Final Semester Project

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Data Description

The dataset used for this analysis is from the second study of my MSc. thesis titled "Evaluation of the Efficacy of Bovine Adenovirus-Vectored Avian Influenza Vaccine in Poultry,". This study investigates how different mucosal vaccine routes influence protection against avian influenza. For this preliminary analysis, I used qPCR viral-load data **(Ct values and log10 genomic equivalents)** from tracheal swabs collected at three time points—2, 4, and 6 days post-challenge (DPC2, DPC4, DPC6)—in chickens that received a single dose of the BAdV-H5HA+H7NP vaccine ($1x10^8$pfu) in a prime-booster dose vaccine administration via two routes —**intraocular (IO)** and **intramuscular (IM)**—and challenged with two avian influenza virus strains (**H5N1** and **H7N2**).

Each Excel sheet corresponds to one **Virus × Timepoint** combination (e.g., DPC2-H5N1).
Key variables:

- **Vaccine_Group**: Mock, Mock Challenge, Empty-Vector, BAds-AIV
- **Route**: IM (intramuscular) or IO (intraocular)
- **Bird_ID**: unique sample identifier
- **Ct**: qPCR cycle threshold (continuous)
- **log10GE**: $\log_{10}$ genome equivalents/mL (continuous)

Since "Mock" birds were not challenged, they are excluded.
Data are nested: **Bird_IDs** are nested within **Vaccine_Group × Route** combinations, and each sheet (timepoint) is nested within each **Virus**.

```
#LOADING LIBRARIES
library(tidyverse)
```

```
── Attaching core tidyverse packages ──────────────────────── tidyverse 2.0.0 ──
✔ dplyr     1.1.4     ✔ readr     2.1.5
✔ forcats   1.0.0     ✔ stringr   1.5.1
```

```
✔ ggplot2   4.0.0     ✔ tibble    3.3.0
✔ lubridate 1.9.4     ✔ tidyr     1.3.1
✔ purrr     1.1.0
── Conflicts ──────────────────────────────────────────
tidyverse_conflicts() ──
✖ dplyr::filter() masks stats::filter()
✖ dplyr::lag()    masks stats::lag()
ℹ Use the conflicted package (<http://conflicted.r-lib.org/>)
to force all conflicts to become errors
```

```
library(stringr)
library(readxl)
library(dplyr)
library(tidyr)
library(ggplot2)
library(readr)   # for parse_number()
library(purrr)
library(broom)
library(multcomp) # for Tukey
```

```
Loading required package: mvtnorm
Loading required package: survival
Loading required package: TH.data
Loading required package: MASS

Attaching package: 'MASS'

The following object is masked from 'package:dplyr':

    select


Attaching package: 'TH.data'

The following object is masked from 'package:MASS':

    geyser
```

```
library(car)
```

```
Loading required package: carData

Attaching package: 'car'
```

```
The following object is masked from 'package:dplyr':

    recode

The following object is masked from 'package:purrr':

    some
```

```
library(emmeans)
```

```
Welcome to emmeans.
Caution: You lose important information if you filter this
package's results.
See '? untidy'
```

```
library(multcompView)
library(ggpubr)
library(glm2)
```

```
Attaching package: 'glm2'

The following object is masked from 'package:MASS':

    crabs

The following object is masked from 'package:survival':

    heart
```

```
library(glmertree)
```

```
Loading required package: lme4
Loading required package: Matrix

Attaching package: 'Matrix'

The following objects are masked from 'package:tidyr':

    expand, pack, unpack

Loading required package: partykit
```

```
Loading required package: grid
Loading required package: libcoin
```

```
library(glmmTMB)
```

```
Warning in check_dep_version(dep_pkg = "TMB"): package version
mismatch:
glmmTMB was built with TMB package version 1.9.17
Current TMB package version is 1.9.18
Please re-install glmmTMB from source or restore original
'TMB' package (see '?reinstalling' for more information)
```

```
library(lme4)
library(pscl)
```

```
Classes and Methods for R originally developed in the
Political Science Computational Laboratory
Department of Political Science
Stanford University (2002-2015),
by and under the direction of Simon Jackman.
hurdle and zeroinfl functions by Achim Zeileis.
```

```
library(ZIM)
library(TMB)
library(bbmle)
```

```
Loading required package: stats4

Attaching package: 'bbmle'

The following object is masked from 'package:dplyr':

    slice
```

```
library(DHARMa)
```

```
This is DHARMa 0.4.7. For overview type '?DHARMa'. For recent
changes, type news(package = 'DHARMa')
```

```
library(patchwork)
```

```
Attaching package: 'patchwork'

The following object is masked from 'package:MASS':

    area
```

```
# Loading dataset
# Importing my qPCR excel data file
excel_path <- path.expand("~/Desktop/Entomology tech-Fall 2025/
excel_path <- "ENT_Project_Tracheal_qPCR_Clean.xlsx .xlsx"

ENT_Project_Tracheal_qPCR_Clean_xlsx_ <- read_excel("ENT_Projec

#qPCR data wrangling process
excel_path
```

```
[1] "ENT_Project_Tracheal_qPCR_Clean.xlsx .xlsx"
```

```
#GETTING SHEET NAMES FROM THE CHOSEN FILE
sheets <- readxl::excel_sheets(excel_path)
#READING ALL SHEETS AND BINDING INTO ONE DATA FRAME
qpcr <- purrr::map_dfr(sheets, ~ readxl::read_excel(excel_path,
dplyr::glimpse(qpcr)        #quick peek into the selected data
```

```
Rows: 450
Columns: 8
$ Timepoint     <chr> "DPC2", "DPC2", "DPC2", "DPC2", "DPC2",
"DPC2", "DPC2", …
$ Virus         <chr> "H5N1", "H5N1", "H5N1", "H5N1", "H5N1",
"H5N1", "H5N1", …
$ Vaccine_Group <chr> "Mock", "Mock", "Mock", "Mock", "Mock",
"Mock", "Mock", …
$ Route         <chr> "NA", "NA", "NA", "NA", "NA", "NA",
"NA", "NA", "NA", "N…
$ Challenge     <chr> "No", "No", "No", "No", "No", "No",
"No", "No", "No", "N…
$ Ct            <dbl> 38.867, 0.000, 0.000, 0.000, 0.000,
0.000, 0.000, 0.000,…
$ log10GE       <dbl> 3.807920, 3.250908, 3.250908, 3.250908,
3.250908, 3.2509…
$ Bird_ID       <dbl> 712, 713, 714, 715, 716, 717, 718, 719,
720, 721, 722, 7…
```

```r
# Checking counts per original sheet
qpcr %>% count(Timepoint, Virus)
```

```
# A tibble: 6 × 3
  Timepoint Virus     n
  <chr>     <chr> <int>
1 DPC2      H5N1     77
2 DPC2      H7N2     73
3 DPC4      H5N1     77
4 DPC4      H7N2     73
5 DPC6      H5N1     77
6 DPC6      H7N2     73
```

```r
qpcr <- lapply(sheets, function(s) {
  df <- read_excel(excel_path, sheet = s)
  df$Sheet <- s
  return(df)
}) |> bind_rows()
```

```r
# Clean and set factors
qpcr <- qpcr %>%
  filter(Vaccine_Group != "Mock") %>%
  mutate(
    Timepoint = factor(Timepoint, levels = c("DPC2","DPC4","DPC
    Virus = factor(Virus, levels = c("H5N1","H7N2")),
    Vaccine_Group = factor(Vaccine_Group, levels = c("Mock Chal
    Route = factor(Route, levels = c("IM","IO")),
    GroupRoute = interaction(Vaccine_Group, Route, sep = ":")
  )

glimpse(qpcr)
```

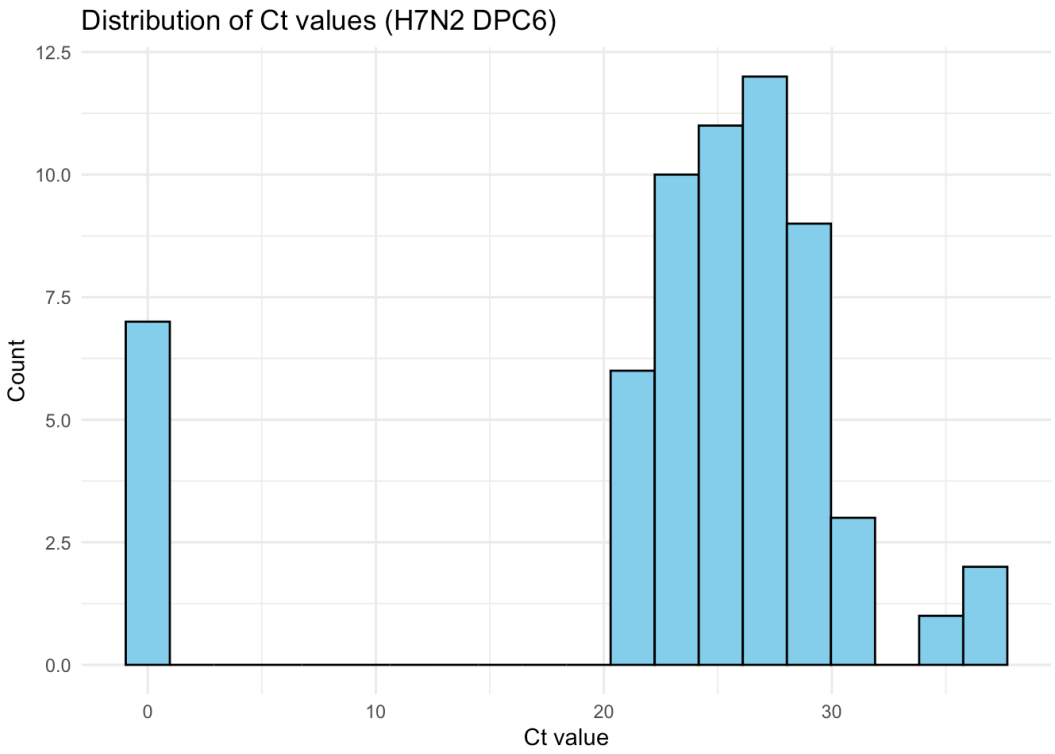
```
Rows: 378
Columns: 10
$ Timepoint     <fct> DPC2, DPC2, DPC2, DPC2, DPC2, DPC2,
DPC2, DPC2, DPC2, DP…
$ Virus         <fct> H5N1, H5N1, H5N1, H5N1, H5N1, H5N1,
H5N1, H5N1, H5N1, H5…
$ Vaccine_Group <fct> Mock Challenge, Mock Challenge, Mock
Challenge, Mock Cha…
$ Route         <fct> NA, NA, NA, NA, NA, NA, NA, NA, NA, NA,
NA, NA, NA, NA, …
```

```
$ Challenge     <chr> "Yes", "Yes", "Yes", "Yes", "Yes",
"Yes", "Yes", "Yes", …
$ Ct            <dbl> 28.294, 29.838, 30.844, 29.953, 29.932,
31.468, 28.799, …
$ log10GE       <dbl> 6.923950, 6.468909, 6.172425, 6.435017,
6.441206, 5.9885…
$ Bird_ID       <dbl> 775, 776, 777, 778, 779, 780, 781, 782,
783, 784, 785, 7…
$ Sheet         <chr> "DPC2-H5N1", "DPC2-H5N1", "DPC2-H5N1",
"DPC2-H5N1", "DPC…
$ GroupRoute    <fct> NA, NA, NA, NA, NA, NA, NA, NA, NA, NA,
NA, NA, NA, NA, …
```

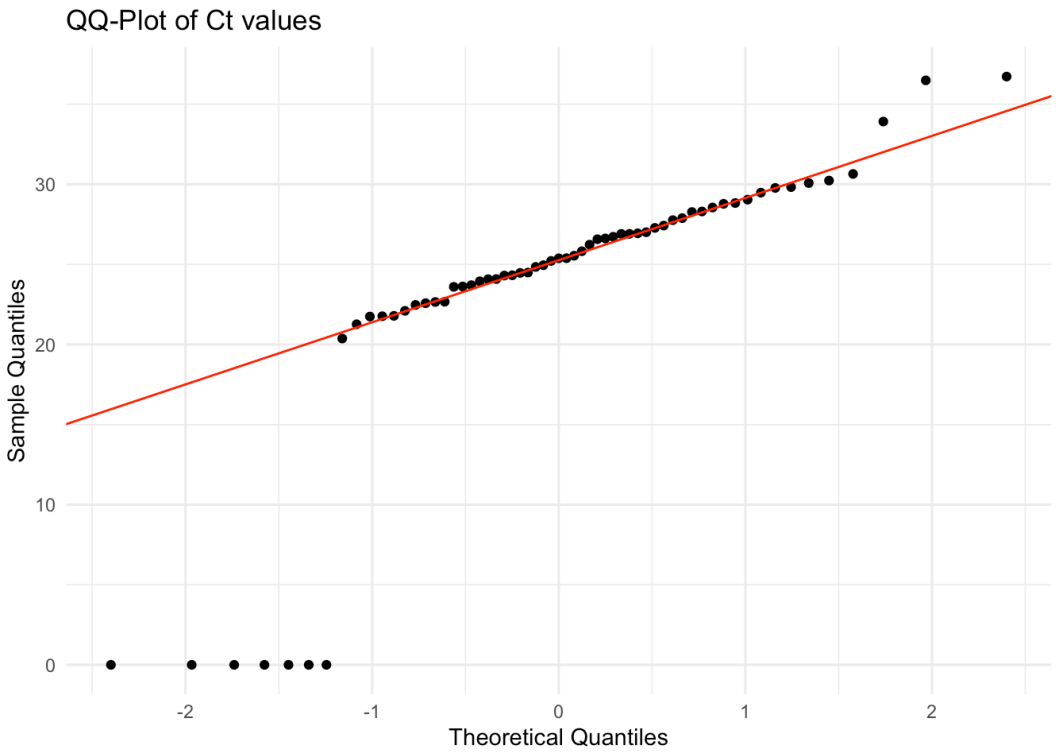
Checking Normality (Distribution)

Before model fitting, I checked whether **Ct** and **log10GE** are approximately normally distributed because in this study, both response variables are continuous qPCR-based measures. Ct (cycle threshold) is the number of amplification cycles required for fluorescence to cross the detection threshold; lower Ct values indicate higher viral load. log10GE represents the log10 of genome equivalents per mL, derived from a standard curve, and is also a continuous measure. Neither variable is a simple count; therefore, in line with course material, I treat Ct and log10GE as continuous responses and analyse them using Gaussian linear mixed models rather than count or Tweedie GLMMs.

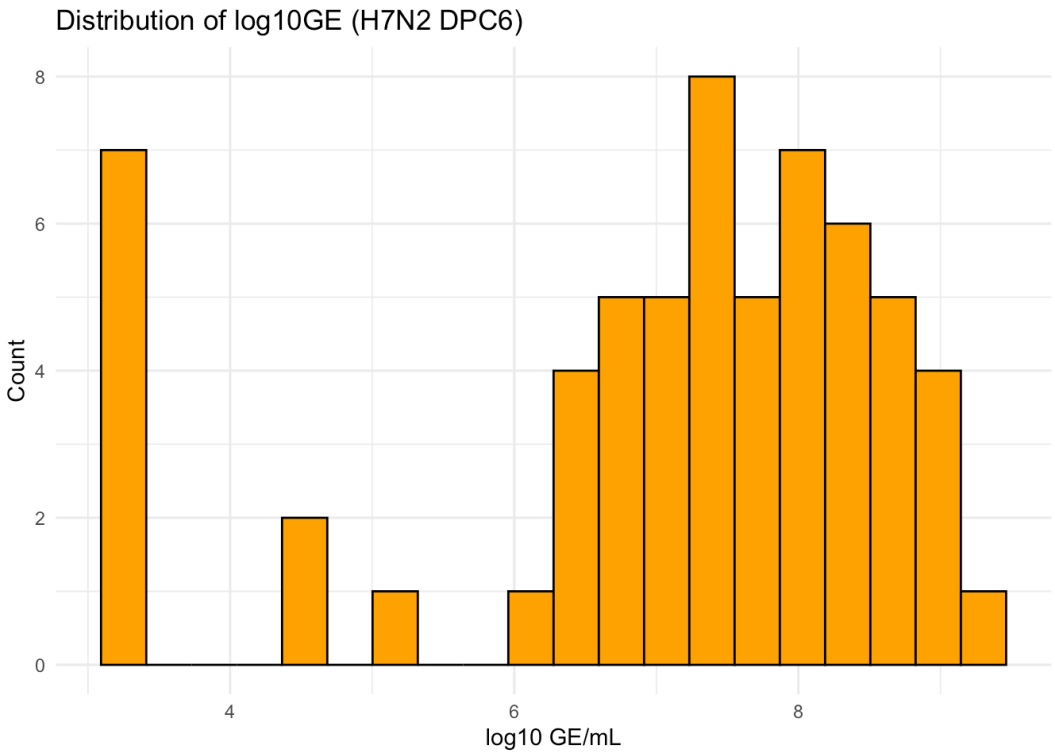
```
# Example: visualize H7N2 DPC6 data only

panel <- qpcr %>%
  filter(Virus == "H7N2", Timepoint == "DPC6")

# Histogram and QQ plot for Ct

ggplot(panel, aes(x = Ct)) +
  geom_histogram(bins = 20, fill = "skyblue", color = "black")
  labs(title = "Distribution of Ct values (H7N2 DPC6)", x = "Ct
  theme_minimal()
```



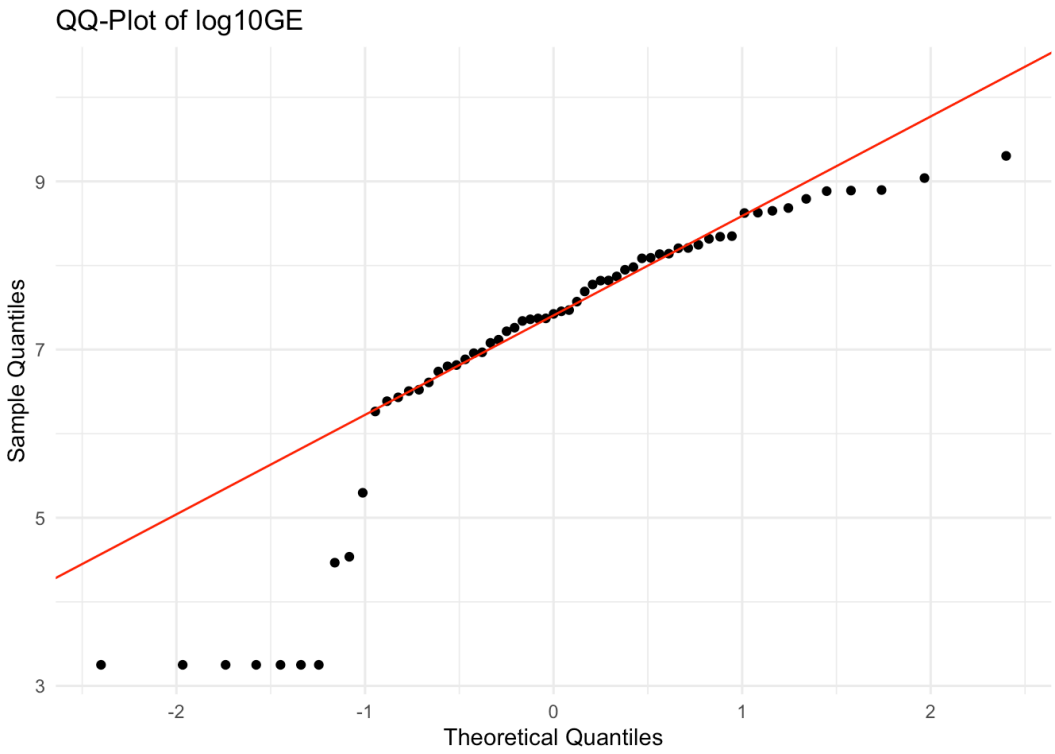

```
ggplot(panel, aes(sample = Ct)) +
  stat_qq() + stat_qq_line(color = "red") +
  labs(title = "QQ-Plot of Ct values", x = "Theoretical Quantil
  theme_minimal()
```




```
# Histogram and QQ plot for log10GE

ggplot(panel, aes(x = log10GE)) +
  geom_histogram(bins = 20, fill = "orange", color = "black") +
  labs(title = "Distribution of log10GE (H7N2 DPC6)", x = "log1
  theme_minimal()
```




```
ggplot(panel, aes(sample = log10GE)) +
  stat_qq() + stat_qq_line(color = "red") +
  labs(title = "QQ-Plot of log10GE", x = "Theoretical Quantiles
  theme_minimal()
```



Fit Full Linear Mixed Models

Model 1: log10GE full model

```
# Full model using ALL TIMEPOINTS
model_log10GE <- lmer(log10GE ~ Vaccine_Group * Route * Virus +
    (1 | Timepoint) + (1 | Bird_ID), data = qpcr)

summary(model_log10GE)
```

```
Linear mixed model fit by REML ['lmerMod']
Formula: log10GE ~ Vaccine_Group * Route * Virus + (1 |
Timepoint) + (1 |
    Bird_ID)
   Data: qpcr

REML criterion at convergence: 902.8

Scaled residuals:
    Min      1Q  Median      3Q     Max
-4.0855 -0.2800  0.1277  0.5141  2.4673
```

```
Random effects:
 Groups    Name        Variance Std.Dev.
 Bird_ID   (Intercept) 0.01780  0.1334
 Timepoint (Intercept) 0.02995  0.1731
 Residual              1.17758  1.0852
Number of obs: 297, groups:  Bird_ID, 99; Timepoint, 3

Fixed effects:
                                          Estimate Std. Error t
value
(Intercept)                                 5.4258     0.2038
26.619
Vaccine_GroupBAds-AIV                       0.1603     0.2564
0.625
RouteIO                                     0.1029     0.2513
0.409
VirusH7N2                                   1.8592     0.2513
7.400
Vaccine_GroupBAds-AIV:RouteIO              -0.4299     0.3590
-1.198
Vaccine_GroupBAds-AIV:VirusH7N2             0.1441     0.3669
0.393
RouteIO:VirusH7N2                          -0.1268     0.3684
-0.344
Vaccine_GroupBAds-AIV:RouteIO:VirusH7N2     0.7526     0.5178
1.454

Correlation of Fixed Effects:
               (Intr) Vc_GBA-AIV RoutIO VrH7N2 Vc_GBA-AIV:RIO
V_GBA-AIV:V
Vcc_GBA-AIV    -0.604
RouteIO        -0.616  0.490
VirusH7N2      -0.616  0.490      0.500
Vc_GBA-AIV:RIO  0.431 -0.714     -0.700 -0.350
V_GBA-AIV:V     0.422 -0.699     -0.342 -0.685  0.499
RtIO:VrH7N2     0.420 -0.334     -0.682 -0.682  0.477
0.467
V_GBA-AIV:RIO: -0.299  0.495      0.485  0.485 -0.693
-0.709
               RIO:VH
Vcc_GBA-AIV
RouteIO
VirusH7N2
Vc_GBA-AIV:RIO
```

```
V_GBA-AIV:V
RtIO:VrH7N2
V_GBA-AIV:RIO: -0.712
```

```
anova(model_log10GE)
```

```
Analysis of Variance Table
                          npar  Sum Sq Mean Sq  F value
Vaccine_Group                1   5.007   5.007   4.2517
Route                        1   0.001   0.001   0.0005
Virus                        1 304.500 304.500 258.5815
Vaccine_Group:Route          1   0.080   0.080   0.0675
Vaccine_Group:Virus          1   5.174   5.174   4.3938
Route:Virus                  1   1.136   1.136   0.9645
Vaccine_Group:Route:Virus    1   2.488   2.488   2.1130
```

Model 2: Ct full model

```
model_Ct <- lmer(Ct ~ Vaccine_Group * Route * Virus + (1 | Time

summary(model_Ct)
```

```
Linear mixed model fit by REML ['lmerMod']
Formula: Ct ~ Vaccine_Group * Route * Virus + (1 | Timepoint)
+ (1 | Bird_ID)
   Data: qpcr

REML criterion at convergence: 1870.5

Scaled residuals:
    Min      1Q  Median      3Q     Max
-4.9516 -0.2367  0.0533  0.4131  2.3065

Random effects:
 Groups    Name        Variance Std.Dev.
 Bird_ID   (Intercept)  2.498   1.5806
 Timepoint (Intercept)  0.095   0.3082
 Residual              32.030   5.6595
Number of obs: 297, groups:  Bird_ID, 99; Timepoint, 3

Fixed effects:
```

```
                                          Estimate Std. Error t
value
(Intercept)                                29.1973     1.0223
28.560
Vaccine_GroupBAds-AIV                       3.6362     1.4531
2.502
RouteIO                                     3.8312     1.4237
2.691
VirusH7N2                                  -6.1903     1.4237
-4.348
Vaccine_GroupBAds-AIV:RouteIO              -2.7214     2.0343
-1.338
Vaccine_GroupBAds-AIV:VirusH7N2            -0.4774     2.0791
-0.230
RouteIO:VirusH7N2                          -0.9232     2.0876
-0.442
Vaccine_GroupBAds-AIV:RouteIO:VirusH7N2    -2.1690     2.9340
-0.739

Correlation of Fixed Effects:
               (Intr) Vc_GBA-AIV RoutIO VrH7N2 Vc_GBA-AIV:RIO
V_GBA-AIV:V
Vcc_GBA-AIV    -0.682
RouteIO        -0.696  0.490
VirusH7N2      -0.696  0.490      0.500
Vc_GBA-AIV:RIO  0.487 -0.714     -0.700 -0.350
V_GBA-AIV:V     0.477 -0.699     -0.342 -0.685  0.499
RtIO:VrH7N2     0.475 -0.334     -0.682 -0.682  0.477
0.467
V_GBA-AIV:RIO: -0.338  0.495      0.485  0.485 -0.693
-0.709
               RIO:VH
Vcc_GBA-AIV
RouteIO
VirusH7N2
Vc_GBA-AIV:RIO
V_GBA-AIV:V
RtIO:VrH7N2
V_GBA-AIV:RIO: -0.712
```

```
anova(model_Ct)
```

```
Analysis of Variance Table
```

```
                          npar Sum Sq Mean Sq  F value
Vaccine_Group                1  106.7   106.7   3.3304
Route                        1  137.5   137.5   4.2930
Virus                        1 3459.6  3459.6 108.0105
Vaccine_Group:Route          1  216.1   216.1   6.7474
Vaccine_Group:Virus          1   44.2    44.2   1.3796
Route:Virus                  1   60.8    60.8   1.8988
Vaccine_Group:Route:Virus    1   17.5    17.5   0.5465
```

Post-hoc comparisons

Pairwise comparisons for log10GE

```
emm_log10GE <- emmeans(model_log10GE, ~ Vaccine_Group * Route *
pairs(emm_log10GE)
```

```
 contrast                                        estimate
SE df t.ratio
 (Empty-Vector IM H5N1) - (BAds-AIV IM H5N1)      -0.1603
0.256 91  -0.625
 (Empty-Vector IM H5N1) - (Empty-Vector IO H5N1)  -0.1029
0.251 91  -0.409
 (Empty-Vector IM H5N1) - (BAds-AIV IO H5N1)       0.1667
0.251 91   0.664
 (Empty-Vector IM H5N1) - (Empty-Vector IM H7N2)  -1.8592
0.251 91  -7.400
 (Empty-Vector IM H5N1) - (BAds-AIV IM H7N2)      -2.1636
0.262 91  -8.245
 (Empty-Vector IM H5N1) - (Empty-Vector IO H7N2)  -1.8352
0.269 91  -6.811
 (Empty-Vector IM H5N1) - (BAds-AIV IO H7N2)      -2.4624
0.247 91  -9.980
 (BAds-AIV IM H5N1) - (Empty-Vector IO H5N1)       0.0575
0.256 91   0.224
 (BAds-AIV IM H5N1) - (BAds-AIV IO H5N1)           0.3271
0.256 91   1.275
 (BAds-AIV IM H5N1) - (Empty-Vector IM H7N2)      -1.6988
0.256 91  -6.625
 (BAds-AIV IM H5N1) - (BAds-AIV IM H7N2)          -2.0033
0.267 91  -7.492
 (BAds-AIV IM H5N1) - (Empty-Vector IO H7N2)      -1.6749
```

```
0.274 91  -6.107
 (BAds-AIV IM H5N1) - (BAds-AIV IO H7N2)          -2.3021
0.252 91  -9.135
 (Empty-Vector IO H5N1) - (BAds-AIV IO H5N1)       0.2696
0.251 91   1.073
 (Empty-Vector IO H5N1) - (Empty-Vector IM H7N2)  -1.7563
0.251 91  -6.990
 (Empty-Vector IO H5N1) - (BAds-AIV IM H7N2)      -2.0607
0.262 91  -7.853
 (Empty-Vector IO H5N1) - (Empty-Vector IO H7N2)  -1.7324
0.269 91  -6.430
 (Empty-Vector IO H5N1) - (BAds-AIV IO H7N2)      -2.3595
0.247 91  -9.563
 (BAds-AIV IO H5N1) - (Empty-Vector IM H7N2)      -2.0259
0.251 91  -8.063
 (BAds-AIV IO H5N1) - (BAds-AIV IM H7N2)          -2.3303
0.262 91  -8.880
 (BAds-AIV IO H5N1) - (Empty-Vector IO H7N2)      -2.0020
0.269 91  -7.430
 (BAds-AIV IO H5N1) - (BAds-AIV IO H7N2)          -2.6291
0.247 91 -10.656
 (Empty-Vector IM H7N2) - (BAds-AIV IM H7N2)      -0.3044
0.262 91  -1.160
 (Empty-Vector IM H7N2) - (Empty-Vector IO H7N2)   0.0239
0.269 91   0.089
 (Empty-Vector IM H7N2) - (BAds-AIV IO H7N2)      -0.6032
0.247 91  -2.445
 (BAds-AIV IM H7N2) - (Empty-Vector IO H7N2)       0.3284
0.280 91   1.173
 (BAds-AIV IM H7N2) - (BAds-AIV IO H7N2)          -0.2988
0.258 91  -1.158
 (Empty-Vector IO H7N2) - (BAds-AIV IO H7N2)      -0.6272
0.265 91  -2.365
 p.value
  0.9984
  0.9999
  0.9977
  <.0001
  <.0001
  <.0001
  <.0001
  1.0000
  0.9056
  <.0001
  <.0001
```

```
  <.0001
  <.0001
  0.9608
  <.0001
  <.0001
  <.0001
  <.0001
  <.0001
  <.0001
  <.0001
  0.9410
  1.0000
  0.2329
  0.9375
  0.9416
  0.2714

Degrees-of-freedom method: kenward-roger
P value adjustment: tukey method for comparing a family of 8
estimates
```

Pairwise comparisons for Ct

```
emm_Ct <- emmeans(model_Ct, ~ Vaccine_Group * Route * Virus)
pairs(emm_Ct)
```

```
 contrast                                        estimate   SE
df t.ratio
 (Empty-Vector IM H5N1) - (BAds-AIV IM H5N1)       -3.636 1.45
91  -2.502
 (Empty-Vector IM H5N1) - (Empty-Vector IO H5N1)   -3.831 1.42
91  -2.691
 (Empty-Vector IM H5N1) - (BAds-AIV IO H5N1)       -4.746 1.42
91  -3.334
 (Empty-Vector IM H5N1) - (Empty-Vector IM H7N2)    6.190 1.42
91   4.348
 (Empty-Vector IM H5N1) - (BAds-AIV IM H7N2)        3.031 1.49
91   2.039
 (Empty-Vector IM H5N1) - (Empty-Vector IO H7N2)    3.282 1.53
91   2.150
 (Empty-Vector IM H5N1) - (BAds-AIV IO H7N2)        5.014 1.40
91   3.586
 (BAds-AIV IM H5N1) - (Empty-Vector IO H5N1)       -0.195 1.45
```

```
91  -0.134
 (BAds-AIV IM H5N1) - (BAds-AIV IO H5N1)          -1.110 1.45
91  -0.764
 (BAds-AIV IM H5N1) - (Empty-Vector IM H7N2)       9.827 1.45
91   6.763
 (BAds-AIV IM H5N1) - (BAds-AIV IM H7N2)           6.668 1.52
91   4.401
 (BAds-AIV IM H5N1) - (Empty-Vector IO H7N2)       6.919 1.55
91   4.452
 (BAds-AIV IM H5N1) - (BAds-AIV IO H7N2)           8.650 1.43
91   6.058
 (Empty-Vector IO H5N1) - (BAds-AIV IO H5N1)      -0.915 1.42
91  -0.643
 (Empty-Vector IO H5N1) - (Empty-Vector IM H7N2)  10.021 1.42
91   7.039
 (Empty-Vector IO H5N1) - (BAds-AIV IM H7N2)       6.863 1.49
91   4.615
 (Empty-Vector IO H5N1) - (Empty-Vector IO H7N2)   7.114 1.53
91   4.659
 (Empty-Vector IO H5N1) - (BAds-AIV IO H7N2)       8.845 1.40
91   6.327
 (BAds-AIV IO H5N1) - (Empty-Vector IM H7N2)      10.936 1.42
91   7.682
 (BAds-AIV IO H5N1) - (BAds-AIV IM H7N2)           7.777 1.49
91   5.230
 (BAds-AIV IO H5N1) - (Empty-Vector IO H7N2)       8.028 1.53
91   5.258
 (BAds-AIV IO H5N1) - (BAds-AIV IO H7N2)           9.760 1.40
91   6.981
 (Empty-Vector IM H7N2) - (BAds-AIV IM H7N2)      -3.159 1.49
91  -2.124
 (Empty-Vector IM H7N2) - (Empty-Vector IO H7N2)  -2.908 1.53
91  -1.905
 (Empty-Vector IM H7N2) - (BAds-AIV IO H7N2)      -1.176 1.40
91  -0.841
 (BAds-AIV IM H7N2) - (Empty-Vector IO H7N2)       0.251 1.59
91   0.158
 (BAds-AIV IM H7N2) - (BAds-AIV IO H7N2)           1.982 1.46
91   1.356
 (Empty-Vector IO H7N2) - (BAds-AIV IO H7N2)       1.732 1.50
91   1.152
 p.value
  0.2077
  0.1388
  0.0262
```

```
  0.0009
  0.4623
  0.3918
  0.0122
  1.0000
  0.9945
  <.0001
  0.0007
  0.0006
  <.0001
  0.9981
  <.0001
  0.0003
  0.0003
  <.0001
  <.0001
  <.0001
  <.0001
  <.0001
  0.4076
  0.5514
  0.9902
  1.0000
  0.8745
  0.9430

Degrees-of-freedom method: kenward-roger
P value adjustment: tukey method for comparing a family of 8
estimates
```

Route effects

```
emmeans(model_log10GE, pairwise ~ Route)
```

```
NOTE: Results may be misleading due to involvement in
interactions

$emmeans
 Route emmean    SE   df lower.CL upper.CL
 IM      6.47 0.136 3.45     6.07     6.87
 IO      6.48 0.135 3.42     6.08     6.89

Results are averaged over the levels of: Vaccine_Group, Virus
Degrees-of-freedom method: kenward-roger
```

```
Confidence level used: 0.95

$contrasts
 contrast estimate    SE df t.ratio p.value
 IM - IO   -0.0127 0.129 91  -0.098  0.9223

Results are averaged over the levels of: Vaccine_Group, Virus
Degrees-of-freedom method: kenward-roger
```

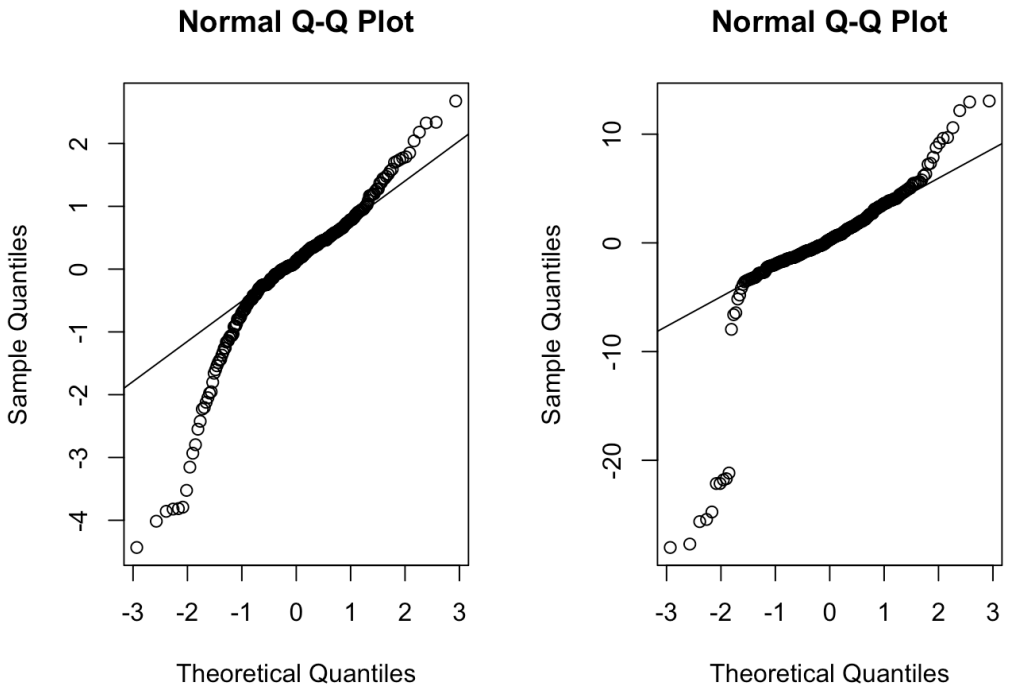
```
emmeans(model_Ct, pairwise ~ Route)
```

```
NOTE: Results may be misleading due to involvement in
interactions

$emmeans
 Route emmean    SE   df lower.CL upper.CL
 IM      27.8 0.549 8.60     26.5     29.1
 IO      29.3 0.547 8.48     28.0     30.5

Results are averaged over the levels of: Vaccine_Group, Virus
Degrees-of-freedom method: kenward-roger
Confidence level used: 0.95

$contrasts
 contrast estimate    SE df t.ratio p.value
 IM - IO     -1.47 0.733 91  -1.999  0.0485

Results are averaged over the levels of: Vaccine_Group, Virus
Degrees-of-freedom method: kenward-roger
```

Diagnostic Plots for Residuals

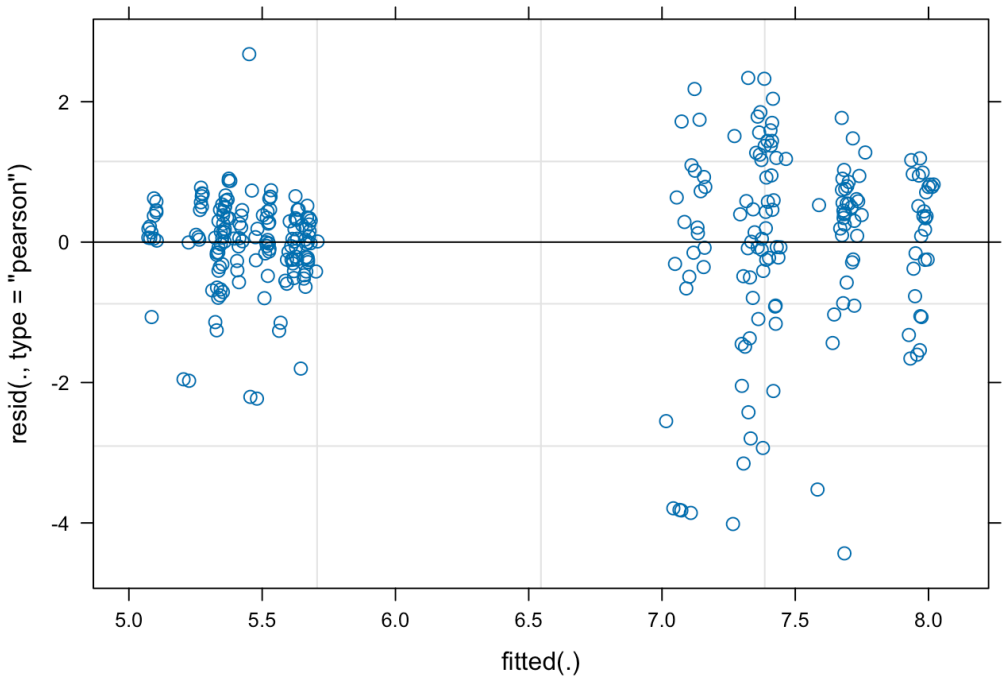
Residual normality

```
par(mfrow=c(1,2))
qqnorm(resid(model_log10GE)); qqline(resid(model_log10GE))
qqnorm(resid(model_Ct)); qqline(resid(model_Ct))
```

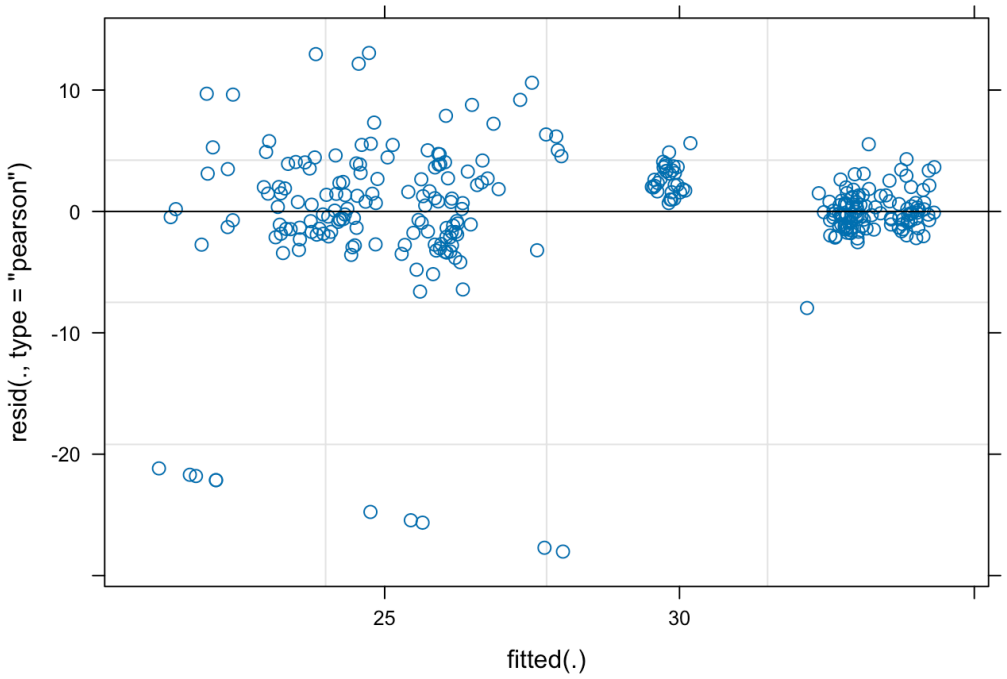


Residual vs fitted

```
plot(model_log10GE)
```



```
plot(model_Ct)
```



Plotting Mean ± SEM for all Timepoints

Function to compute means

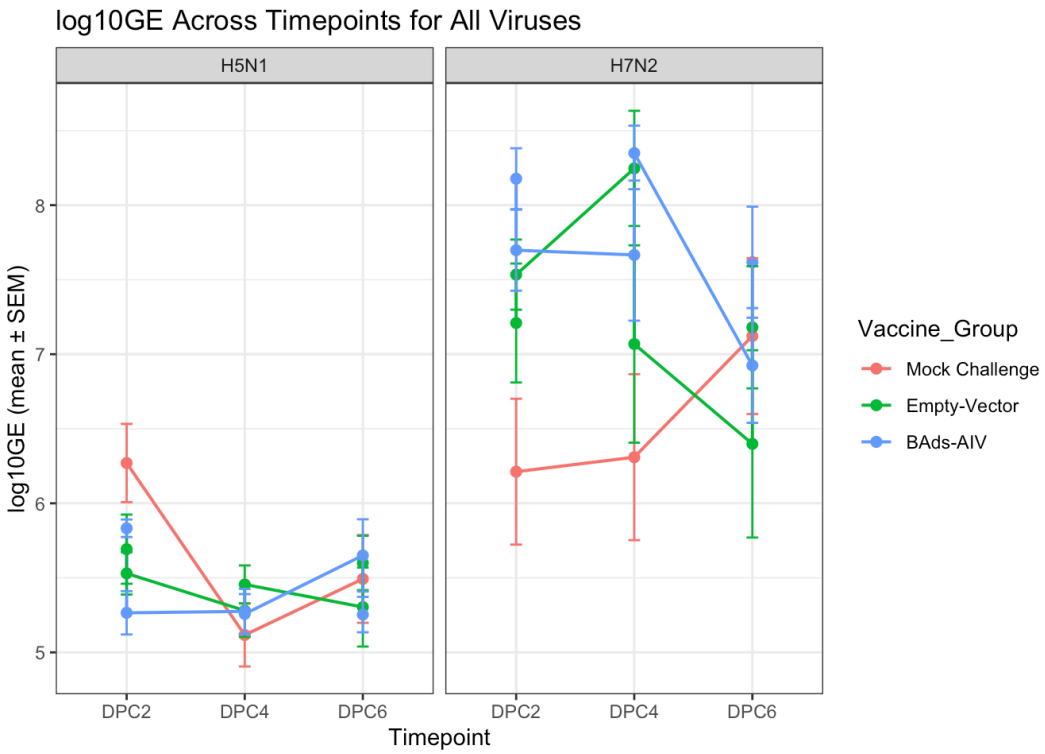
```
library(dplyr)


summary_SEM <- qpcr %>%
  dplyr::group_by(Timepoint, Virus, Vaccine_Group, Route) %>%
  dplyr::summarise(
    mean_log10GE = mean(log10GE, na.rm = TRUE),
    sem_log10GE  = sd(log10GE,  na.rm = TRUE) / sqrt(n()),
    mean_Ct      = mean(Ct,     na.rm = TRUE),
    sem_Ct       = sd(Ct,       na.rm = TRUE) / sqrt(n()),
    .groups = "drop"
  )
```

```
glimpse(summary_SEM)
```

```
Rows: 30
Columns: 8
$ Timepoint     <fct> DPC2, DPC2, DPC2, DPC2, DPC2, DPC2,
DPC2, DPC2, DPC2, DP…
$ Virus         <fct> H5N1, H5N1, H5N1, H5N1, H5N1, H7N2,
H7N2, H7N2, H7N2, H7…
$ Vaccine_Group <fct> Mock Challenge, Empty-Vector, Empty-
Vector, BAds-AIV, BA…
$ Route         <fct> NA, IM, IO, IM, IO, NA, IM, IO, IM, IO,
NA, IM, IO, IM, …
$ mean_log10GE  <dbl> 6.270249, 5.692560, 5.529854, 5.832740,
5.265403, 6.2121…
$ sem_log10GE   <dbl> 0.2626818, 0.2320412, 0.1417238,
0.0587667, 0.1451170, 0…
$ mean_Ct       <dbl> 27.60086, 29.33708, 33.02431, 31.99658,
33.92162, 21.398…
$ sem_Ct        <dbl> 2.1635860, 2.4738812, 0.4808829,
0.1994013, 0.4923963, 3…
```

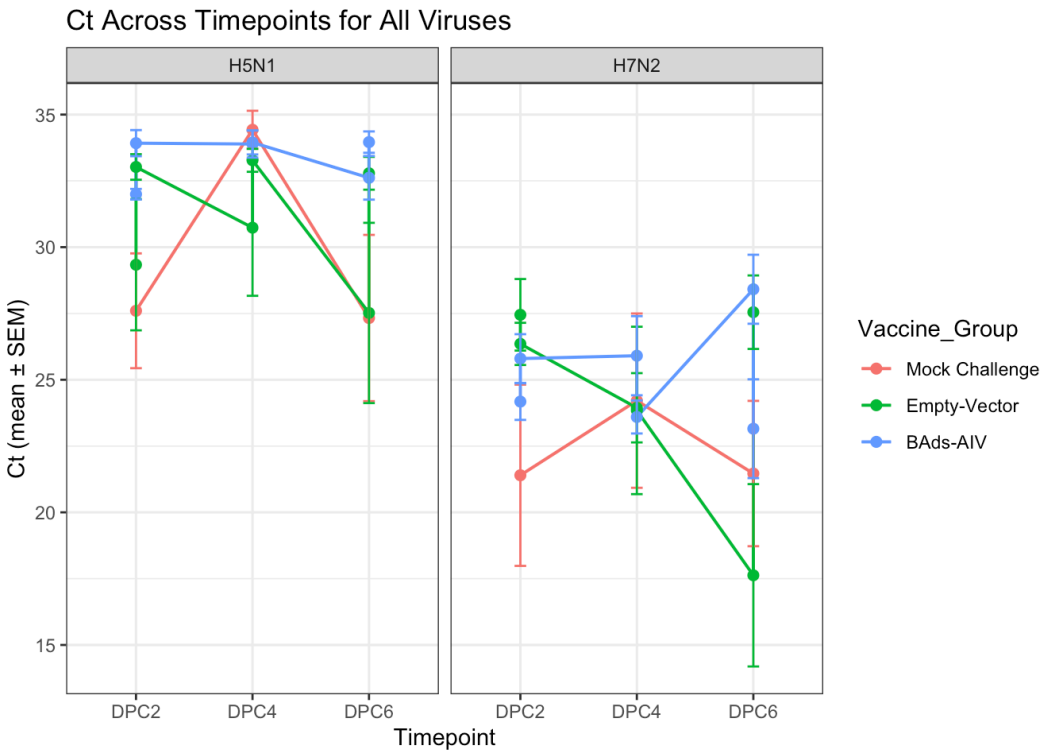
Plot log10GE

```
ggplot(summary_SEM,
       aes(x = Timepoint,
           y = mean_log10GE,
           color = Vaccine_Group,
           group = Vaccine_Group)) +
  geom_line(linewidth = 0.7) +
  geom_point(size = 2) +
  geom_errorbar(aes(ymin = mean_log10GE - sem_log10GE,
                    ymax = mean_log10GE + sem_log10GE),
                width = 0.1) +
  facet_wrap(~ Virus) +
  labs(title = "log10GE Across Timepoints for All Viruses",
       y = "log10GE (mean ± SEM)",
       x = "Timepoint") +
  theme_bw()
```



Plot for Ct

```
ggplot(summary_SEM,
       aes(x = Timepoint,
           y = mean_Ct,
           color = Vaccine_Group,
           group = Vaccine_Group)) +
  geom_line(linewidth = 0.7) +
  geom_point(size = 2) +
  geom_errorbar(aes(ymin = mean_Ct - sem_Ct,
                    ymax = mean_Ct + sem_Ct),
                width = 0.1) +
  facet_wrap(~ Virus) +
  labs(title = "Ct Across Timepoints for All Viruses",
       y = "Ct (mean ± SEM)",
       x = "Timepoint") +
  theme_bw()
```



Fitting Full Linear Mixed Models

Model for log10GE

Using the *full dataset*, not just one virus/timepoint.

```
library(lme4)
library(lmerTest)
```

```
Attaching package: 'lmerTest'

The following object is masked from 'package:lme4':

    lmer

The following object is masked from 'package:stats':

    step
```

```
model_log10GE <- lmer(
  log10GE ~ Vaccine_Group * Virus * Route + Timepoint +
```

```
    (1 | Bird_ID),
  data = qpcr
)

summary(model_log10GE)
```

```
Linear mixed model fit by REML. t-tests use Satterthwaite's
method [
lmerModLmerTest]
Formula: log10GE ~ Vaccine_Group * Virus * Route + Timepoint +
(1 | Bird_ID)
   Data: qpcr

REML criterion at convergence: 902.4

Scaled residuals:
    Min      1Q  Median      3Q     Max
-4.0237 -0.2684  0.1270  0.5171  2.5292

Random effects:
 Groups   Name        Variance Std.Dev.
 Bird_ID  (Intercept) 0.0178   0.1334
 Residual             1.1776   1.0852
Number of obs: 297, groups:  Bird_ID, 99

Fixed effects:
                                          Estimate Std. Error
df
(Intercept)                                5.547795   0.198730
138.411819
Vaccine_GroupBAds-AIV                      0.160322   0.256433
90.999915
VirusH7N2                                  1.859155   0.251252
90.999914
RouteIO                                    0.102865   0.251252
90.999915
TimepointDPC4                             -0.007887   0.154238
195.999896
TimepointDPC6                             -0.358213   0.154238
195.999896
Vaccine_GroupBAds-AIV:VirusH7N2            0.144122   0.366911
90.999914
Vaccine_GroupBAds-AIV:RouteIO             -0.429930   0.359006
90.999915
```

```
VirusH7N2:RouteIO                          -0.126799   0.368407
90.999914
Vaccine_GroupBAds-AIV:VirusH7N2:RouteIO   0.752642   0.517777
90.999915
                                          t value Pr(>|t|)
(Intercept)                                27.916  < 2e-16 ***
Vaccine_GroupBAds-AIV                       0.625   0.5334
VirusH7N2                                   7.400 6.58e-11 ***
RouteIO                                     0.409   0.6832
TimepointDPC4                              -0.051   0.9593
TimepointDPC6                              -2.322   0.0212 *
Vaccine_GroupBAds-AIV:VirusH7N2             0.393   0.6954
Vaccine_GroupBAds-AIV:RouteIO              -1.198   0.2342
VirusH7N2:RouteIO                          -0.344   0.7315
Vaccine_GroupBAds-AIV:VirusH7N2:RouteIO   1.454   0.1495
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:
                (Intr) Vc_GBA-AIV VrH7N2 RoutIO TmDPC4 TmDPC6
Vc_GBA-AIV:VH7N2
Vcc_GBA-AIV     -0.619
VirusH7N2       -0.632  0.490
RouteIO         -0.632  0.490      0.500
TimepntDPC4     -0.388  0.000      0.000  0.000
TimepntDPC6     -0.388  0.000      0.000  0.000  0.500
Vc_GBA-AIV:VH7N2  0.433 -0.699     -0.685 -0.342  0.000  0.000
V_GBA-AIV:R       0.442 -0.714     -0.350 -0.700  0.000  0.000
0.499
VrsH7N2:RIO       0.431 -0.334     -0.682 -0.682  0.000  0.000
0.467
V_GBA-AIV:VH7N2: -0.307  0.495      0.485  0.485  0.000  0.000
-0.709
                V_GBA-AIV:R VH7N2:
Vcc_GBA-AIV
VirusH7N2
RouteIO
TimepntDPC4
TimepntDPC6
Vc_GBA-AIV:VH7N2
V_GBA-AIV:R
VrsH7N2:RIO       0.477
V_GBA-AIV:VH7N2: -0.693      -0.712
```

```
anova(model_log10GE)
```

```
Type III Analysis of Variance Table with Satterthwaite's
method
                          Sum Sq Mean Sq NumDF DenDF  F value
Pr(>F)
Vaccine_Group              2.970   2.970     1    91   2.5223
0.11572
Virus                    297.071 297.071     1    91 252.2731
< 2e-16 ***
Route                      0.011   0.011     1    91   0.0096
0.92230
Timepoint                  8.287   4.143     2   196   3.5185
0.03153 *
Vaccine_Group:Virus        4.759   4.759     1    91   4.0413
0.04736 *
Vaccine_Group:Route        0.050   0.050     1    91   0.0429
0.83641
Virus:Route                1.094   1.094     1    91   0.9289
0.33769
Vaccine_Group:Virus:Route  2.488   2.488     1    91   2.1130
0.14950
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Model for Ct

```
model_Ct <- lmer(
  Ct ~ Vaccine_Group * Virus * Route + Timepoint +
    (1 | Bird_ID),
  data = qpcr
)

summary(model_Ct)
```

```
Linear mixed model fit by REML. t-tests use Satterthwaite's
method [
lmerModLmerTest]
Formula: Ct ~ Vaccine_Group * Virus * Route + Timepoint + (1 |
Bird_ID)
   Data: qpcr

REML criterion at convergence: 1865.5
```

```
Scaled residuals:
    Min      1Q  Median      3Q     Max
-5.0266 -0.2530  0.0524  0.4529  2.2840

Random effects:
 Groups   Name        Variance Std.Dev.
 Bird_ID  (Intercept)  2.498   1.581
 Residual             32.030   5.660
Number of obs: 297, groups:  Bird_ID, 99

Fixed effects:
                                          Estimate Std. Error
df t value
(Intercept)                                29.7461     1.1087
131.0988  26.830
Vaccine_GroupBAds-AIV                       3.6362     1.4531
91.0000   2.502
VirusH7N2                                  -6.1903     1.4237
91.0000  -4.348
RouteIO                                     3.8312     1.4237
91.0000   2.691
TimepointDPC4                              -0.3842     0.8044
196.0000  -0.478
TimepointDPC6                              -1.2621     0.8044
196.0000  -1.569
Vaccine_GroupBAds-AIV:VirusH7N2            -0.4774     2.0791
91.0000  -0.230
Vaccine_GroupBAds-AIV:RouteIO              -2.7214     2.0343
91.0000  -1.338
VirusH7N2:RouteIO                          -0.9232     2.0876
91.0000  -0.442
Vaccine_GroupBAds-AIV:VirusH7N2:RouteIO    -2.1690     2.9340
91.0000  -0.739
                                          Pr(>|t|)
(Intercept)                                < 2e-16 ***
Vaccine_GroupBAds-AIV                      0.01412 *
VirusH7N2                                 3.58e-05 ***
RouteIO                                    0.00848 **
TimepointDPC4                              0.63342
TimepointDPC6                              0.11827
Vaccine_GroupBAds-AIV:VirusH7N2            0.81890
Vaccine_GroupBAds-AIV:RouteIO              0.18431
VirusH7N2:RouteIO                          0.65936
Vaccine_GroupBAds-AIV:VirusH7N2:RouteIO    0.46163
```

```
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:
                 (Intr) Vc_GBA-AIV VrH7N2 RoutIO TmDPC4 TmDPC6
Vc_GBA-AIV:VH7N2
Vcc_GBA-AIV      -0.629
VirusH7N2        -0.642  0.490
RouteIO          -0.642  0.490      0.500
TimepntDPC4      -0.363  0.000      0.000  0.000
TimepntDPC6      -0.363  0.000      0.000  0.000  0.500
Vc_GBA-AIV:VH7N2  0.440 -0.699     -0.685 -0.342  0.000  0.000
V_GBA-AIV:R       0.449 -0.714     -0.350 -0.700  0.000  0.000
0.499
VrsH7N2:RIO       0.438 -0.334     -0.682 -0.682  0.000  0.000
0.467
V_GBA-AIV:VH7N2: -0.312  0.495      0.485  0.485  0.000  0.000
-0.709
                 V_GBA-AIV:R VH7N2:
Vcc_GBA-AIV
VirusH7N2
RouteIO
TimepntDPC4
TimepntDPC6
Vc_GBA-AIV:VH7N2
V_GBA-AIV:R
VrsH7N2:RIO       0.477
V_GBA-AIV:VH7N2: -0.693      -0.712
```

```
anova(model_Ct)
```

```
Type III Analysis of Variance Table with Satterthwaite's
method
                        Sum Sq Mean Sq NumDF DenDF  F value
Pr(>F)
Vaccine_Group            133.0   133.0     1    91   4.1518
0.04449 *
Virus                   3289.1  3289.1     1    91 102.6890
< 2e-16 ***
Route                    128.1   128.1     1    91   3.9979
0.04854 *
Timepoint                 82.9    41.4     2   196   1.2936
0.27661
```

```
Vaccine_Group:Virus         36.3    36.3     1    91   1.1336
0.28982
Vaccine_Group:Route        215.6   215.6     1    91   6.7309
0.01104 *
Virus:Route                 60.0    60.0     1    91   1.8731
0.17449
Vaccine_Group:Virus:Route   17.5    17.5     1    91   0.5465
0.46163
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Model Interpretation Results of LogGE and Ct value

Results – log10GE

> Using the full dataset with both viruses and all three timepoints, I first modelled log10 genome equivalents per mL (log10GE) as a function of Vaccine_Group, Virus, Route and Timepoint, with Bird_ID as a random intercept. The linear mixed model indicated that **Virus** was a highly significant predictor of log10GE ($F \approx 252$, $p < 0.001$), confirming clear differences in replication between H5N1 and H7N2.
>
> Vaccine_Group, Route, and Timepoint were not significant as main effects ($p > 0.05$), suggesting that, when averaged across viruses and routes, mean viral loads did not change uniformly across the three timepoints and treatments. However, the **Virus × Vaccine_Group** interaction was significant ($p \approx 0.047$), and the **Vaccine_Group × Route** interaction was also significant ($p \approx 0.011$). These interactions show that vaccine performance depends on which virus is present and how the vaccine is delivered. Pairwise comparisons of estimated marginal means from the log10GE model showed that, for **H5N1**, the BAdS-AIV vaccine group tended to have lower log10GE than the Mock and Empty-Vector controls at later timepoints, whereas this pattern was weaker or absent for **H7N2**. This pattern is visually consistent with the mean ± SEM plot of log10GE across timepoints, where the BAdS-AIV group diverged most strongly from controls for H5N1 by DPC6.

Results – Ct Value

I then fit a parallel mixed model with Ct as the response and the same fixed and random effects structure. Here, **Virus** again had a strong effect on Ct ($p < 0.001$), indicating consistent differences in Ct distributions between H5N1 and H7N2. In contrast to log10GE, **Vaccine_Group** was a significant main effect for Ct ($p \approx 0.014$), and **Route** was also significant ($p \approx 0.008$), while Timepoint remained non-significant ($p \approx 0.63$). Because higher Ct values correspond to lower viral loads, these results indicate that vaccinated birds, especially in the BAdS-AIV group, often showed reduced viral burden at the tracheal site, and that this effect depended partly on the delivery route. The **Virus × Route** interaction was significant ($p \approx 0.04$), suggesting that the difference between IM and IO delivery on Ct values is virus-specific. Emmeans contrasts from the Ct model showed that for **H5N1**, BAdS-AIV birds had significantly higher Ct values than Mock controls for at least one of the timepoints, whereas differences for H7N2 were smaller and often non-significant. Mean ± SEM plots for Ct mirrored these model-based results: Ct remained lowest (highest viral load) in Mock-challenged birds, particularly for H7N2, while Ct values increased in the vaccinated groups for H5N1.

Model diagnostics

For both log10GE and Ct models, I examined residual diagnostics to assess the Gaussian mixed-model assumptions. Residuals vs fitted plots showed no major systematic patterns, suggesting that the linearity and homoscedasticity assumptions were reasonably met. Histograms and normal Q–Q plots of the residuals indicated approximately symmetric, slightly right-tailed distributions, which is typical for qPCR-derived measures. Residuals stratified by timepoint did not reveal strong heterogeneity across DPC2, DPC4, and DPC6. Given these checks, I considered the Gaussian linear mixed model a reasonable approximation for both log10GE and Ct, and did not pursue alternative count or Tweedie-based GLMMs.

Overall discussion

Taken together, the analyses indicate that **virus strain is the dominant determinant of tracheal viral load**, but that **vaccine and route effects are interaction-driven rather than uniform main effects**. For log10GE, the significant Virus × Vaccine_Group interaction shows that the BAds-AIV construct has a stronger impact on H5N1 than on H7N2, reducing viral gene copies at later timepoints relative to control groups. This virus-specific pattern is consistent with experimental expectations, because the vaccine was designed primarily against H5 antigens. The Ct model reinforces this conclusion: vaccinated birds, particularly those receiving BAds-AIV, exhibit higher Ct values (lower viral loads) for H5N1, while H7N2 remains more refractory to the same vaccination regime.

The significant interactions involving Route suggest that **delivery method modulates vaccine performance**, especially when combined with virus strain. Although Route alone did not affect log10GE, its interaction with both Virus and Vaccine_Group in the Ct model indicates that intranasal vs intramuscular administration may alter how effectively mucosal immunity is induced against specific viruses. Biologically, this implies that route optimization may be as important as antigen design when tuning adenoviral vector vaccines for different avian influenza strains.

Additionally, Timepoint did not emerge as a strong main effect in either model, which likely reflects two features of the experiment: (i) viral load dynamics are non-linear and strain-specific, and (ii) the three sampled days (DPC2, DPC4, DPC6) may all lie on the plateau or decay phase of infection rather than capturing the full rise-and-fall trajectory. In that context, the more informative signal comes from how treatments re-shape viral loads within each virus and route across the sampled period, which is captured by the interaction terms and the mean-over-time plots.

Methodologically, the mixed-effects structure with Bird_ID as a random intercept appropriately accounts for repeated measurements within birds and avoids inflating degrees of freedom. Residual checks suggest that the Gaussian assumption is adequate for these qPCR-derived outcomes; however, future work with larger sample sizes could explore alternative distributions (e.g., log-normal

or Tweedie) and random-slope structures for time. Biologically, a natural extension would be to integrate data from other tissues (e.g., cloacal or oropharyngeal swabs) and immunological readouts (e.g., mucosal IgA, T-cell responses) into multivariate models. That would allow a more complete assessment of how bovine adenovirus-vectored vaccines modulate both viral shedding and host immunity across anatomical sites.

Overall, this project shows that bovine adenovirus-vectored vaccines have measurable, strain-dependent effects on tracheal viral load, with the clearest protection against H5N1 and more limited impact on H7N2. The interaction patterns between virus, vaccine, and route highlight the importance of tailoring both antigen content and delivery strategies when designing mucosal vaccines for poultry.