# IBV persistence in the chicken: a long -term study

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# استمرارية الإصابة بفيروس التهاب الشعب الهوائية في الدجاج دراسة مطولة

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**الملخص:**

قد وجد تحليل تسلسل جين بروتين السنبلة للفيروس المكتشف في الكلى بعد 46 يومًا من الإصابة مطابقًا للفيروس الأصلي ، مما يشير إلى عدم حدوث طفرة في الفيروس المسبب للعدوى خلال تلك الفترة الزمنية، على الأقل في ذلك الجزء من الجينوم. ولاستكشاف هذا الأمر بشكل أكبر، في التجربة الموصوفة في هذه الدراسة، تم الاحتفاظ بالطيور لفترة أطول بكثير، حتى بعد النضج الجنسي.

ففي هذه الدراسة تم تقديم جميع "العوامل المسببة للتوتر"، المحبط للجهاز المناعى (السايكلوسبورين) (أكثر من مرة)، والحركة، والنضج الجنسي. وكان من المأمول فحص الفيروس المفرز عند النضج الجنسي أو بعده لتحديد ما إذا كان التغيير في التسلسلات في جين السبايك بروتين S1 يشير إلى طفرة بعد الاستمرار الطويل.

ومن خلال نتائج عزل الفيروس وتفاعل البوليميراز المتسلسل العكسي كان الفيروس موجودًا في الأنسجة كما هو موضح بواسطة عزل الفيروس وتفاعل البوليميراز المتسلسل من الأنسجة المتوقعة أثناء المرحلة الحادة في(الأيام 1-21). كانت نتائج عزل الفيروس وتفاعل البوليميراز المتسلسل العكسي متطابقة للمرحلة الحادة.

بعد علاج السيكلوسبورين لاحباط الجهاز المناعي، تم عزل الفيروس من الكلى فقط بعد 12 يوم من علاج السيكلوسبورين وكانت هذه النتيجة مماثلة لتلك الموجودة في النتائج السابقة، بعد علاج السيكلوسبورين، على الرغم من طول التجربة و"الضغوط" المختلفة المطبقة على الطيور، فإن الفيروس الوحيد الذي تم اكتشافه خارج المرحلة الحادة سجل لمرة واحدة بعد بدء علاج السيكلوسبورين، وكان من المأمول أن يكون تثبيط المناعة في الخلايا والحركة والنضج الجنسي كافيين لتحفيز إعادة تنشيط الفيروس، إن عدم القدرة على اكتشاف الفيروس خارج المرحلة الحادة إلا في حالة واحدة يمثل خيبة أمل كبيرة، لعدة أسباب. استغرقت هذه الدراسة عدة أسابيع، وتضمنت معالجة شاقة للعديد من العينات والمراقبة اليومية للطيور.

**الكلمات المفتاحية:** *عزل الفيروس، سلسلة التفاعل البلمري، المقايسة الامتصاصية المناعية للإنزيم المرتبط، السيكلوسبورين، الجهاز المناعي لدى الطيور.*

**Abstract:**

The sequence analysis of the spike protein gene of virus detected in the kidney at 46 days post-infection was found identical to the original virus, suggesting that that there was no mutation of the infecting virus over that period of time, at least in that part of the genome. To explore this further, in the experiment described in this study, birds were kept for much longer, indeed until after sexual maturity.

Virus isolation and RT-PCR results are shown in Table.1 and.2. Virus was present in the tissues as shown by VI and PCR from expected tissues during the acute phase (days 1-21). VI and RT-PCR results were identical for the acute phase.

After CSA treatment, virus was isolated from kidney only at 12 days post CSP treatment and this result were similar to that found in the previous chapter.

After CSA treatment, virus was isolated from kidney only at 12 days post CSP treatment and this result were similar to that f Despite the length of the experiment and the various 'stresses' applied to the birds, the only virus detected outside the acute phase was one a single occasion after the onset of CSA treatment. It was to be hoped that T-cell immunosuppression, movement and sexual maturity would be sufficient to induce the reactivation of the virus. The inability to detect virus outside the acute phase except on one occasion, represents a huge disappointment, for several reasons.

This study lasted many weeks, involved the laborious processing of numerous samples and the daily monitoring of the birds.

**Keywords**: *VI, RT-PCR, ELISAs, CSA, TOC, Infectious bronchitis virus, IBV, Chicken.*

**Introduction:**

IBV virus could persistent in the kidney of the chicken after infection at day old. Those findings were in agreement with those of Bhattacharjee et al. (1995) and Dhinakar Raj et al. (1997). We also showed that persistent infection can be demonstrated by experimentally inducing re-activation by treating chickens with CSA beyond the acute phase post infection (Bhattacharjee et al., 1995; Dhinakar Raj et al., 1997).

The sequence analysis of the spike protein gene of virus detected in the kidney at 46 days post-infection was found identical to the original virus, suggesting that that there was no mutation of the infecting virus over that period of time, at least in that part of the genome. To explore this further, in the experiment described in this study, birds were kept for much longer, indeed until after sexual maturity.

Virus may remain persistently infected until at least sexual maturity (Jones and Ambali, 1987). In that work, the molecular nature of the virus re-excreted at maturity was not examined. The present study was planned to examine the nature of persistent IBV in chickens infected at day old, until sexual maturity and beyond, with the aim of determining whether persistent virus changes over time in the bird. Additionally, chickens were treated with CSA on more than one occasion.

Hughes et al (1989), working with ILTV, found that rehousing of infected chicks with unfamiliar chicks after the acute phase of the infection had subsided resulted in re-activation of the virus. ILTV is a herpesvirus and as such, becomes latent, but it was considered worthwhile examining the effect of rehousing on non-excreting IBV- infected chickens, to see if this stress could similarly induce viral reactivation. In addition, Hughes et al. (1989) also found that onset of lay caused the re-appearance of infectious virus after infection at eight weeks of age.

Thus in the present study, all three 'stressors' were given, i.e. CSA (more than once), movement and sexual maturity. It was hoped to examine virus excreted at or after sexual maturity to determine if alteration in sequences in the S1 spike gene would indicate mutation after the long persistence.

**Materials and methods**

**Chicks, virus, details of VI, RT-PCR, ELISAs and CSA application**

**1. Chickens**

SPF eggs of white leghorn (WLH) chickens were obtained from a commercial source. They were hatched in our laboratory and the chickens maintained in complete isolation. They were fed and watered *ad-libitum.*

**2. Viruses**

Chicks were inoculated by a combination of intranasally and intraoculary application of 100µl of allantoic fluid containing 4.9 $\log_{10}$ $CID_{50}$ of IBV/ml of the Massachusetts M41strain.

**3. Experimental designs**

Fifty-six one-day old SPF chicks were infected with IBV strain M41. Following infection, samples of trachea, lung, kidney, caecal tonsil, brain, thymus, testes (from males) and duodenum were collected on day 3, 7, 10, 14, 21, 28 and 35 pi for VI in TOC and RT-PCR. Each time, five infected birds and three controls were examined. Twenty-nine chicks were kept as controls. After the birds had recovered fully from the acute phase of the disease, at 5 weeks of age, a course of intramuscular CSA injections (see below) was given, starting from day 34 pi and then, every three days until day 49, in attempt to induce re-excretion of virus.

Samples of trachea, lung, kidney, caecal tonsil, bursa, brain, thymus, and duodenum were collected 12, 15, 18 and 21 days post the onset of CSA treatment from three treated birds and two controls for examination by VI and PCR. Effectively, these were days 46, 49, 52 and 55 days of age.

**4. Chicken embryo tracheal organ culture (TOC)**

These were prepared and used for virus isolation and titration as described by Cook et al. (1976),. The method for preparing TOC was as described by Cook et al. (1976). Briefly, embryos were removed from fertile 19 or 20-day-old SPF eggs and tracheas were collected from them after decapitation. They were freed from excess fat and other tissues and placed in a Petri dish contain warm (37$^{o}$C) TOC medium Tracheas were cut in to 0.6mm thick rings using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd, Gomshall, Surrey, UK) and each ring was placed in a sterile glass tube (Nunc Life Technologies, Paisley, Scotland) containing 0.7ml of TOC medium. Tubes were replaced in a rotating rack and turned at 8 revolutions per hour at 37$^{o}$C.

After 24 hours the rings were checked for ciliary movement. Those showing poor or no ciliary activity were discarded.

**5. Serology**

Blood samples were collected from the brachial veins of 5 chickens from each infected group and from 2 chickens of the control group at 14, 21 and 28 days p.i. to determine the levels of antibodies in serum using ELISA.

**6. Enzyme linked immunosorbent assay (ELISA)**

The indirect ELISA was used to measure anti IBV specific isotopic antibody responses in serum as described by Cook and Hugein (1986). An indirect ELISA was used to measure

anti IBV specific isotypic antibody responses in serum and local secretion as described by Cook and Mockett (1986). ELISA plates were coated with optimally diluted purified IBV antigen in 50mM carbonate-bicarbonate buffer (PH 9.6) overnight at $4^0$C. IBV coated wells were blocked with PBS contain 1% bovine serum albumin. Doubling dilutions of serum or secretion samples were prepared in PBS-contain 0.05% Tween/20) and added to the wells of virus-coated plate for one hour at $37^0$C. After washing, virus specific chicken isotypic antibodies were detected employing optimally diluted isotype-specific murine monoclonal antibodies against chicken IgM, IgG and IgA (kindly provided by Dr T.F. Davision Animal Health Compton Laboratory, Newbury, Berks) for 1 hour at $37^0$C. These were followed by application of goat antimouse IgG peroxidase conjugate (Sigma) for one hour at $37^0$C. Enzymic reaction was visualized by O-phenylendiamine (OPD) as substrate (2.5 mM in 0.1 M carbonate phosphate buffer, PH 5.0 with 0.015% $H_2O_2$). After 10-15 minutes, the reaction was stopped by 50µl of 2.5 N sulphuric acid and plates were read at 490nm in a micro- ELISA reader (Dynatech). Between each individual incubation steps, the ELISA plates were washed each time six times with PBS-Tween20.

Checkerboard titrations were performed to determine optimal dilutions of IBV antigen, monoclonal antibodies against chicken IgG, IgM and IgA and anti-mouse IgG peroxidase conjugate to be used in the test proper. A volume of 50µl /well of reagent was used throughout the test.

**7. Polymerase chain reaction**

The polymerase chain reaction was performed with viral RNA and a pair of oligonucleotides spaced 400 bases apart in a conserved region of the spike protein gene of the infectious bronchitis virus dry trachea swabs or swabs from chopped tissue at autopsy were treated as pools and dipped into 400µl of guanidine isothiocynate (solution D) and 2- mercaptoethanol to lyse cells for each swab. Then, the mixture was vortexed and left to stand at room temperature for 10 seconds.500µl of a 10 parts to 3 of a mixture of phenol and chloroform (pH 7.5) were then added to each tube and the mixture was vortexed vigorously for 10 seconds. The two phases were separated by centrifugation for 5 minutes and the supernatant was transferred to fresh tube containing 400µl of 3M sodium acetate (1:10 volume) and 900ul (volume) of cold absolute alcohol. The tube was vortexed briefly, stored at $-20^o$C overnight and then centrifuged for 5 minutes to pellet precipitated RNA. The supernatant was removed and the pellet air-dried prior to resuspension in 50µl of sterile deionised water. One µl of this solution was used as a template for each PCR reaction.

***RNA extraction*** The pool was centrifuged to extract the fluid, which was frozen overnight. RNA was purified by phenol/chloroform extraction and precipitated overnight at $-20^o$C in isopropanol.

***Reverse transcriptase (R-T)*** The precipitate was washed in ethanol and suspended in water. The reverse-transcriptase step was done using enzyme 'Superscript' (Invitrogen, Life technology, USA) and one outer IBV olignucleotide. SX2- (equimolar mixture of 5' tccacctctataaacacctttac 3' and 5'tccacctctataaacaccttac 3)

*PCR* The first PCR was done with two outer oligos (*SX1+ (equimolar mixture of 5'cacctagaggtttgttagcatg 3' and 5'cacctagaggtttgcttgcatg 3') and SX2- (equimolar mixture of 5' tccacctctataaacacctttac 3' and 5'tccacctctataaacaccttac 3)* and

Taq,(Promega Corporation, USA) followed by a second using two universal oligos for IBV so that any strain could be detected**.**

*Running the gel* Samples were run on an agarose gel for 30 minutes at 90 volts, stained with ethidium bromide for 25 minutes, examined on the transilluminator and photographed

**8. T-cell suppression**

T-cell suppression was induced by the application of CSA 100mg per ml in maize oil (Neoral, Sandoz Ltd, Bordon, Hants, UK). The drug was given intramuscularly into the thigh or breast muscle at a dose of 100mg per kg bodyweight every three days, a dose found satisfactory by Dhinakar Raj and Jones, (1996).

**9. Virus isolation and titration**

Aseptically collected tissues from euthanised birds were processed following the method of Cook et al., (1991) and used for virus isolation and titration in TOC.

**10. Collection and separation mononuclear cells from blood samples**

This was done during this experiment and as a corollary to it, but the results are described in Chapter 8. Peripheral blood samples were collected in heparinized tubes at days 0, 3, 7, 10, 14, 21 and 28-post infection. These samples were used to establish the distribution of lymphocyte subpopulations. Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood samples, by density gradient centrifugation over Ficoll-Paque (Pharmacia, Fin Chemicals, Uppsala, Sweden) as described earlier (Chapter 3) Cell viability was determined by the trypan-blue exclusion technique and the cells were resuspended in RPMI medium to give a concentration of $1x10^7$ viable cells/ml.

Similar samples were also collected pre the first CSA treatment (day 35 pi) and during first post-treatment on day 3,7,10,12, and 15 (effectively days 38, 42, 45, 47 and 50) of age.

**11. Experimental design**

This is detailed in the 'flow diagram in Table.2, parts 1 and 2.

***Acute phase.*** The unsexed day-old chicks were each infected by i/n and i/o routes with 100µl of allantoic fluid containing 4.9 $\log_{10}$ $CD_{50}$ of IBV strain M41. Five birds were killed at intervals between days 3-21 and samples of trachea, lung, kidney, caecal tonsils, bursa, thymus, brain and rectum were collected for virus isolation and swabs for RT-PCR. For both techniques, tissue samples from each occasion were pooled. Appropriate controls were kept.

***First CSA treatment.*** When the birds had recovered from the acute phase of the disease, CSA injections were given from 35 dp.i.every three days. Another group of birds was housed separately and not treated with CSA. For each group, swabs from the trachea and cloaca were collected from birds on days 0, 3, 6 and 9 after the start of CSA, while five birds were killed on each of days 12,15,18 and 21 after the start of treatment. All were examined by VI and RT-PCR.

***Re-housing*** when the birds reach 8 weeks of age, the two infected groups were re-housed in the same pen. Birds in the CSA-treated group were kept on the floor on litter, while the untreated group were housed in the same pen but in a large cage. All birds were swabbed via the trachea and cloaca twice weekly until the second CSA treatment. Examination for virus was done by RT-PCR only on dry tracheal and cloacal swabs.

***Second CSA treatment.*** This was begun at 110 days (15.5 weeks of age and p.i.) and again swabbing was done twice weekly until the third CSA treatment. Examination for virus was done by RT-PCR only on dry tracheal and cloacal swabs only.

***Onset of lay*** Females began laying eggs from 22 weeks of age. At this time, the CSA-treated group contained 8 females and 4 males, while the untreated group contained 12 males. Swabbing was continued through the onset of lay. Examination for virus was done by RT-PCR on dry tracheal and cloacal swabs only.

***Third CSA treatment*** A third course of CSA treatment was given commencing at 29 weeks of age (203days). All birds were swabbed 3, 6 and 9 days later. On the 10th day, all birds were killed and tissues were examined for virus by RT-PCR.

Table.1. Flow chart of the experiment.

| Part 1 | | | |
|---|---|---|---|
| *Day* | *Event* | *Protocols* | |
| 0 | **IBV infection** | 80 chicks Infected with IBV | 25 chicks Uninfected controls |
| 3-21 | Acute phase | Tissues sampled for VI and PCR | |
| 35 [5.wk] | First CSA | *separated* | |
| | | **CSA treated** | **Untreated** |
| | | 24 birds every 3d for 15d | 24 birds |
| | | All swabbed via trachea and cloaca up to 15d post onset of CSA treatment | |
| 56 | Re-housing | Re-housed in the same pen [8wk] | |
| | | **CSA** Caged | **Untreated** on litter |
| | | All swabbed via trachea and cloaca twice weekly. | |

Table. 2. Flow chart of the experiment (/continued).

| Part 2 | | | |
|---|---|---|---|
| *Day* | *Event* | *Protocols* | |
| 110 [15.5wk] | Second CSA | **CSA** every 3d for 15d | **Untreated** |
| | | All swabbed via trachea and cloaca up to 15d post onset of CSA treatment | |
| 154 | Onset of lay | Twice weekly swabbing continued [22 wk approx] | |
| 203 [29wk] | Third CSA | **CSA** every 3d for 15d | **Untreated** |
| | | All swabbed via trachea and cloaca on 3, 6 and 9 days post onset of CSA treatment. | |
| 213 | End of experiment | All killed: tissues examined by VI and PCR [30.5wk] | |

**Results:**

**1. Clinical signs**

Typical IBV signs were observed in infected birds. The injection of CSA every third day was not associated with any overt toxicity in inoculated chickens. All the CSA- treated birds were clinically normal and no mortality was seen. The only significant signs observed were yellowish diarrhoea and cessation of egg production during the CSA treatment in lay.

**2. Post mortem lesions**

During the acute phase the carcasses of the birds showed expected lesions. After treatment with CSA the first lesion observed was watery diarrhoea and very congested intestine. When the birds reach sexual maturity, initially, egg production was good but after treatment with CSA, egg production stopped and at post mortem, the ovaries were found to be atrophied.

**3. IBV detection in the tissues**

**Acute phase and first CSA treatment**

Virus isolation and RT-PCR results are shown in Table.1 and.2. Virus was present in the tissues as shown by VI and PCR from expected tissues during the acute phase (days 1-21). VI and RT-PCR results were identical for the acute phase and similar to results given in Chapter 6.

After CSA treatment, virus was isolated from lkidney only at 12 days post CSP treatment and this result were similar to that found in the previous chapter.

Table.3 Virus detection by VI during the acute and first CSA phase.

| | Days post infection | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Tissue | 3 | 7 | 10 | 14 | 21-35* | 46 | 49 | 52 |
| Trac hea | 2[a] | 2 2 | 0[b] | 0 | 0 | 0 | 0 | 0 |
| Lung | 2 | 2 | 2 | 0 | 0 | 3 | 0 | 0 |
| Kidney | 3 | 2 | 2 | 0 | 0 | 3 | 0 | 0 |
| B. of Fabricius | 3 | 3 | 3 | 0 | 0 | 0 | 0 | 0 |
| Thymus | 3 | 3 | 3 | 0 | 0 | 0 | 0 | 0 |
| Brain | 3 | 3 | 3 | 0 | 0 | 0 | 0 | 0 |
| Rectum | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 |

[a]: No of passages before a sample found positive; [b]: negative

*CSA treatment started at 35 days post infection

Table.4 Virus detection by RT-PCR during the acute and first CSA phase.

| Tissue | Days post infection 3 | 7 | 10 | 14 | 21-35* | 46 | 49 | 52 |
|---|---|---|---|---|---|---|---|---|
| Trachea | +[a] | + | + | _[b] | – | – | – | – |
| Lung | + | + | + | – | – | + | – | – |
| Kidney | + | + | + | – | – | + | – | – |
| B. of Fabricius | + | + | + | – | – | – | – | – |
| Thymus | + | + | + | – | – | – | – | – |
| Brain | + | + | + | – | – | – | – | – |
| Rectum | + | + | + | – | – | – | – | – |

[a]: positive sample; [b]: negative

*CSA treatment started at 35 days post infection

**Sequence of the S1 Spike gene of virus detected on day 46**

This was found to be identical to that of the original inoculated virus,

**Rehousing, Second CSA treatment, Onset of lay, Third CSA treatment, End of the experiment**

No virus was detected by RT-PCR in any swabs or tissues from any of the groups tested after the above treatments.

**4. Serology**

The results of ELISAs for IgM and IgG from sera collected during the acute phase and after treatment with CSA are shown in Figures 7.1 and 7.2.

Fig. 1. Depicts the IBV-specific IgM in sera. IgM titres were generally lower in birds treated with CSA compared to untreated chickens.

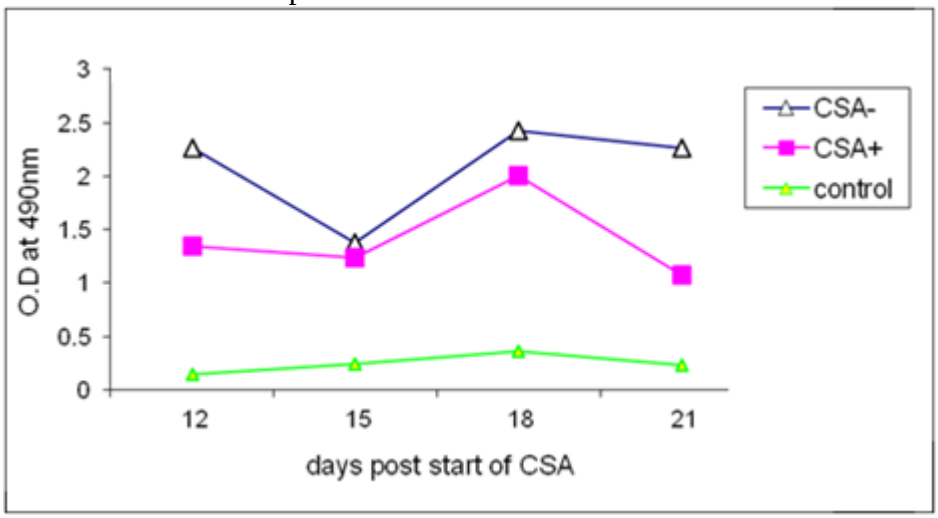


Figure.1. IBV-specific IgM in sera of chickens infected with IBV M41 at day old and (i) treated with CSA (CSA+) or (ii) not treated (CSA-).

Fig.2. Depicts the IBV-specific IgG profile. in the serum. IgG levels were generally lower in CSA treated birds, apart from day 14, where they were the same.

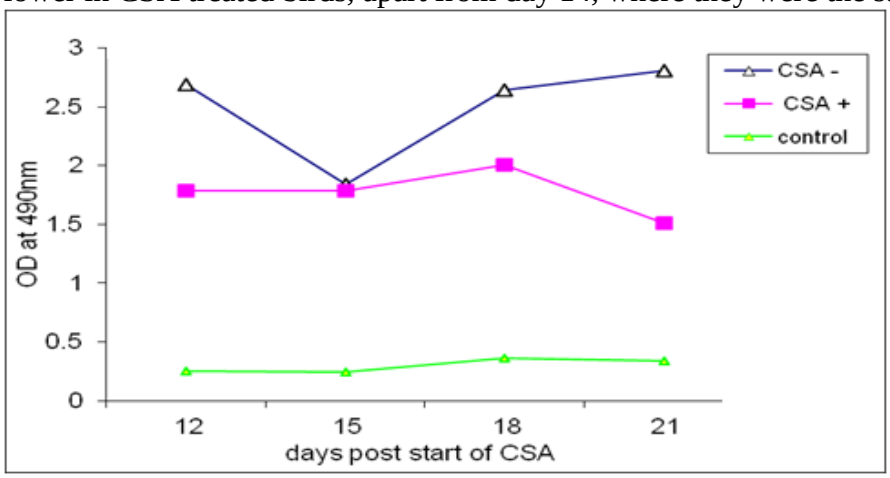


Fig. 2. IBV-specific IgG in the serum of chickens infected with IBV M41 at day old and (i) treated with CSA (CSA+) or (ii) not treated (CSA-).

**Discussion:**

Despite the length of the experiment and the various 'stresses' applied to the birds, the only virus detected outside the acute phase was one a single occasion after the onset of CSA treatment. It was to be hoped that T-cell immunosuppression, movement and sexual maturity would be sufficient to induce the reactivation of the virus. The inability to detect virus outside the acute phase except on one occasion, represents a huge disappointment, for several reasons.

This study lasted many weeks, involved the laborious processing of numerous samples and the daily monitoring of the birds. Unfortunately, it was not possible to undertake a repeat, because of problems with the Isolation Facility of the Department and time constrains. Beyond the first CSA stage, in view of the excellent correlation between VI and RT-PCR, it was decided to use RT-PCR only. In retrospect, it would have been valuable to use VI also and this could still be done on the samples.

To add to the frustration, after this experiment was near completion, a paper was published by Naqi et al. (2003) in the American journal Avian Diseases. This paper effectively described similar experimental work to that described here, but virus was detected with ease for several weeks after infection of chicks, without the need for immunosuppression or stress factors. In the first of two experiments, intranasal inoculation of 6-week old antibody-free chickens with IBV vaccine resulted in intermittent shedding of virus from both tracheas and cloacas of individuals for up to 63 days. Also, virus was recovered from internal organs (spleen, gonad, kidney, lung, caecal tonsil and bursa) of 6/8 birds killed at intervals between 27 and 163 days (23 weeks) post inoculation. In the second experiment, exposure of antibody-positive chicks lead to periodic shedding from the trachea and cloaca of all chickens. However, antibody appeared to protect the internal organs, since fewer isolations were made in these birds at 175 days pi.

Interestingly, reisolated viruses from the later stages of the latter experiment were compared with the original virus for their S1 spike gene sequences. They were found to be the identical. This result is in accord with our findings, albeit over a shorter interval,

suggesting that unlike mammalian coronaviruses, the propensity for frequent genetic change may not be inherent in the IBV genome and such change is probably not associated with long-term persistence.

This finding contrasts with the situation with some mammalian coronaviruses. For example in mice infected with murine hepatitis virus, a number of variants with deletions in the S gene have been isolated from persistently infected animals (Rowe et al., 1997). In that study, 11/20 mice had spike deletion variants. On the other hand, in a study of feline coronavirus infections, Addie et al. (2003) found that the consensus partial S sequence of isolates recovered from persistently infected cats at time intervals spanning years was generally conserved.

Despite the successful results of Naqi et al. (2003), these authors did not address the subject of the site of persistence nor did they refer to the Dhinakar Raj and Jones (1997) paper, which suggested that the kidney and not the caecal tonsils are the true site of persistence of IBV. Naqi et al. (2003) also did not examine the late recovered virus for virulence. It was hoped that these aspects could have been investigated here. It was originally planned that tissues or swabs that proved positive by RT-PCR would be re-tested by isolation. In practice, all samples beyond the first CSA treatment were negative by RT-PCR but there was no time to re-examine them by isolation. This could still be done, although previously, RT-PCR has proved as sensitive as isolation for detection in the earlier work described in this thesis.

The site or sites and nature of IBV persistence and the pathogenic capability of re-isolated virus after long persistence are still important aspects to be investigated.

It is worth considering the reasons why the present study was not successful in detecting virus while that of Naqi et al. (2003) was. A number of possible variables can be considered.

*1.Viruses.* The American paper used Massachusetts vaccine strains. Alexander and Gough (1977) had found persistence with Massachusetts vaccine H120. A virulent strain, M41 as was chosen for use here, since this had proved capable of persistence previously. Doses of virus and routes of infection in the two works were virtually identical (approximately 5.0 $\log_{10}$ $EID_{50}$ and ocular or oculo-nasal).

*2.Chicks.* White leghorns, either antibody free or with maternal antibodies to IBV were used in the published report and white SPF leghorns were used here. The antibody-free birds probably had similar susceptibility to ours to IBV.

*3. Age at infection.* In the American paper, 6-week old antibody free or 1 day old antibody positive chicks were used. Previous work in this department indicated that age at infection could be important and that day old infection was likely to provide greatest success (Dhinakar Raj and Jones, 1997). Persistence of IBV was not found when chicks were infected at two weeks old. This discrepancy cannot easily be explained.

*4.Virus detection.* TOC were used here during the acute phase and the first CSA treatment. In the American paper, repeated passages in fertile eggs were used throughout. In some instances, eggs seem more sensitive, but not others. In this thesis work, RT-PCR was used for all attempted virus identification after the first CSA phase. In the Dhinakar Raj and Jones (1997) work, isolation in eggs was done and virus was then only detected briefly after CSA treatment. Jones and Ambali (1987) used eggs and detected virus in all

birds after sexual maturity. Thus, the difference in IBV detection methods is a possible explanation for the discrepancies in isolation rates.

Nonetheless, the striking aspect of the American work is the apparent ease and frequency of isolation, compared to our results. Given time, the samples from this experiment could still be examined by egg passage, but is would be a long time- consuming process, and there would be no guarantee of success.

Since this long experiment can be considered to have been unsuccessful in achieving the aims as originally set out, and with no time to repeat it, chapters 9 and 10 in this thesis cover different aspects of IB.

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