected birds per cohort in a broiler house.

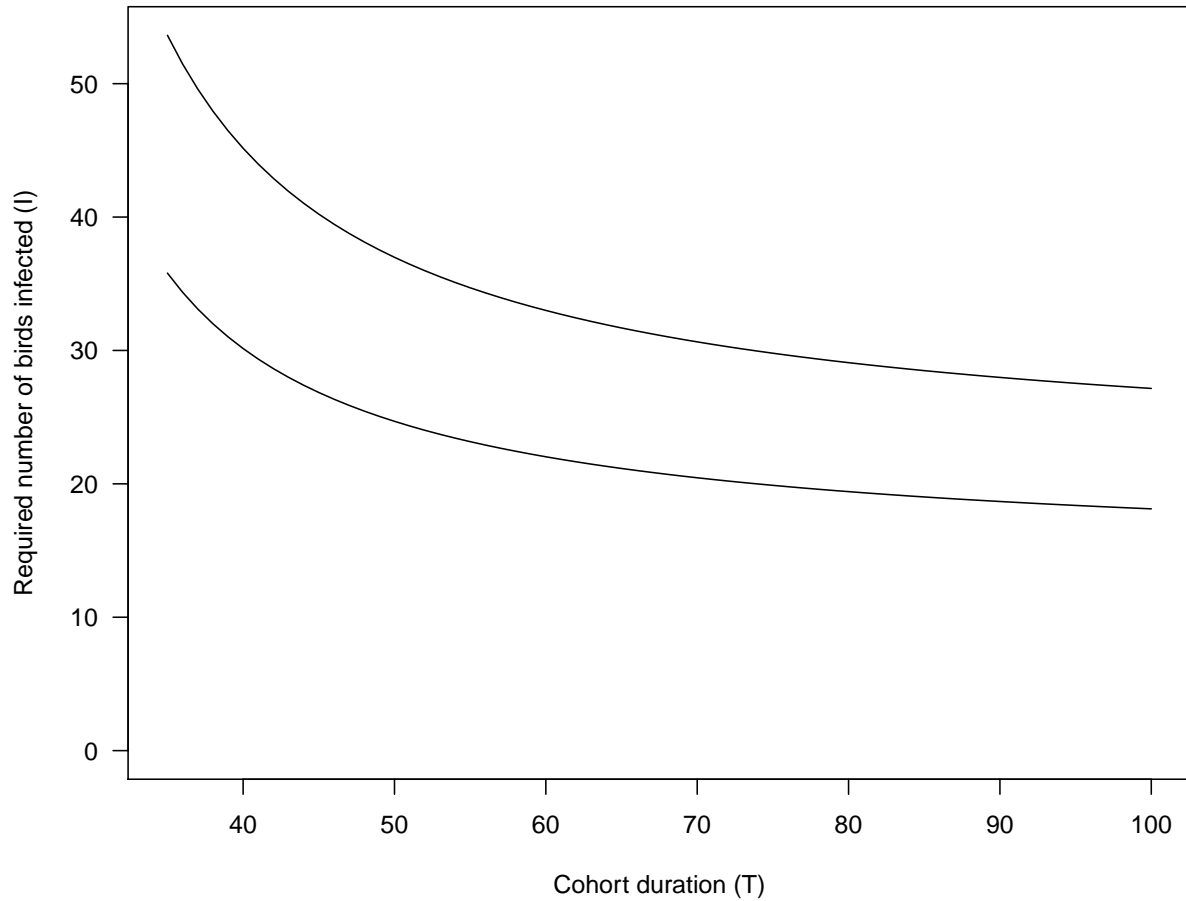


Figure S6: Estimated number of birds that would need to be infected to detect virus by our qPCR assay. The two different lines show detection limits for the two different virus strains characterized by Islam and Walkden-Brown (10). For both cases, over a wide range of cohort durations, our assay was sufficiently sensitive to detect virus when even a small number of birds were infected.

A.6 Feather tip data analysis

A complication in analyzing the feather tip data was accounting for background virus con-
232 tamination. As shown from the air tube data, in some chicken houses, virus DNA was
sufficiently prevalent that it was detectable even without being intentionally introduced. To
account for this potential contamination, control tubes were used during the collection of
feather tips. Each set of feather collections were associated with a “short open” tube, which
236 was left open for the amount of time that it took to collect a single feather from a single
bird (generally less than 10 seconds), and a “long open” tube, which was left open for the
amount of time that it took to collect two feathers from each of ten birds (approximately
30 minutes). This latter tube was open substantially longer than the former because of the
240 time associated with bird selection and capture. This control was therefore likely to be a
substantial overestimate of potential virus contamination.

To determine the fraction of birds with detectable virus in feather tips, we compared the
qPCR virus copy number measurement of sample tubes to that of the control tubes. If at
244 least one of the two feather samples from a bird was higher than both control tubes, that
bird was considered to have detectable virus in its feather tips, and otherwise not. Of course,
had we chosen a different cutoff our estimates would have differed. Fig. S7 shows this, by
using a more conservative requirement that both feather samples needed to give higher copy
248 number reads than both controls, and a less conservative cutoff that at least one of the two
feather samples needed to give a higher copy number read than the “short open” control.

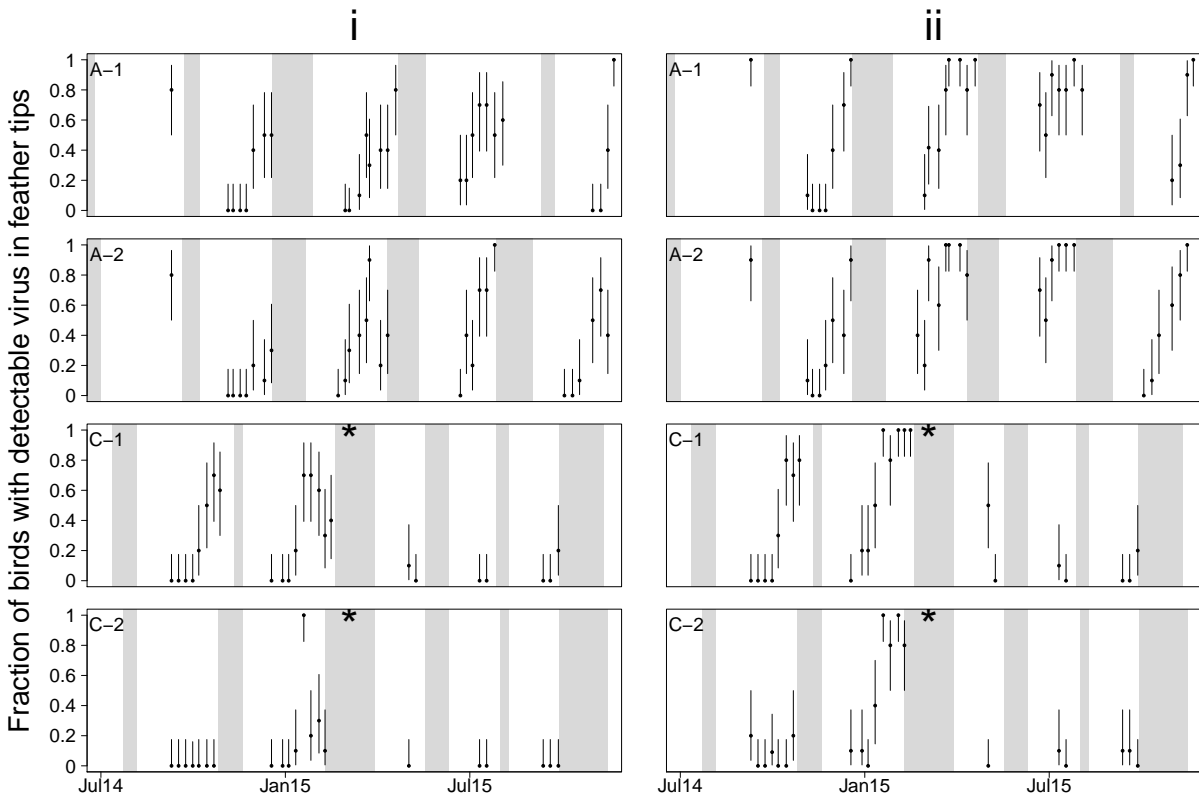


Figure S7: Fraction of birds with detectable virus in feather tips. Column i shows the data when using the highly conservative requirement that both feather samples must yield higher virus copy number estimates than both controls to be considered a virus positive bird. Column ii shows the data using a less conservative requirement that at least one feather must yield a higher virus copy number than the “short open” control. Together these two sets of plots bound our uncertainty due to potential contamination.

A.7 Model details

Data were analyzed using Bayesian generalized linear mixed effects models (5). There are several advantages to this approach, including readily interpretable confidence intervals on all model parameters (7), the ability to incorporate many random effect levels (3), and the ability to perform model parameter estimation in the presence of quasi-complete separation

(6). A caveat of this approach was that every parameter in the model including both fixed

256 and random effects required the specification of a prior distribution.

To account for the lack of balance in our sampling scheme, we analyzed our data using generalized linear mixed effects models (5). For this analysis, we excluded the data that

were collected from five farms at regular intervals (i.e. the longitudinal data), because we

260 were concerned that these farms would have outsized importance on factors such as seasonal patterns and flock to flock variation. We additionally excluded samples where bird age

was unavailable (103 samples). Our models had random effects for “Operation”, “Farm”,

“House”, “Flock”, and “Sample” to account for these levels of clustering in the data. We

264 were also interested in whether virus dynamics differed between broiler, broiler breeder, and layer farms, and so we included a fixed effect of “Production type”. We allowed for seasonal

variation in virus dynamics by including fixed effects on the sine and cosine of the collection date, transformed such that one year corresponds to a period of 2π . This method fit a

268 sigmoidal curve with a flexible amplitude and offset that was constrained to have a cycle period of one year. Lastly, we included an effect of cohort age by using a spline function

on bird age, because preliminary analysis suggested that the effect of age was non-linear (Appendix A.12). This approach allowed us to fit a non-linear effect of age while nonetheless

272 taking advantage of the computational benefits of generalized linear models (8, 9). This

spline contained knots at cohort ages of 21, 42, 100, and 315 days. These ages were selected

for their biological and empirical relevance: virus concentration in shed dust first peaks

around 21 days post infection (10, 15), commercial broiler cohorts in our data were typically

276 processed at around 42 days of age, the longer-lived broilers in our data were typically
processed at around 100 days, and the halfway point for our oldest flock sampled was 315
days.

A.8 Sensitivity to priors

280 Following Hadfield (7), prior distributions for fixed effects were univariate normal distribu-
tions, and for random effects were inverse Wishart distributions. For each fixed effect we
used mean 0, and standard deviation 7. For each random effect, we used scale 5 and degrees
of freedom 3. These prior distributions were set for practical, rather than biological reasons,
284 because less informative priors resulted in models that failed to converge in a reasonable
number of MCMC iterations. To explore whether our results were driven by our choice of
prior, we reran our analysis with three different sets of priors. In the main text we used the
parameters scale $V = 3$ and degrees of freedom $\nu = 5$ for random effects, and the parameters
288 mean $\mu = 0$ and variance $\sigma^2 = 49$ for fixed effects (prior set “A”). We explored the effect
of changing the random effects prior to $V = 5$, $\nu = 1$, while leaving the fixed effects prior
unchanged (prior set “B”), and we explored the effects of changing the random effects prior
to $\mu = 0$, $\sigma^2 = 16$ (prior set “C”). All three sets of priors are shown in fig. S8. The results
292 of our analyses were similar for all three sets of priors, and the model ranks according to
DIC were unchanged. This provided evidence that our conclusions are robust to our choice
of prior.

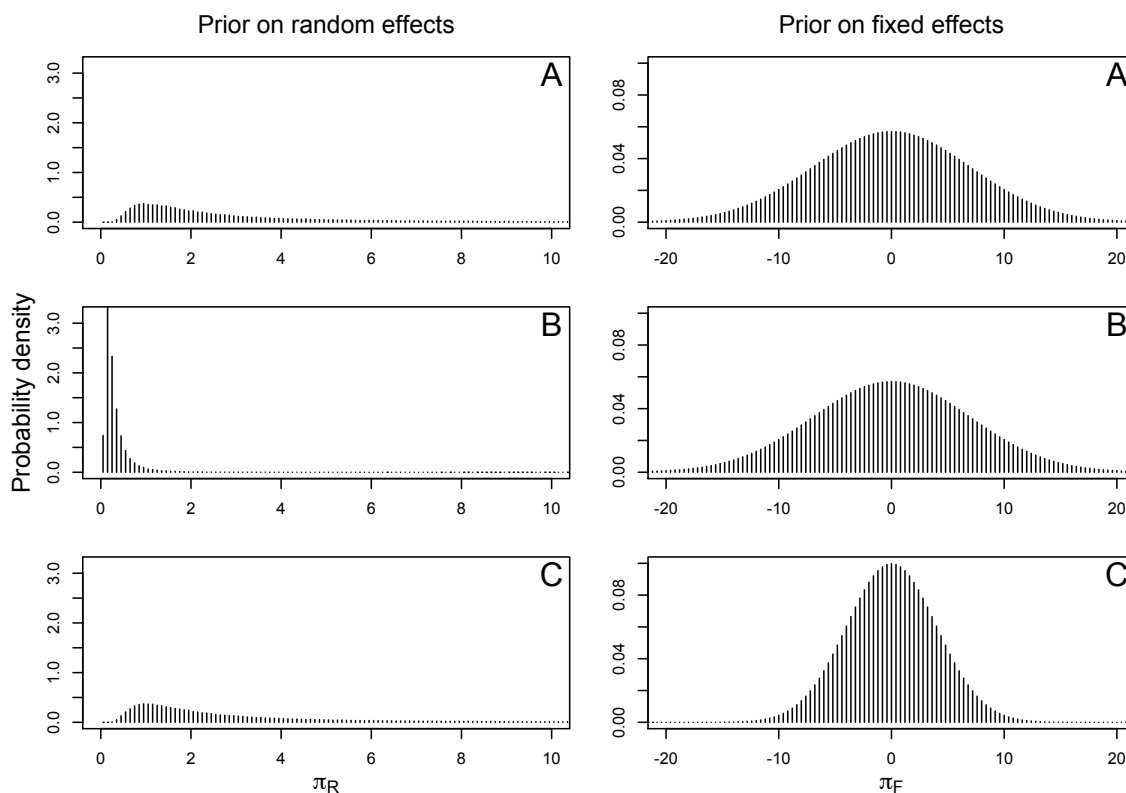


Figure S8: Three sets of priors explored, labelled “A”, “B”, and “C”, with the priors for random effects on the left, and the priors for fixed effects on the right. “A” is the set of priors used in the main text.

A.9 Assay precision

296 For each of our collected dust samples, duplicate technical replicates of dust samples were weighed out and assayed independently. We used these duplicates to determine the technical error in our methods. The error in qPCR tends to occur on a log scale and so means and variances for these data were calculated on the log scale. In practice, this was done by

300 estimating a variance for each set of duplicates. Estimating a variance is of course impossible

when virus levels are undetectable, and so we only used those samples with detectable virus. We therefore calculated sample variances for each of our sets of samples with detectable virus. We then averaged these values together and took the square root to determine our best estimate of the sample standard deviation. This analysis included 1374 sets of samples. The sample standard deviation on a \log_{10} scale was determined to be 0.319 with 95% confidence intervals [0.308, 0.331]. This meant that the standard deviation on a single technical replicate was between about 2.03 and 2.14 fold.

To calculate the variation between biological replicates, we then repeated the above analysis, using our biological replicates instead of our technical replicates. That is, we calculated the sample variances between sets of samples collected from the same house of the same farm in the same collection trip. This analysis included 465 sets of samples. The sample standard deviation was determined to be 0.556 \log_{10} units with 95% confidence intervals [0.513, 0.599]. This related to a standard deviation of between about 3.26 and 3.97 fold.

In a further analysis we tested whether these variances changed with the mean. This was done by regressing point-wise estimates of the standard deviation against the mean virus concentration in those samples, and performing a likelihood ratio to test whether mean virus concentration was a significant predictor of the standard deviation. In both the technical replicates ($\chi^2 = 250.0, d.f. = 1, p < 0.001$) and the biological replicates ($\chi^2 = 16.0, d.f. = 1, p < 0.001$), we observed a significant effect of the mean on the standard deviation in the data, such that the standard deviation was inversely correlated with the mean (fig. S9). For

the analyses done in this paper, ignoring this heteroscedasticity would have been unlikely to have much effect. One place that this would have had a consequence was in the error bars
324 in figs. 6 and 7, which should have been slightly larger when virus concentrations were low, and slightly smaller when virus concentrations were high. Nevertheless, the smoothness of the data and error bars in this figure over time suggested that the assumption of normality was providing reasonable estimates.

328 We nevertheless further explored the relationship between the mean and variance in the technical and biological replicates by calculating means and variances for the data on the natural scale, as opposed to the log scale. Regressing the log of the variance against the log of the mean for technical replicates revealed that the slope of this relationship was 1.71 ± 0.02 ,
332 suggesting that these data followed a compound Poisson-gamma distribution (fig. S10, (13)). Note that because this was performed on a log scale, the 5 of 1374 sets of samples with an estimated variance of 0 needed to be excluded from the analysis. Repeating the analysis for our biological replicates, we found that the relationship between the log of the variance
336 and the log of the mean was not statistically different from 2 (fig. S10, likelihood ratio test, $\chi^2 = 1.04, d.f. = 1, p = 0.31$), suggesting that these data followed a gamma distribution (18). For the purposes of the analyses in this paper, however, the assumption of normality on the log scale was probably sufficient.

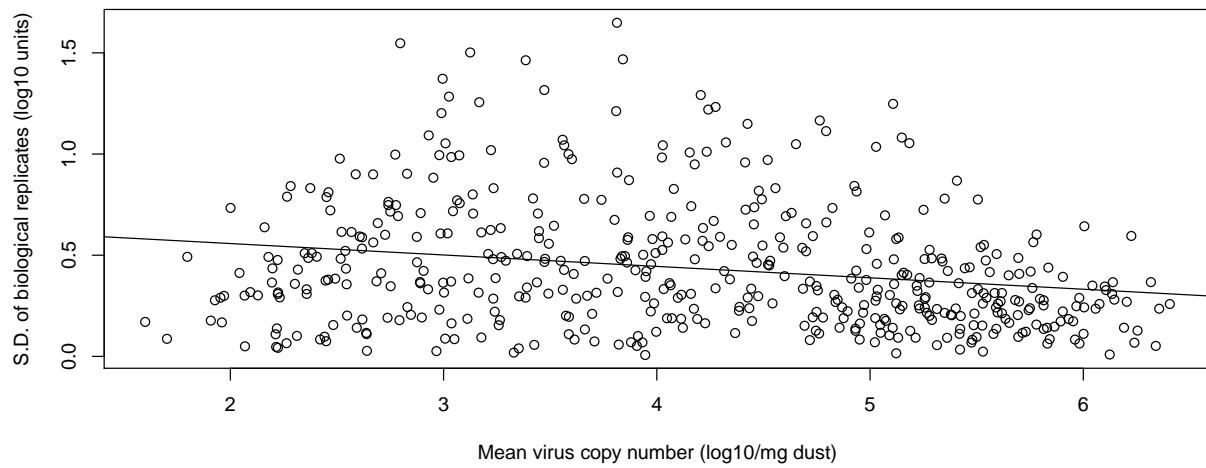
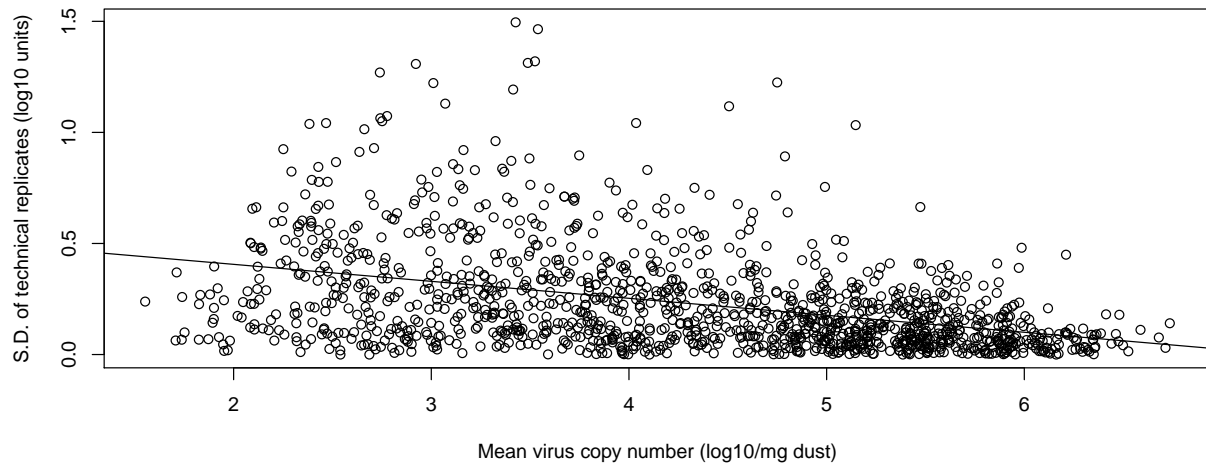


Figure S9: The relationship between the mean, and the standard deviation of technical (top) and biological (bottom) replicates using our qPCR assay. Means and standard deviations were calculated on the log₁₀ scale. Solid black lines show the best fit regression line.

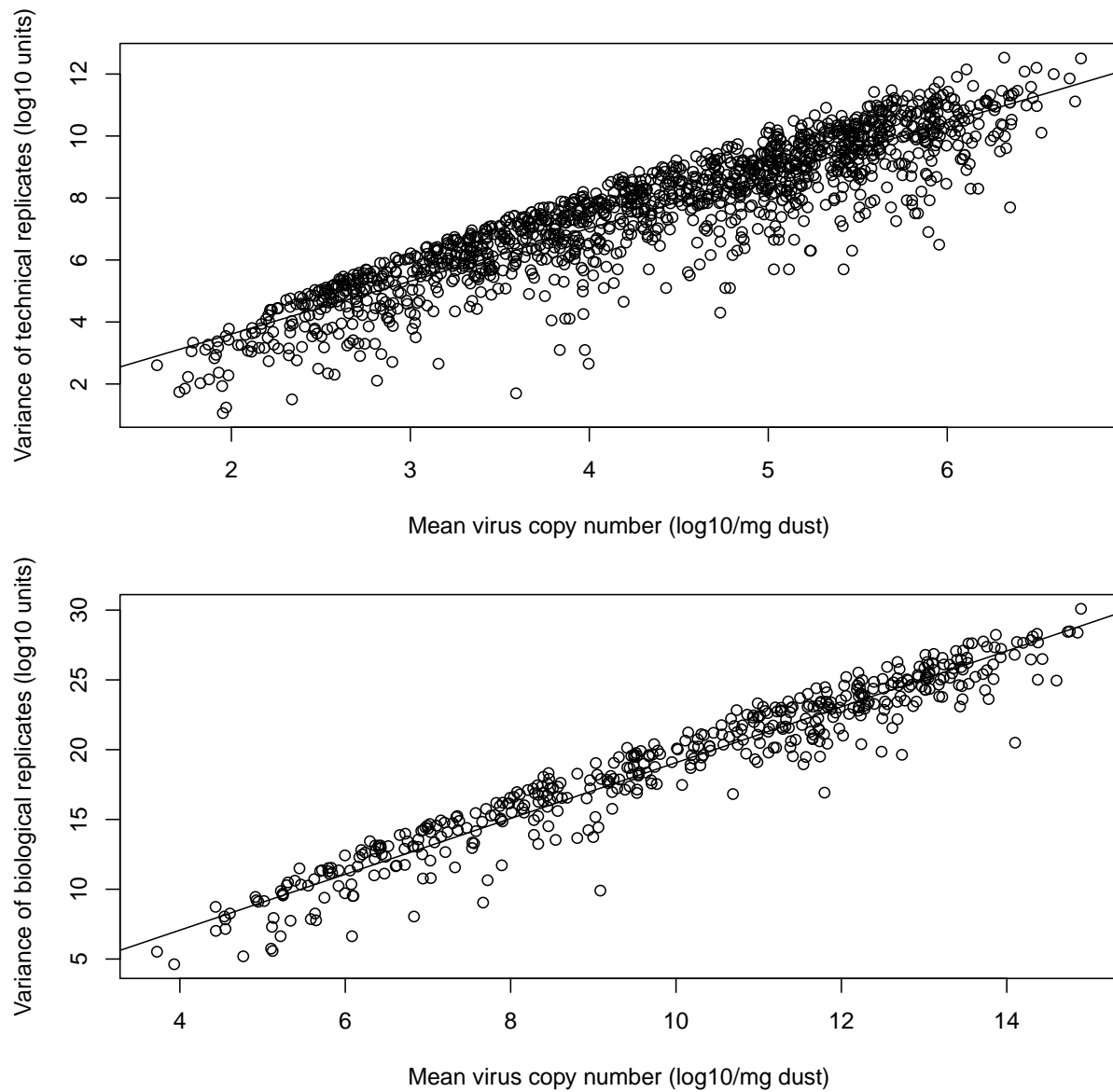


Figure S10: The relationship between the \log_{10} of the mean, and the \log_{10} of the variance, when mean and variance are calculated on the natural scale. The top panel shows the data from technical replicates with the solid line depicting the best fit regression line. The bottom panel shows the data from biological replicates with the solid line depicting the best fit regression line where the value of the slope is fixed at 2.

340 **A.10 Summary plots of marginal prevalence**

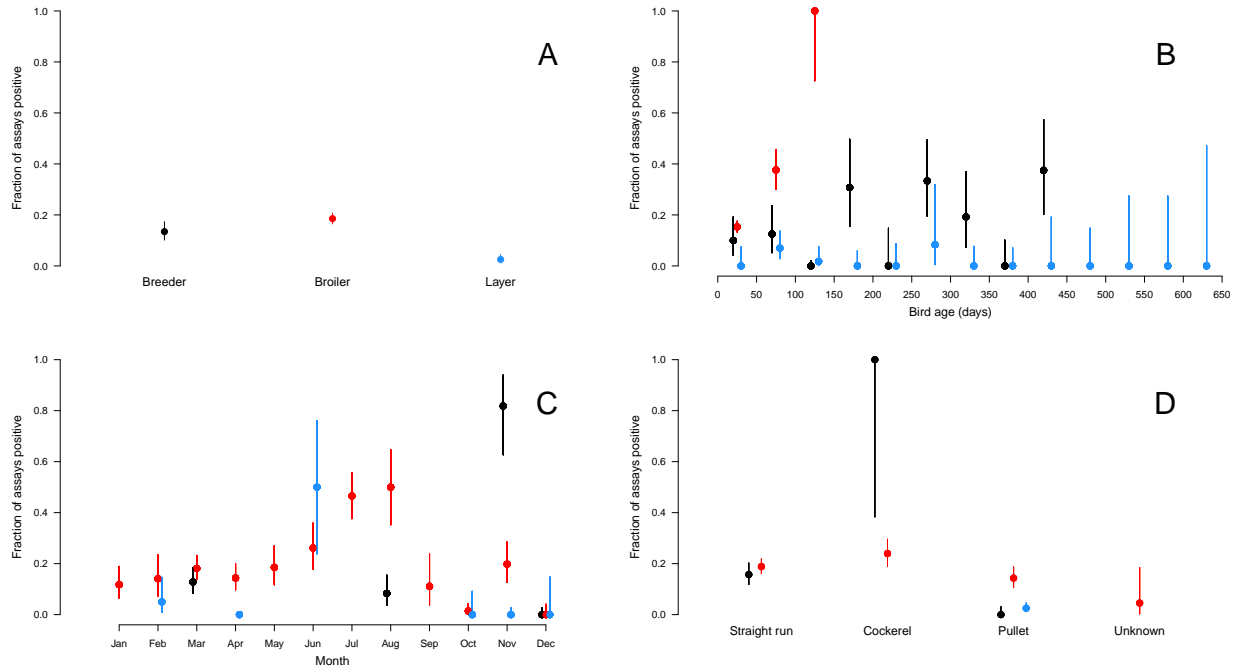


Figure S11: Summary plots depicting the fraction of total assays that were positive for Marek's disease virus as a function of production type (A), bird age (B), month of the year (C), and sex (D). As in fig. 2, black denotes breeder facilities, red denotes broiler facilities, and blue denotes layer facilities. Error bars are 95% confidence intervals on the estimate of the fraction of positive assays. Note, however, that these error bars were generated under the assumption that the data were perfectly binomially distributed data, which was almost certainly untrue for these data because they were marginalized over other factors that we showed to be important to virus prevalence. For these reasons, we were cautious not to over interpret these plots.

A.11 Correlation between production type and operation

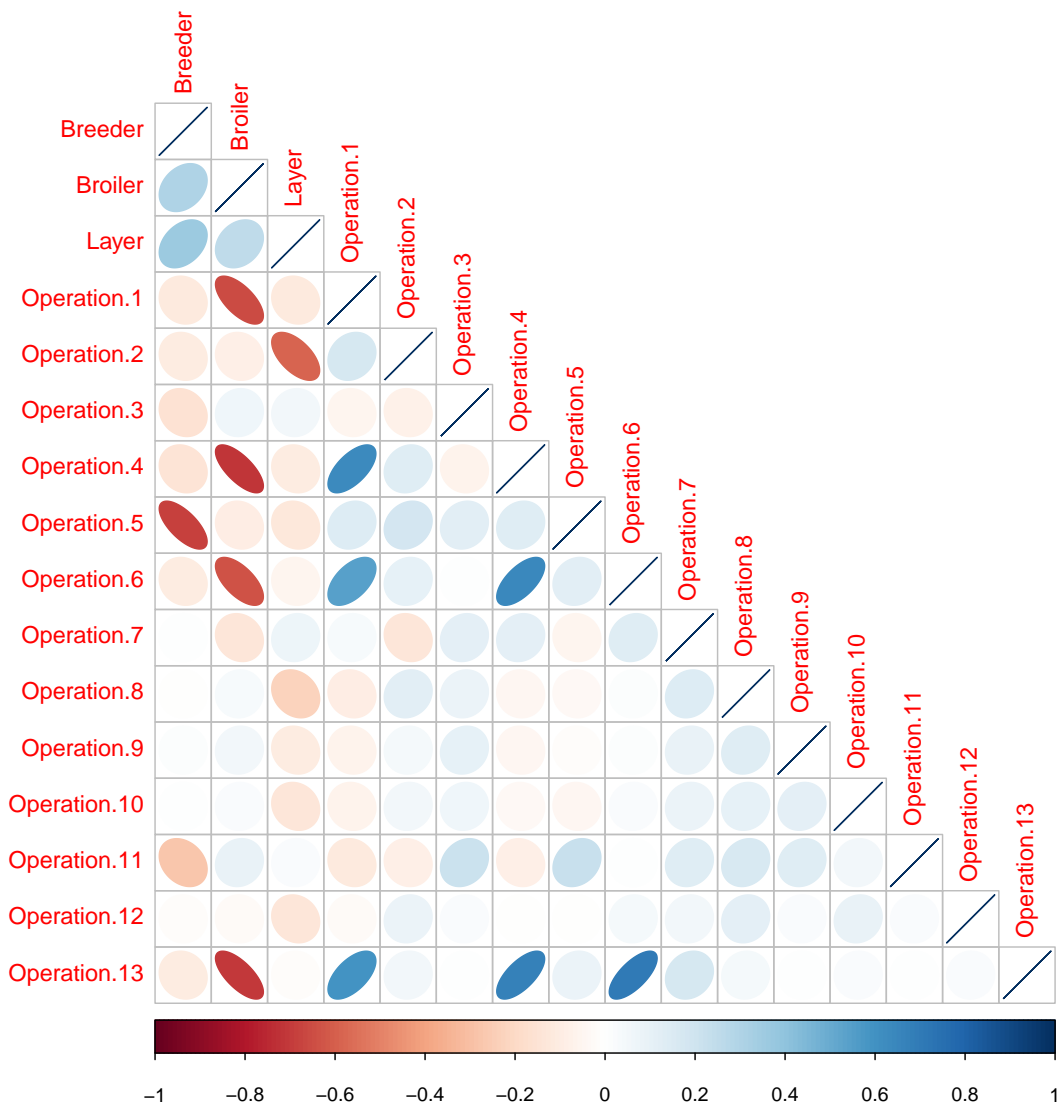


Figure S12: The correlation in estimated effect sizes between production type and operation. Correlations between production types and operations are indicated by the shape and color of ellipses. Note that the correlation between production types and operations are almost always negative. Several of these are strong negative correlations indicating a lack of power to distinguish between production type effects and company effects.

A.12 Longitudinal cohort spline

To look for effects of cohort age on virus concentration, we fit cubic smoothing splines to the
344 longitudinal data on virus concentration. We treated data from each farm individually, but
within each farm, all data across houses, flocks, and samples were used to infer the effect of
cohort age. As mentioned in the methods, we used 4 knots, but we explored every number
of knots up to 9 and saw nearly identical results. For this analysis, we fit the splines on a log
348 scale, and we treated samples that were negative by qPCR first as values of 0, and second
as values at our qPCR limit of detection. These two sets of analyses gave qualitatively
similar outcomes. In fig. S13 we show the results for 4 knots, treating negative samples as
though they were at a value of 10^2 virus copies per mg of dust. Note the decrease in virus
352 concentration that occurred early in cohorts, and the subsequent increase that occurred as
cohorts aged.

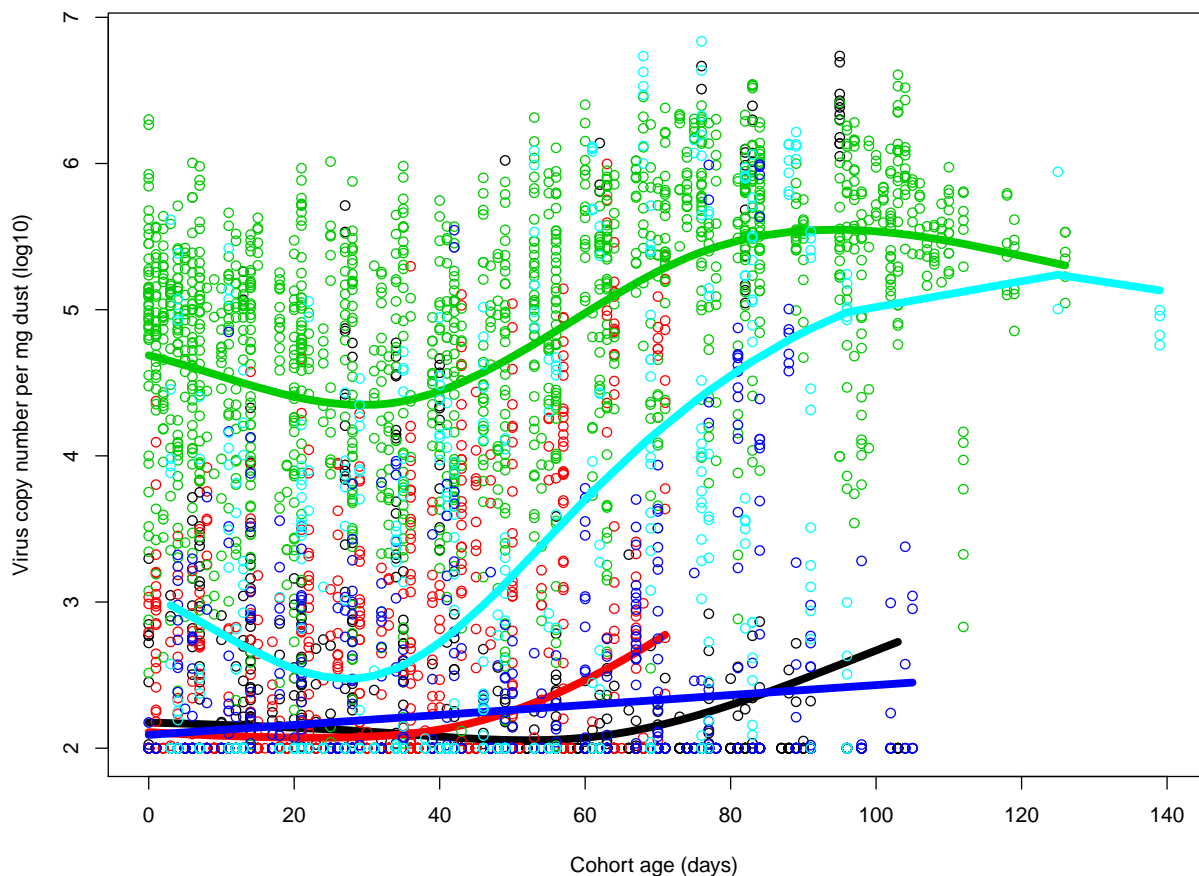


Figure S13: The effect of cohort age on virus concentration per mg of dust. All data points from our longitudinal surveillance are plotted as open circles with different colors representing data from different farms. The smoothing splines are plotted as solid lines matching the color of the points for the respective farms.

A.13 Sample humidity or qPCR inhibitors

In the main text, we stated that the “U” shape pattern observed in virus concentration
 356 within a cohort was likely to be driven either by dust dilution or virus DNA degradation
 followed by re-concentration. As an alternative to these biological explanations, we explored

whether this pattern could be caused by either of two technical factors. First, water content in dust samples might have changed over the course of a cohort resulting in substantial
360 changes to the dry mass of the dust included in DNA extractions. Second, qPCR inhibitor concentration in dust might have similarly changed over the cohort limiting our ability to detect virus DNA when present. Here, we show that neither of these explanations can explain the observed pattern in the data.

364 To explore whether changing water content could explain the “U” shape pattern in the data, we used dust samples collected from the fourth and fifth cohorts of farm “A”, house 2, and from the eighth cohort of farm “A”, house 2 (fig. 6). These cohorts were chosen because their dynamics were among the most and least extreme fluctuations in virus concentration.

368 For each collection trip in each of these flocks, we randomly chose one representative dust sample. A 2 mg aliquot of this dust sample was used in our standard qPCR assay to re-determine virus concentration. In addition, a 10 mg aliquot was transferred into a clean 1.5 ml centrifuge tubes. As controls, we had one tube with 10 mg of previously desiccated dust,
372 one tube with 21 μ l of distilled water, and one tube with both 2.5 mg of desiccated dust and 9.8 μ l of water. All tubes were opened and placed on a VWR Analog Heatblock for 5 hours at 37 °C. This was sufficiently long for all visible water to evaporate from control tubes. The mass of the controls changed as expected, suggesting that our protocol was
376 sufficient to desiccate the samples. The 10mg dust samples on average lost 1.2 mg of mass through desiccation, with a range of -0.2 mg to 6.8 mg. We divided the mass remaining after desiccation by the original mass to determine the fraction of each dust sample that

was dry mass. We then divided the virus concentration determined through qPCR by these
380 fractions to explore whether this would eliminate the “U” shape in our data. We found that
the dynamics were nearly unchanged (fig. S14), meaning that water content alone could not
explain the pattern observed in the data.

To explore whether qPCR or DNA extraction inhibitors might have created this “U”
384 shape pattern in the data, we mixed together pairs of samples with high virus concentration
and low virus concentration. Because we were interested in whether low virus concentration
samples contained inhibitors, we mixed these samples at a ratio of 9:1, such that an 18
mg sample with low virus concentration was combined with a 2 mg sample with high virus
388 concentration. If true virus dynamics in a house were essentially flat, and the pattern
observed was due to inhibitors, then mixing these samples together should result in virus
concentrations consistently lower than expected. Alternatively, if the virus concentration
truly changed over this time period, these mixtures would have given qPCR reads similar to
392 those expected by simple averaging. We found the latter to be true (fig. S15), suggesting that
inhibitors were unlikely to drive the virus dynamics seen in the data. We were therefore left
to conclude that the dynamics seen in the data were probably caused by biological, rather
than technical, factors.

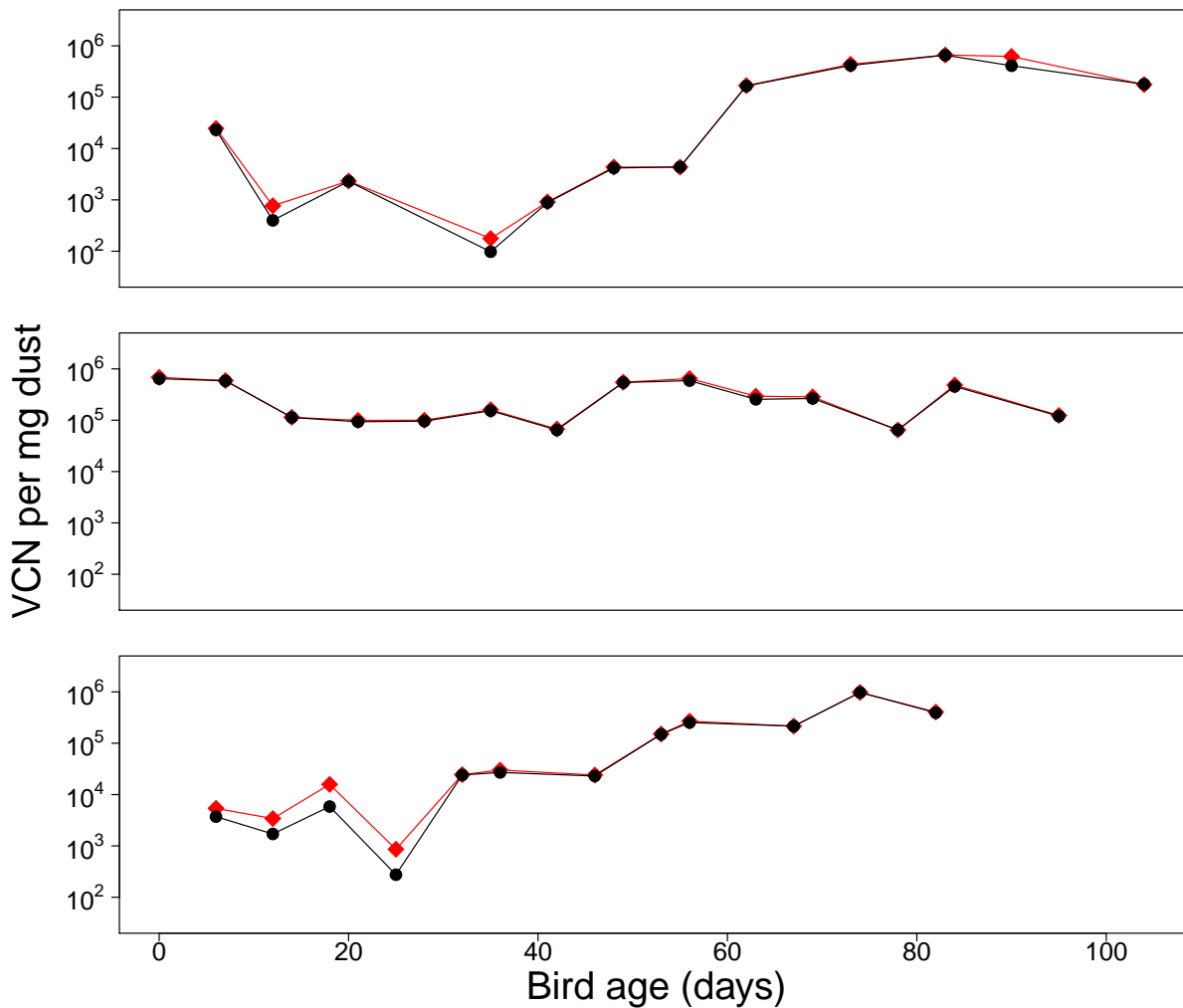


Figure S14: Virus copy number per mg of dust and per mg of desiccated dust. The three plots show data from three different flocks as a function of bird age. Black circles show virus copy number per mg of dust. Red diamonds show the data corrected to be virus copy number per mg of dry mass. The values differed only slightly, and we thus concluded that water content was unable to explain the “U” shape seen in virus concentration within cohorts.

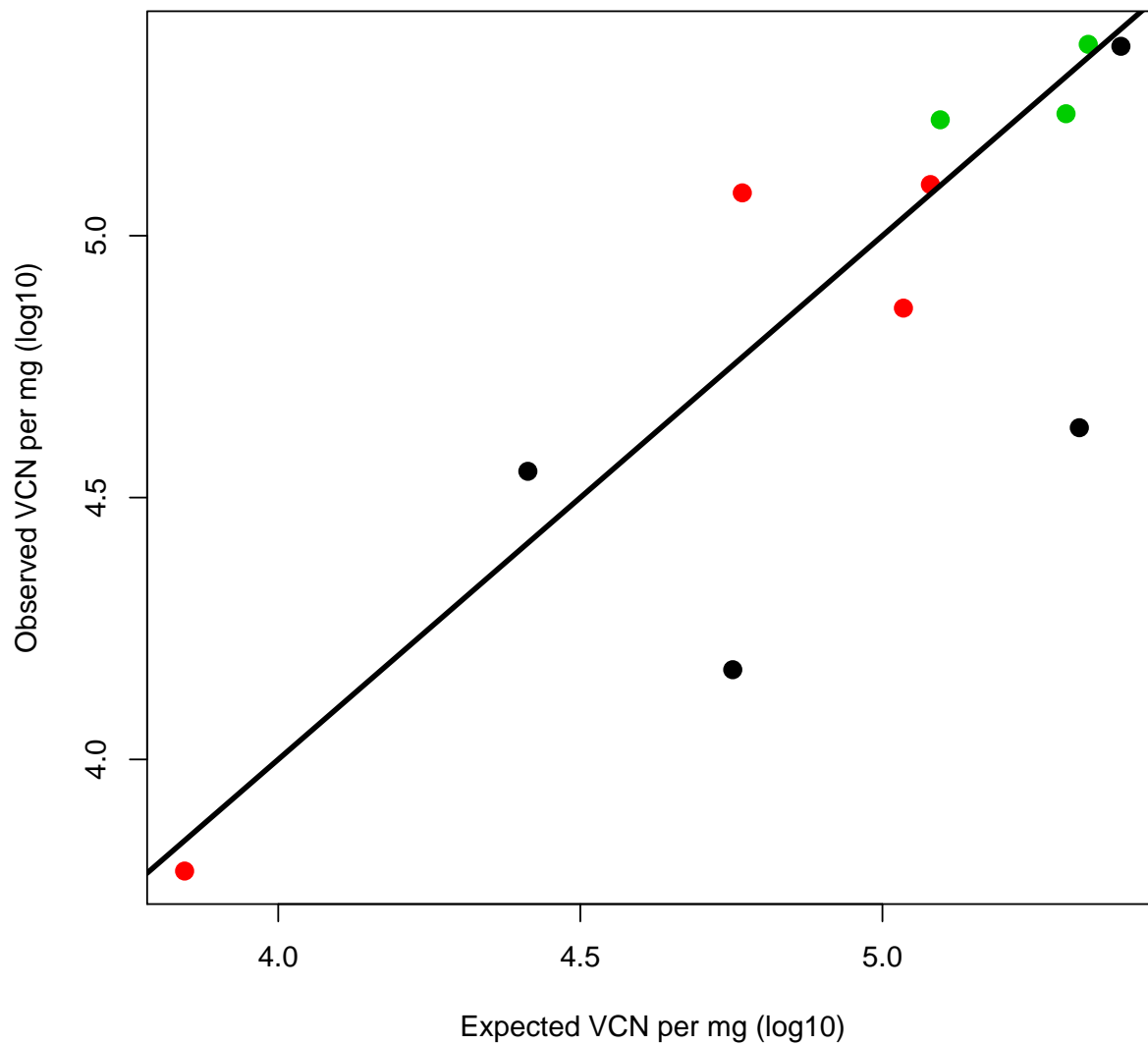


Figure S15: Expected vs. observed virus concentration in mixed samples. Each point shows a mixture of a different pair of samples. Each color depicts samples collected from different flocks. The solid black line is a one to one line.

396 **A.14 Autocorrelation within houses**

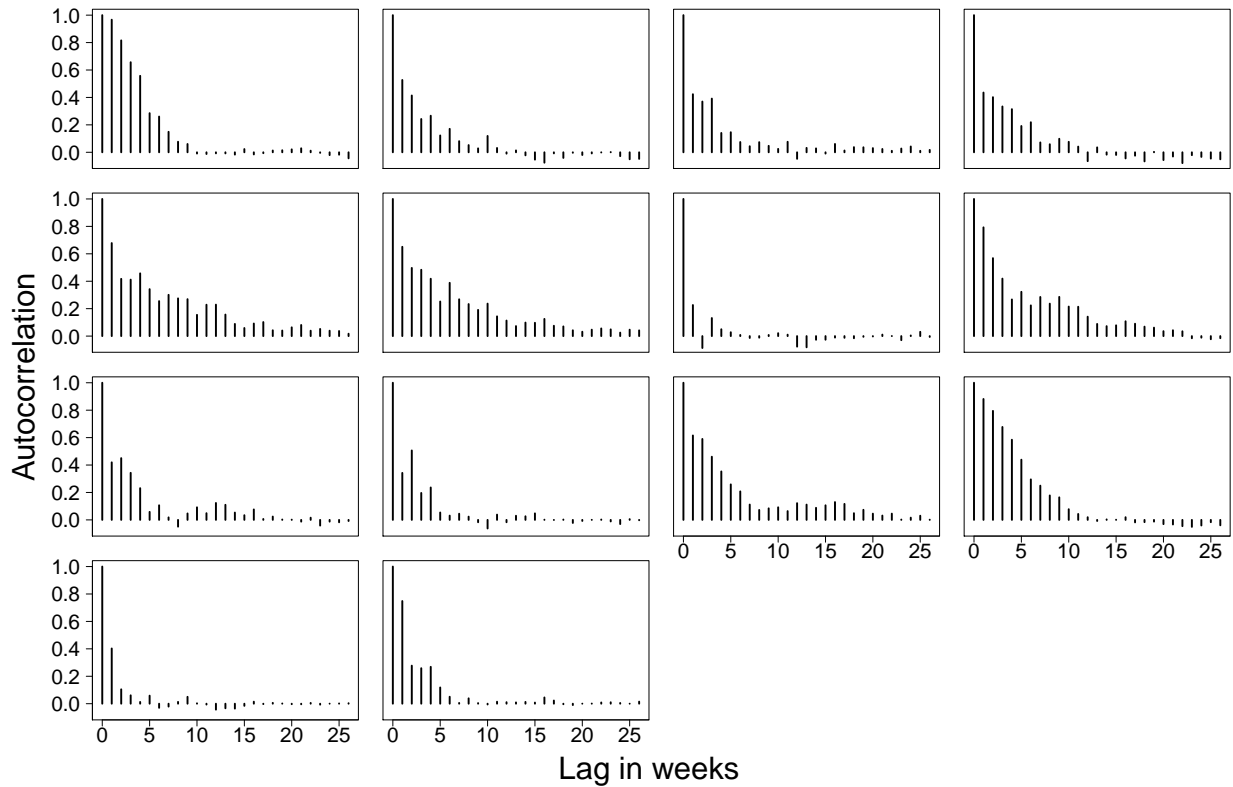


Figure S16: Plots showing the autocorrelation in virus concentration in the longitudinal data. Each plot shows data from a different house.

A.15 Estimating virus concentrations and 95% confidence intervals

Confidence bounds for all prevalence data were calculated using likelihood. 95% intervals
400 spanned the ranges that were within 1.92 log likelihood units of the maximum likelihood
estimate.

For our quantitative data, the standard practice of using sample standard errors to gener-

ate confidence intervals on data points was impossible, because samples were routinely below
404 the qPCR limit of detection. We were therefore forced to estimate virus concentrations and
95% confidence intervals in a slightly more complex way. First, we calculated the standard
deviation of our samples as described in subsection Assay repeatability. We then used this
point estimate of the standard deviation in the following way.

408 There were two possible outcomes when running our qPCRs, we either observed a virus
concentration ($X = x$), or the virus concentration was so low that it was undetectable
($X < C$) where C was our limit of detection. For our calculations below, we assumed a limit
of detection of $C = 100$ target copy number per mg of dust which was a slightly conservative
412 estimate of our limit of detection in qPCR runs (Appendix A.4). In cases where virus
was quantifiable by qPCR, we assumed the likelihood of the data on a log scale followed
a normal distribution. These values were calculated in the R programming language using
the function ‘dnorm’. In cases where virus could not be quantified by qPCR, we extended
416 this methodology by using a Bernoulli distribution for the likelihood function, where the
probability of not detecting virus was exactly equal to the cumulative distribution of a
normal distribution up to the limit of detection $\log_{10}(C)$. In R, these values were calculated
using the function ‘pnorm’. The sum of the log of these values was a likelihood function. We
420 thus generated a maximum likelihood estimate of virus concentration by maximizing this
likelihood function using the R function ‘optimize’. Upper and lower confidence intervals
for virus concentrations were then calculated by solving for the values of virus concentration
for which the likelihood function was 1.92 log likelihood points worse than the maximum

424 likelihood. This cutoff was chosen so that we would have 95% confidence intervals. Note
that the data did not exactly follow the normal distribution (Appendix A.9). In practice,
this meant that our estimates of the confidence intervals were slightly inflated for points at
high virus concentrations, and slightly deflated for points at low virus concentrations.

428 **A.16 Rispens virus interference**

The Rispens vaccine is an attenuated strain of Marek’s disease virus, and being a live virus,
it is shed from vaccinated hosts (12, 16). In Pennsylvania, this vaccine is rarely used in
broiler chicken flocks, but it is commonly used in broiler-breeder and egg-laying chickens.
432 Due to its genetic similarity to wild-type virus, our qPCR assay was not able to perfectly
discriminate between Rispens virus and wild-type virus. We assessed the degree of this
interference through a lab experiment.

In this experiment we generated samples to test for interference. Each sample contained
436 one of eight concentrations of wild-type Marek’s disease DNA from our virus standard rang-
ing by tenfold dilutions from 2.41×10^7 to 2.41×10^0 DNA copies per $4 \mu\text{l}$ volume, or a control
that lacked this DNA. The samples also contained one of eight concentrations of DNA from
our Rispens virus standard ranging by tenfold dilutions from 1.60×10^7 to 1.60×10^0 DNA
440 copies per $4 \mu\text{l}$ volume, or a control. By making every combination of the above, we gen-
erated 81 total samples. We then ran our wild-type virus assay and Rispens virus assay on
each sample to quantify potential interference. The wild-type assay was run using the meth-
ods described in the main text. The Rispens virus assay was run in an identical manner,

444 except that we used the Rispens virus DNA specific probe pp38-CVI from Baigent et al.
(1). We used the samples lacking either Rispens or wild-type Marek's disease virus DNA as
standards for DNA quantification.

This analysis showed that in both the Rispens virus and wild-type virus assays inter-
448 ference was negligible when the target DNA was more prevalent than the non-target DNA
(fig. S17). Quantification was fairly accurate, although counts were slightly biased low, when
non-target DNA was approximately tenfold more prevalent than target DNA. Beyond this
ratio, interference from the non-target DNA began to severely bias quantification. This bias
452 was more extreme in the Rispens assay than in the wild-type assay.

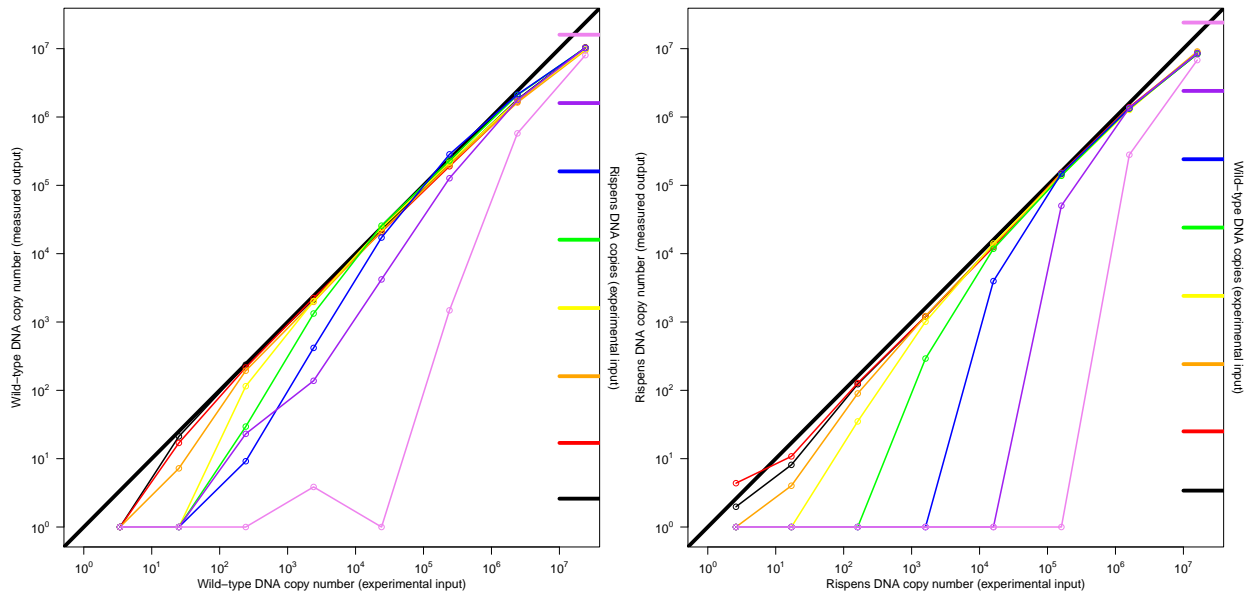


Figure S17: Interference plots for the wild-type virus assay (left), and Rispens virus assay (right). Each plot shows the quantification of target DNA over 7 orders of magnitude in the presence of contaminating wild-type or Rispens virus DNA. The diagonal black line is a 1:1 line representing perfect agreement between the assay and the experimental target value. The greater the deviance from this line, the less accurate the quantification. The data are shown as colored dots connected by same colored lines, where each color is a different level of interfering DNA. The quantity of interfering DNA is shown on the right side of the plot. Note that for both assays, quantification was fairly accurate when interfering DNA was less prevalent than target DNA, and only slightly biased when interfering DNA was 10-fold more prevalent.

A.17 Rispens virus in field samples

In Pennsylvania and many other parts of the world, the Rispens virus vaccine is used more commonly for breeder and layer birds than for broiler birds. As expected, we detected
 456 Rispens virus more often in samples collected from breeder and layer flocks than from broiler flocks (fig. S18). This, coupled with the observation that Rispens virus DNA can interfere with detection of wild-type DNA in qPCR, suggested that observed differences in wild-type

virus prevalence between different production types might result from a detection bias. From
460 our model comparison, we concluded that wild-type virus was more prevalent in dust col-
lected from broiler flocks than layer flocks. We thus performed a bootstrap analysis to show
that this difference cannot be explained by a detection bias caused by qPCR interference.

In Appendix A.16 we showed that interference for both the Rispens and wild-type assays
464 was negligible when target DNA was at least as prevalent as the contaminating DNA, and
interference was minor when the non-target DNA was 10-fold greater than the target. We
therefore wanted to determine whether the wild-type virus DNA detected in broiler samples
would have reached or exceeded these ratios if they had a distribution of Rispens virus DNA
468 similar to that seen in the layer samples.

To determine the distribution of Rispens virus concentration for our bootstrap analy-
sis, we discarded all layer samples in which the wild-type virus concentration exceeded the
Rispens virus concentration, and retained the measurements of Rispens virus concentration
472 in all other layer samples. This left us with 311 measures of Rispens virus concentration. To
determine the distribution of wild-type virus concentration, we retained the measurements
of wild-type virus concentration from all broiler samples. This left us with 1304 measures
of wild-type virus concentration. In our original analysis we had 317 measurements from
476 layer samples, 2.5% of which had detectable wild-type virus. We drew 317 pairs of Rispens
virus concentration and wild-type virus concentration with replacement and determined the
fraction of these pairs that had “detectable wild-type virus”. This process was repeated
100,000 times. Detectable wild-type virus can be interpreted to mean wild-type virus that

480 is no more than 10-fold less prevalent than Rispens virus. However, to be conservative, we
reran the analysis under the assumption that wild-type virus would only be detectable if it
were more common than Rispens virus. In all 100,000 runs of both analyses, wild-type virus
was detectable in a larger fraction of samples than that of the layers (fig. S19). This analysis
484 showed that even with Rispens contamination, the prevalence of wild-type virus in layer
samples was below that seen in broiler samples, suggesting that qPCR interference could not
explain the difference in virus detection between layer samples and broiler samples.

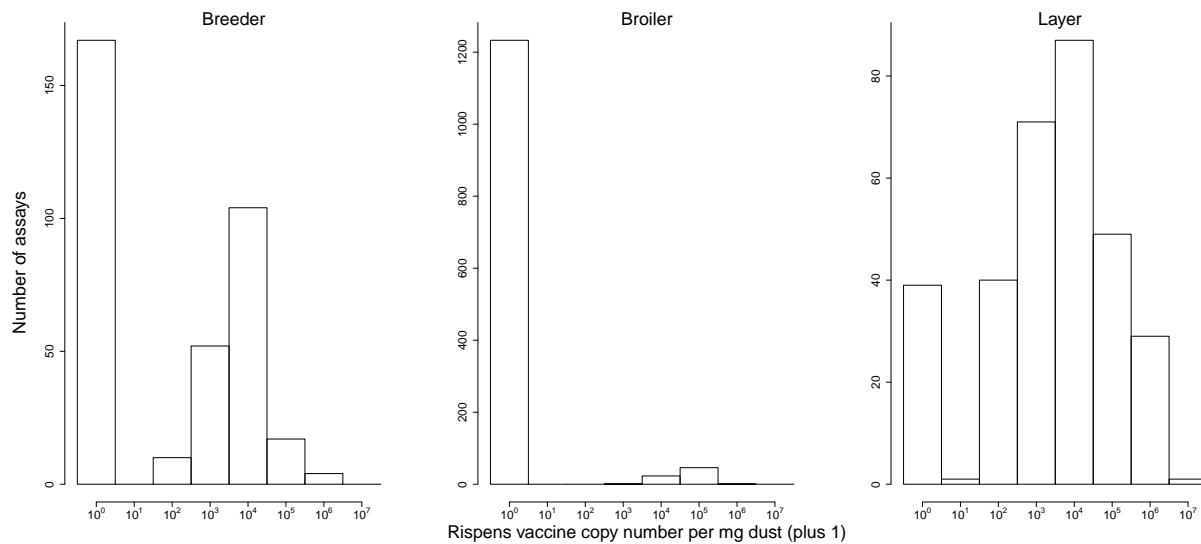


Figure S18: Histograms of Rispens virus DNA in samples. All breeder and layer samples, and all broiler samples suspected to contain Rispens virus, were tested for the Rispens virus. Note, that for many of the broiler samples, qPCR curves were examined to determine whether Rispens virus was likely to be present at high concentrations. When no evidence of Rispens virus was present, these samples were assumed to have no Rispens virus present, because Rispens virus vaccine was not used in these chicken flocks.

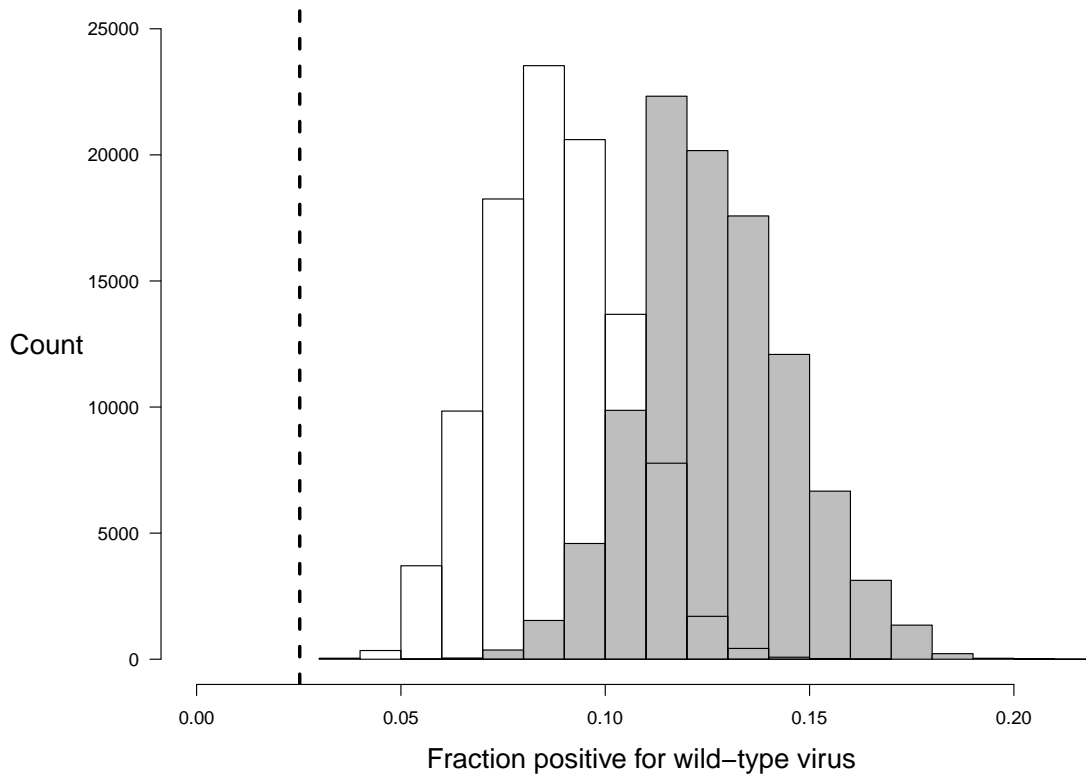


Figure S19: Bootstrap analysis results showing fraction of broiler samples that would have had “detectable virus” in a background of Rispens virus contamination similar to that seen in the layer samples. Grey bars show the distribution where “detectable virus” was defined as wild-type virus no more than 10-fold less concentrated than Rispens virus. White bars show the distribution where the threshold for detection was assumed to be equal concentrations. The dotted line is the fraction of layer samples that had detectable wild-type virus by qPCR.

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