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An ex vivo chicken primary bursal-cell culture model to study IBDV pathogenesis

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May 18th 2018

To whom it may concern,

We have the pleasure of submitting our manuscript entitled:
"An *ex vivo* chicken primary bursal-cell culture model to study IBDV pathogenesis".

Here, we describe the isolation of chicken primary bursal cells from the bursa of Fabricius, the culture and infection of the cells with IBDV, and quantification of viral replication. This model has the potential to expand our understanding of how avian B-cell tropic viruses interact with their host cells while reducing the number of birds used in i*n vivo* infection studies. The techniques can be applied to multiple lymphoid organs, multiple viruses and potentially multiple species of birds, making it an attractive model that can contribute to the avian virology and immunology fields.

We hope that the manuscript will make a valuable addition to your journal and look forward to hearing from you in due course.

Yours faithfully,

Andrew Broadbent
MA VetMB MSc PhD MRCVS

Research Fellow
The Pirbright Institute

TITLE:

An *ex vivo* Chicken Primary Bursal-cell Culture Model to Study Infectious Bursal Disease Virus Pathogenesis

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KEYWORDS:

Chicken, primary cells, bursa of Fabricius, B cells, virus, infectious bursal disease virus, IBDV

SUMMARY:

Here we describe the isolation of chicken primary bursal cells from the bursa of Fabricius, the culture and infection of the cells with infectious bursal disease virus, and the quantification of viral replication.

ABSTRACT:

Infectious bursal disease virus (IBDV) is a birnavirus of economic importance to the poultry industry. The virus infects B cells, causing morbidity, mortality, and immunosuppression in infected birds. In this study, we describe the isolation of chicken primary bursal cells from the bursa of Fabricius, the culture and infection of the cells with IBDV, and the quantification of viral replication. The addition of chicken CD40 ligand significantly increased cell proliferation fourfold over six days of culture and significantly enhanced cell viability. Two strains of IBDV, a cell-culture adapted strain, D78, and a very virulent strain, UK661, replicated well in the *ex vivo* cell cultures. This model will be of use in determining how cells respond to IBDV infection and will permit a reduction in the number of infected birds used in IBDV pathogenesis studies. The model can also be expanded to include other viruses and could be applied to different species of birds.

INTRODUCTION:

The global poultry industry is essential to secure enough food for an expanding human population. However, immunosuppression is the threat to food security and the welfare of

affected birds and represents a key economic challenge to the industry. The majority of cases of immunosuppression in chickens are caused by the infection with immunosuppressive viruses, with those most responsible for impairing acquired immunity having a tropism for T and B lymphocytes[1]. In birds, the majority of B cells are located within an organ known as the bursa of Fabricius (BF). B cells are susceptible to infection with several immunosuppressive viruses, including those that cause lysis, such as IBDV and Marek's disease virus (MDV), and those that cause transformation, such as avian leukosis virus (ALV) and reticuloendotheliosis virus (REV).

In order to develop better strategies for controlling these infections, it is essential to characterize the interaction of the viruses with chicken B cells. However, when B cells are removed from a bird, they do not survive well in *ex vivo* culture[2], making it difficult to perform a thorough analysis of the interactions of IBDV with chicken B cells, or the early events following ALV or REV infection. Consequently, many host cell-virus interactions have been studied *in vivo*[3-10]. Although these studies are informative, they involve the use of infected birds which suffer from diseases that can be severe.

The CD40 ligand is a molecule that induces B cell proliferation[11]. Following identification of the gene encoding chicken CD40L (chCD40L), a soluble fusion protein was engineered that, when added to the culture media, induced the proliferation of chicken primary B cells *ex vivo*[12]. In 2015, B cells cultured in this fashion were found to support the replication of MDV[2] and in 2017, we and others found that primary bursal cells stimulated with chCD40L could be used as a model to study IBDV replication[13,14]. Here, we describe the isolation and culture of chicken primary bursal cells, the infection of the cells with IBDV, and the quantification of viral replication. Although we describe the method in the context of the BF, this could be applied to isolate and culture cells from different lymphoid organs.

PROTOCOL:

All procedures with animals must be ethically approved in advance. In our institution, all procedures are performed in accordance with the UK Animal (Scientific Procedures) Act 1986 under Home Office Establishment, Personal and Project licenses, after the approval of the internal Animal Welfare and Ethical Review Board (AWERB).

1. Preparation of chCD40L

1.1 Culture HEK 293-msCD8-CD40L cells that stably express a soluble chCD40L construct in 1x Roswell Park Memorial Institute (RPMI) medium supplemented with 10% heat-inactivated (hi) fetal calf serum (FCS) and 1 µg/mL puromycin at 37 °C in an atmosphere containing 5% CO_2.

Note: These cells are available from The Pirbright Institute following the signing of the appropriate material transfer agreement.

1.2 Split the cells at a 1:5 density when confluent. Collect the supernatant (containing the soluble chCD40L construct) each time the cells are split, centrifuge it at 400 x *g* for 5 min to remove

cellular debris, and store it at 4 °C.

1.3 When 500 mL of the supernatant has been collected, pool the liquid and filter-sterilize it through a 0.2-µm filter.

1.4 Concentrate the supernatant using centrifugal protein concentrators with a molecular-weight cutoff of 10 K according to the manufacturer's instructions. Extract the concentrated supernatant from each column, pool it together, and filter-sterilize it by passing it through a 0.22-µm syringe filter.

1.5 Determine the final concentration to be used in experiments by serially diluting the chCD40L solution in 1x Iscove's modified Dulbecco's medium (IMDM) (described in step 2.4) and culturing primary bursal cells in the presence of the dilutions. Determine the number and percentage viability of the cells daily for up to a week.

Note: The lowest concentration where cell proliferation and viability are adequate is the concentration to use in the assay. This is likely to be between 1:20 and 1:50.

2. Preparation of Solutions for Chicken Primary Bursal Cell Isolation

2.1 Prepare 1x Hanks' balanced salt solution (HBBS) with calcium (Ca) by adding 10 mL of 10x HBBS with Ca to 90 mL of sterile H_2O and 0.47 µL of 7.5% $NaHCO_3$.

2.2 Prepare collagenase D stock solution at 8 mg/mL in 1x HBBS with Ca. Filter-sterilize the solution through a 0.2-µM filter.

Note: It is advisable to prepare 5-mL aliquots and freeze them at -20 °C.

2.3 Prepare 1x RPMI medium supplemented with 5% hi FCS. Store the media at 4 °C.

2.4 Prepare 1x 500 mL of IMDM supplemented with 8% hi FCS, 2% hi chicken serum, 50 mM β-mercaptoethanol, 50 µL of insulin-transferrin-sodium-selenite, and 1% penicillin/streptomycin. Store the media at 4 °C.

Note: Prepare all the above-mentioned solutions in advance.

2.5 Prepare 1x HBBS with Ca. Store the solution on ice.

2.6 Prepare 1x HBBS without Ca by adding 10 mL of 10x HBBS without Ca to 90 mL of sterile H_2O, 0.47 µL of 7.5% $NaHCO_3$, and EDTA at a final concentration of 10 mM. Store the solution on ice.

2.7 Prepare 1x collagenase D solution by adding 5 mL of collagenase D stock solution to 13 mL of HBBS with Ca to make a total of 18 mL. Store the solution on ice.

Note: Prepare the solutions mentioned in steps 2.5 - 2.7 on the day of the experiment.

3. Removal of the Bursa of Fabricius (BF)

3.1 Rear and hatch chickens in an appropriate, approved facility and humanely cull them at 2 - 3 weeks of age.

Note: Use institute-approved methods for culling.

3.2 Collect the BF from each chicken using aseptic techniques.

Note: Use the protocols in place at the institution.

3.2.1 Place the carcass in dorsal recumbency and sterilize the skin and feathers overlaying the abdomen and thorax with a solution of 70% ethanol, diluted in water.

3.2.2 Make a ventral midline incision in the lower abdomen using a sterilized scalpel or scissors.

3.2.3 Locate the bursa of Fabricius, which is connected to the caudal section of the colon, cranial to the cloaca.

3.2.4 Using sterilized forceps and scissors, cut the bursa of Fabricius free from the colon. Take care to avoid puncturing the gut.

3.3 Place the organ in cold PBS and transfer it to the laboratory on ice.

Note: Primary cells should be isolated as soon as possible after the organ harvest.

4. Isolation of Chicken Primary Bursal Cells

4.1 Working in a microbiological safety cabinet, wash the BF at least 3x in 30 mL of cold PBS.

4.2 Transfer the tissue to a Petri dish (92 mm in diameter, 21 mm in height) and add 5 mL of 1x collagenase D solution.

4.3 Using sterile scissors or a scalpel blade, cut the BF into pieces of less than 5 mm in diameter.

4.4 Incubate the tissue at 37 °C with periodic gentle agitation for 30 min.

Note: The collagenase solution will begin to digest the tissue.

4.5 Using a sterile Pasteur pipette, repeatedly aspirate the mixture to encourage disintegration of the tissue. If necessary, cut the tissue into smaller pieces.

4.6 Add another 5 mL of 1x collagenase D solution to the tissue and incubate it at 37 °C with periodic gentle agitation for another 30 min.

4.7 Repeat steps 4.6 and 4.7 until the tissue is completely digested.

Note: There will be small granules that do not dissolve further.

4.8 Pass the cell suspension through a 100-µm cell strainer into 20 mL of 1x HBBS without Ca.

4.9 Centrifuge the cell suspension at 400 x *g* for 5 min.

4.10 Discard the supernatant and resuspend the pellet in 10 mL of 1x RPMI with 5% FCS.

4.11 Either overlay 10 mL of the cell suspension on top of 5 mL of density gradient media containing polysucrose and sodium diatrizoate or underlay 5 mL of density gradient media beneath the 10-mL cell suspension. Ensure there is a clear interface between the two.

4.12 Centrifuge the overlay at 900 x *g* for 20 min at 4 °C. Lower or remove the centrifuge break.

Note: The cells should form a band at the interface between the cell media and the density gradient media.

4.13 Using a sterile Pasteur pipette, remove the cells and place them in cold PBS. Wash the cells 3x by centrifuging them at 400 x *g* for 5 min and resuspending them in cold PBS.

4. Culture of Chicken Primary Bursal Cells

4.1 Centrifuge the cell suspension at 400 x *g* for 5 min and resuspend them in 1x complete IMDM.

4.2 Take an aliquot of the cell suspension, add it to a Trypan blue solution and count the number of viable cells that exclude the Trypan blue. Determine the number of cells and the percentage viability.

4.3 Centrifuge the cell suspension at 400 x *g* for 5 min and resuspend it in complete IMDM supplemented with a 1:20 dilution of chicken CD40L at a density of 1 x 10^7 cells/mL. Titrate the concentrated supernatant containing the chCD40L to determine the optimal dilution, which is likely to lie in the range of 1:10 to 1:50.

4.4 Culture the cells in either 96- or 24-well plates at 37 °C for 48 - 72 h. U-bottomed 96-well plates are preferable to flat-bottomed plates.

5. Infection of Chicken Primary Bursal Cells with IBDV

5.1 48 - 72 h post-isolation, thaw an aliquot of the virus, vortex the sample, and store it on ice.

5.2 Resuspend the primary bursal cells, take a 10-µL aliquot of the cell suspension, add it to 10 µL of a Trypan blue solution, and determine the number of cells and percentage viability.

5.3 Dilute the virus in 1x complete IMDM to the appropriate multiplicity of infection (MOI) to make the virus inoculum and vortex.

5.4 Centrifuge the cell suspension at 400 x *g* for 5 min.

5.5 Remove the supernatant and resuspend the cells in the virus inoculum.

5.6 Incubate the cell suspension at 37 °C for 1 h with periodic agitation.

5.7 Centrifuge the cell suspension at 400 x *g* for 5 min, remove the virus inoculum, and wash the cells in 1x complete IMDM media.

5.8 Centrifuge the cell suspension at 400 x *g* for 5 min, remove the supernatant, and resuspend the cells in complete IMDM media supplemented with chicken CD40L at a density of 1 x 10^7 cells per mL.

5.9 Culture the cells in either 96- or 24-well plates at 37 °C.

6. Quantification of IBDV Replication in Chicken Primary Bursal Cells

6.1 At the desired time-point postinfection, resuspend the cells, transfer them to an appropriate tube, centrifuge them at 400 x *g* for 5 min and harvest the supernatant for virus titration by either plaque assay or $TCID_{50}$ assay as per the Reed-Muench method[15].

6.2 Wash the cells in 1 mL of PBS and prepare them either for immunostaining with an antibody specific to IBDV, or extract RNA using an appropriate kit (following the manufacturer's instructions) and perform reverse transcription quantitative polymerase chain reaction (RT-qPCR) using primers specific for an IBDV gene (Forward, GAGGTGGCCGACCTCAACT; Reverse, GCCCGGATTATGTCTTTGAAG). Mock-infected cell cultures should be used as a control.

REPRESENTATIVE RESULTS:

Chicken Primary Bursal Cells Can Be Cultured in the Presence of Chicken CD40L

When chicken primary bursal cells were cultured in the presence of soluble chCD40L, the number of cells increased fourfold from 9.02 x 10^5 to 3.63 x 10^6 per mL over a period of 6 days, in contrast to when it was absent ($p < 0.05$) (**Figure 1A**). Cell viability was also significantly improved, for example from 25% at day 3 post-culture in the absence of chCD40L to 48% in the presence of chCD40L ($p < 0.05$) (**Figure 1B**)[13].

Chicken Primary Bursal Cells Can Support the Replication of Both Cell-culture Adapted and Very

Virulent Strains of IBDV

Mock-infected and infected cell cultures were fixed 18 hours postinfection, labeled with a monoclonal antibody against IBDV VP2 and a secondary antibody conjugated to Alexa Fluor 488, and counterstained with DAPI. Infected cells had evidence of green fluorescence around the nucleus (**Figure 2A**), consistent with the presence of IBDV in the cytoplasm. This was evident for two strains of IBDV, a cell-culture adapted strain, D78, and a very virulent strain, UK661 (**Figure 2A**). At 5, 18, 24, and 48 hours postinfection, RNA was extracted from infected cultures and subjected to RT-qPCR with primers specific to a conserved region of the IBDV VP4 gene. The expression of VP4 was first normalized to the house-keeping gene TBP and then expressed as fold change relative to mock samples in a ΔΔCt analysis. IBDV VP4 expression increased to 16,603 copies at 48 hours postinfection with D78 and 38,632 copies at 48 hours postinfection with UK661. Taken together, these data demonstrate that the chicken primary bursal cells could support the replication of cell-culture-adapted and very virulent IBDV strains[13].

FIGURE AND TABLE LEGENDS:

Figure 1: Chicken primary bursal cells can be cultured in the presence of chicken CD40L. Chicken primary bursal cells were cultured in the presence or absence of chCD40L (black bars and white bars, respectively). (**A**) The number of live cells and (**B**) the percentage of viable cells were determined at the indicated time-points postinfection. The data shown are representative of at least three replicate experiments, the error bars represent the standard deviation of the mean, and the statistical significance was determined using a paired Student's *t*-test at each time-point, * $p < 0.05$. This figure has been modified with permission from Dulwich *et al.*[13].

Figure 2: Chicken primary bursal cells can support the replication of both cell-culture adapted and very virulent strains of IBDV. (**A**) Chicken primary bursal cells were mock-infected or infected with either D78 or UK661 and a sample from each culture was fixed, labeled and imaged: IBDV VP2, green; nuclei, blue. The scale bar is 7 µm. (**B**) RNA was extracted at the indicated time-points postinfection, reverse-transcribed, and a conserved region of the IBDV VP4 gene was amplified by quantitative PCR. The $\log_{10}$ fold change in VP4 copy number was normalized to the TBP housekeeping gene and expressed relative to mock-infected samples as per the $2^{-\Delta\Delta CT}$ method. The data shown are representative of at least three replicate experiments, and the error bars represent the standard deviation of the mean. This figure has been modified with permission from Dulwich *et al.*[13].

DISCUSSION:

In this study, we describe the successful culture of chicken primary bursal cells *ex vivo* in the presence of soluble chCD40L and demonstrate that these cells can support the replication of an attenuated strain and a very virulent strain of IBDV. This *ex vivo* model can be used to determine how the cells respond to an IBDV infection[13], which has distinct advantages over *in vivo* and *in vitro* studies.

When harvesting the BF, it is critical to not puncture the gut so as to avoid bacterial contamination of the isolated bursal cells. In addition, it is important to isolate the primary cells

as soon as possible after the organ harvest to limit cell death. The need to use chCD40L is a limitation of the technique; however, work conducted by Soubies *et al.* shows that the use of phorbol 12-myristate 13-acetate (PMA) to prolong bursal cell viability instead of chCD40L[14] may enable the model to be adopted by a greater number of laboratories. The protocol outlined above determines the optimal concentration of chCD40L empirically, by culturing primary B cells in serially diluted concentrations of the molecule and observing cell proliferation and viability. One potential modification to the protocol could be to purify the chCD40L molecule and to add a specific concentration to the cell culture media to avoid batch-to-batch variability.

In vivo studies have shown that following IBDV infection, there is an increase in the expression of genes involved in pro-inflammatory cytokine responses, Type I IFN responses, and apoptosis in the BF[5,9,10]. However, following infection, there is an influx of inflammatory cells and effector T cells into the BF, which differ in the genes they express compared to the infected B cell population[9]. It is, therefore, difficult to interpret how infected cells respond to IBDV. To address this, some research groups have characterized the transcriptional response of cells infected with IBDV in culture[16-20]. These *in vitro* studies have the advantage of well-defined MOIs and time-points postinfection. However, *in vitro* studies have typically been characterized in either fibroblast cells[16,17,20] or dendritic cells[18]. While providing some insight into host cell-IBDV interactions, the current belief is that the infection of B cells is crucial to the pathogenesis of IBDV and, therefore, the relevance of the data cannot be overinterpreted. Prior to our *ex vivo* bursal cell culture model, only one study had characterized the cellular response of B cells to IBDV infection[19]; however, this study utilized an immortalized B cell line that was transformed due to infection with ALV, limiting the conclusions that could be made. In contrast, the *ex vivo* model of IBDV infection described here allows researchers to retain the advantages of *in vitro* studies, such as defined MOIs and time-points, while studying the interactions of the virus with its relevant host cell. As the primary bursal cells are obtained from uninfected BF tissue, there are no inflammatory or T cells present, and we have demonstrated by flow cytometry (using standard conditions) that, following chCD40L stimulation, 97% of the cell population is positive for the B cell marker Bu-1 (data not shown). Given that 3% of the cells are Bu-1 negative, it will be interesting to determine whether these cells become infected with IBDV and explore their gene expression and contribution to the pathogenesis.

We anticipate that the *ex vivo* chicken primary bursal cell culture model can also be expanded to study the host cell-virus interactions of other B-cell tropic viruses infecting chickens, such as ALV or REV, and could also be expanded to other avian species (*e.g.*, ducks or turkeys). The ability to culture primary bursal cells *ex vivo* also opens up the possibility to study aspects of the pathogenesis and immunosuppression caused by these viruses without the need to infect birds. As *in vivo* studies cause significant morbidity, this will have a substantial impact on the replacement, refinement, and reduction of the use of animals in research.

In summary, the *ex vivo* chicken primary bursal cell culture model described here has the potential to expand the understanding of how avian B-cell tropic viruses interact with their host cells while reducing the number of birds used in *in vivo* infection studies. The techniques can be applied to multiple lymphoid organs, multiple viruses, and, potentially, multiple species of birds,

making it an attractive model that can contribute to the avian virology and immunology fields.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

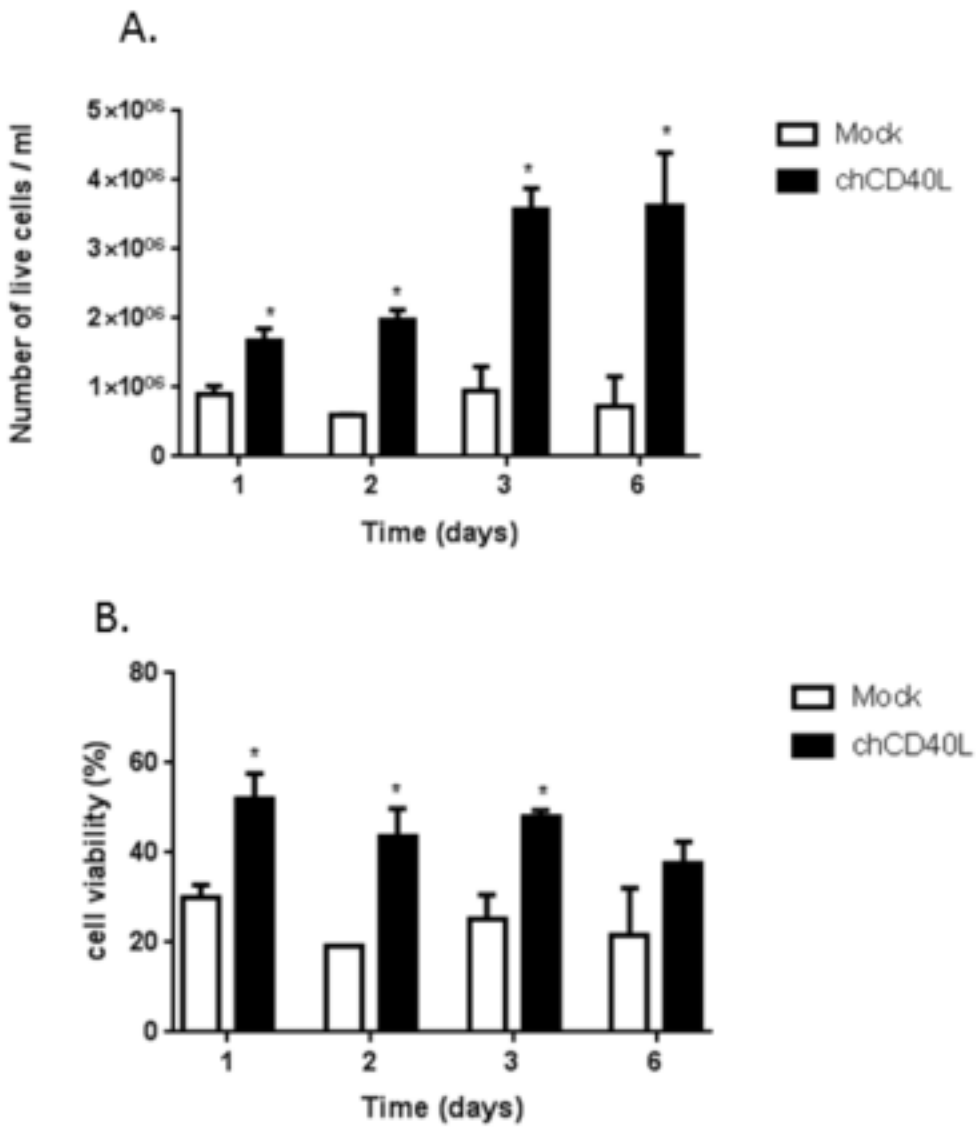
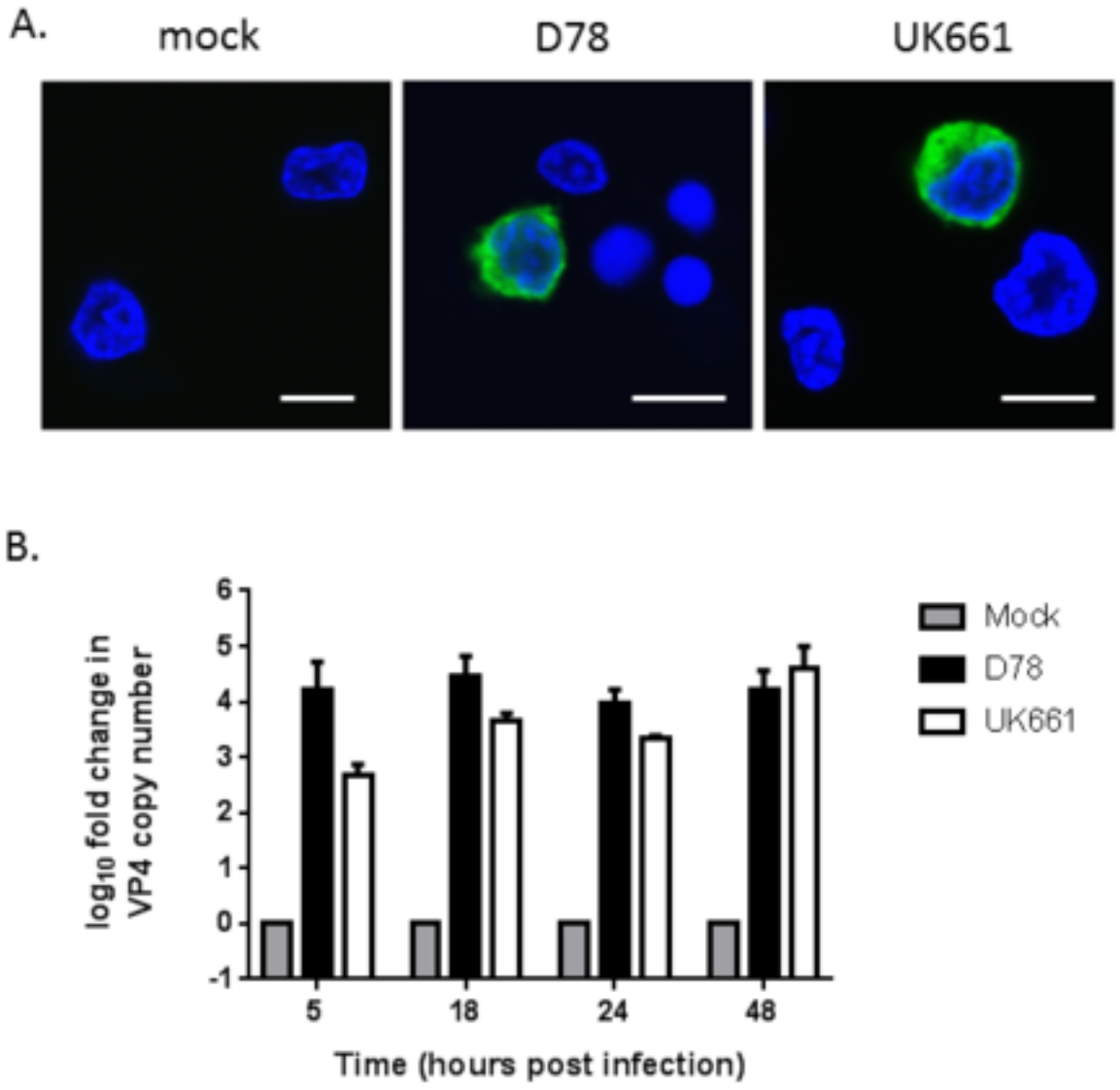


Figure 2



Materials Table

Click here to download Table of Materials JoVE Materials list.xlsx

Name of Material/ Equipment
RPMI-1640 Medium
FBS
Puromycin Dihydrochloride
Nalgene Rapid-Flow Sterile Disposable Filter with a PES membrane
Pierce Protein Concentrator PES (10K MWCO, 20ml)
0.22µm Millex-GP Syringe Filter
Hanks' Balanced Salt Solution (HBBS) + CaCl2 + MgCl2
Hanks' Balanced Salt Solution (HBBS) - CaCl2 - MgCl2
Ethylenediaminetetraacetic acid solution (EDTA)
Iscove's Modified Dulbecco's Medium (IMDM) (1 X) + GlutaMAX
Chicken Serum
2-Mercaptoethanol 50mM
insulin-transferrin-sodium-selenite
Collagenase D
100µm Cell Strainer
Histopaque-1083
Trypan Blue solution
Nunc96 Well-Polystyrene Round Bottom Microwell Plates
Falcon 24-well Clear Flat Bottom TC-treated Multiwell Cell Culture Plate, with Lid, Sterile
Rneasy Mini Kit

Company	Catalog Number	Comments/Description
Merck	R8758-500ML	
gibco by Life Technologies	10099-141	heat-inactivate at 56°C for 1 hour
Thermo Fisher Scientific	A1113802	
Thermo Fisher Scientific	168-0045	
Thermo Fisher Scientific	88528	
Merck	F7648	
gibco by Life Technologies	14060-040	
gibco by Life Technologies	14180-046	
Merck	03690-100ML	
gibco by Life Technologies	31980-030	
Merck	C5405-100ML	
gibco by Life Technologies	31350-010	
gibco by Life Technologies	41400-045	
Roche Diagnostics GmbH	11088882001	
Corning	431752	
Merck	10831-100ML	
Merck	T8154-20ML	
Thermo Fisher Scientific	163320	
Corning	353047	
Qiagen	74104	

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AUTHOR:

Name: ANDREW BROADBENT

Department: Avian Viral Diseases

Institution: The Pirbright Institute

Article Title: An ex vivo chicken primary bursal-cell culture model to study IBDV pathogenesis

Signature: A. J. Broadbent Date: 18/5/18

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We have checked the manuscript for errors.

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We emailed The Journal of General Virology regarding permissions and received the following reply: "The copyright transfer agreement/licence to publish that was signed by the authors gives express permission for authors to reuse their own content, provided that the journal is acknowledged as the original source. No further permission is required." We have uploaded a copy of this email and updated the figure legends.

3. Figure 2B: Please capitalize the first word in the labels on the x axis (i.e., Time).

This has been addressed.

4. Please remove the titles and Figure Legends from the uploaded figures. The information provided in the Figure Legends after the Representative Results is sufficient.

This has been addressed.

5. Please provide an email address for each author.

Katherine L. Dulwich: kate.dulwich@pirbright.ac.uk
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6. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

This has been addressed.

7. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

This has been addressed.

8. Please use centrifugal force (x g) for centrifuge speeds.

This was already the case.

9. Please move the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

This has been addressed.

10. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

This has been addressed.

11. Please revise the protocol to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

This has been addressed.

12. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

This has been addressed.
13. Line 86: Please specify the culture conditions.
This has been addressed.
14. Line 94: Please mention the pore size of the filter.
This has been addressed.
15. Line 100: What is used to titrate the chCD40L solution?
As described, the chCD40L is titrated using primary bursal cells. Briefly, the chCD40L is serially diluted and the cells cultured in the presence of the chCD40L dilutions. The lowest concentration where cell proliferation and viability is adequate should be used in the infection experiments. The text has been modified to clarify this in the protocol and discussion.
16. Lines 129-130: Please add more details to this step. For instance, mention how animals are anesthetized and how proper anesthetization is confirmed. Please specify all surgical instruments used. This step does not have enough detail to replicate as currently written. Alternatively, add references to published material specifying how to perform the protocol action.
No animals are anaesthetized. Animals are humanely culled and tissues removed at necropsy. Describing the culling steps is beyond the scope of our protocol. The labs that are trying to adopt this protocol will already have procedures in place to humanely cull their animals. In addition, legal methods of culling differ from country to country and it would be inappropriate to be prescriptive in our protocol. There are also multiple ways to remove the organ and the lab should use the protocols in place at their institution. The text has been modified to describe one such method if no protocols are in place.
17. Line 131: What volume of PBS is used to wash?
This has been addressed.
18. Line 132: How large is the petri dish?
This has been addressed.
19. Line 134: Please specify the instrument used to cut.
This has been addressed.
20. Line 159: Please specify the volume of cell suspension and Trypan Blue solution used.
This has been addressed.
21. Lines 187-192: Please add more details to your protocol steps. For instance, how are plaque assay or TCID50 assay performed? Please ensure that for all PCR, conditions and primers are listed. Alternatively, add references to published material specifying how to perform the protocol action.
A reference has been added.
22. Please include single-line spaces between all paragraphs, headings, steps, etc. After that, please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
This has been addressed.
23. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
This has been addressed.
24. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I.,

LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.
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25. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.
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Reviewers' comments:

Reviewer #1:
Manuscript Summary:
Dulwich and coworkers describe the isolation of chicken B cells from the bursa of Fabricius for cell culture and infection with Infectious Bursal Disease Virus (IBDV). The protocol will be very useful for the study of primary chicken B cells and studies on several other immunosuppressive chicken viruses that cause major losses in poultry farming. Furthermore, this protocol may prove very useful for the isolation and culture of B cells of other avian species as monoclonal antibodies for positive or negative selection for B cell isolation are largely lacking for other avian species. The title and abstract are succinct. The protocol is detailed with all reagents needed and steps well explained and appears straightforward to allow successful implementation by other groups. The data is convincing with appropriate controls included and findings nicely illustrated.

Major Concerns:
None

Minor Concerns:
None

We thank the reviewer for their comments.

Reviewer #2:
Manuscript Summary:
IBDV is an important immunosuppressive pathogen responsible for the Infectious Bursal Disease(IBD). The very virulent strain of IBDV is still difficult to grow in currently available celllines, which hampers the research progress in pathogenesis of IBDV. The authors describe the isolation of chicken primary bursal cells from the bursa of Fabricius, the culture and infection of the cells with IBDV, and quantification of viral replication. The addition of chicken CD40 ligand significantly increased cell proliferation and enhanced cell viability. Importantly, in additon to D78, a cell-culture adapted strain of IBDV, the very virulent strain UK661 replicated well in the ex vivo cell cultures. This model will be very helpful to the investigators working on the pathogenesis of IBDV. Overall, the article was well and clearly written and very informative to those working in this field. However there is a minor concern to be addressed.

Major Concerns:
No

Minor Concerns:
Lines 100-103. There is still much room in Step 5 for improvement. The purity of CD40L is a potential issue. It would be much better if the quantity of CD40L was more accurately determined with SDS-PAGE using a purified non-relevant protein of similar mass as a standard control for comparison, followed by the measurement of the protein band of interest with densitometry. Thus, the final concentration of CD40L in the culture medium could be calculated as microgram/mL instead of dilution. In this case, the different concentrations of CD40L purified from batch to batch could be properly diluted when CD40L was used in cell culture.

We thank the reviewer for their comments. We agree that the protein could be purified from the supernatant in the manner they describe. This is something that we could do going forward. For our current needs, simply concentrating the supernatant and adding it as a 1:20 - 1:50 dilution to the culture media works adequately.

Reviewer #3:
Manuscript Summary:
The manuscript described an interesting method to isolate chicken primary bursal cells from the bursa of Fabricius and then successfully infected the cells with different virulent IBDVs. The protocols were clearly described. The only concerns is that the origin of the important ingredient "chCD40L" that support the cell growing is complicated to obtain, which means it is still difficult for other researchers to follow the protocol even though the authors noted that phorbol 12-myristate 13-acetate could be used as well instead of chCD40.

We thank the reviewer for their comments. The chCD40L is available to labs upon the signing of an appropriate MTA. However, we have acknowledged the limitation of this in the discussion and provide a reference where PMA is used to stimulate the cells which the reader can try.

Reviewer #4:
Title: An ex vivo chicken primary bursal-cell culture model to study IBDV pathogenesis

Review report
The manuscript is well written and designed and has relevance in the field of infectious diseases in poultry. Following are my concerns that should be addressed in the revised manuscript.
1. Bursal tissue contains heterogeneous cell population. So the characterisation of B-cells should be mentioned properly like markers, antibodies used. Although flowcytometry procedure in the discussion portion (line 271) mentioning the increased B-proliferation by the chCD40L stimulation has been indicated. But should be properly explained.

We thank the reviewer for their comments and we agree that the population of cells is heterogeneous. We have therefore been careful to use the term ‘bursal’ cell and not ‘B’ cell throughout the manuscript. Flow cytometry was used to characterise the cell population before and after chCD40L stimulation, using standard conditions, but we have not yet characterised the infected cell population with cellular markers. When staining with the chicken B cell marker anti-Bu-1 (SouthernBiotech), we found that 97% of our cultures

stained positive following chCD40L stimulation. The text has been adjusted to reflect this. It is also important to determine whether the Bu-1 negative population are infected with IBDV, and we have added this statement to the manuscript text. This work is ongoing in the laboratory.

2. It could have been more informative and conclusive if the primary bursal tissue cells infected with IBDV(untreated) would have been compared with the infected primary bursal tissue cells (treated with chCD40L) to demonstrate specific B-cell infection.

As shown in figure 1, the number of cells is low and the viability poor when the cells are not treated with chCD40L. We did not infect these cultures with IBDV as it would be difficult to make meaningful conclusions as the cell population is dying before infection and may be altering the expression of cellular genes. It might be interesting, however, to do these experiments in the future to determine whether the Bu-1 negative population are infected. However, as we have stated 'bursal' cell population in this manuscript, these experiments are beyond the scope of the current study.

3. Line 57-62 Reference should be incorporated

This text has been removed as a result of revisions

4. Line 76 Indicate type of cell

We have modified the text to state that the cells are primary bursal cells stimulated with chCD40L.

5. Line 99 Provide molecular weight of protein. Also mention how supernatant containing protein is sterilised filtered.

The molecular weight of the protein is 55kDa (see Tregaskes et al., Dev & Comp Imm, 2005). The supernatant from the stably expressing cell-line is first concentrated and then passed through a 0.2 µm syringe filter to ensure no bacterial contamination is present.

6. Line 107-108 indicate amount of calcium

HBSS supplemented with calcium is available to buy from ThermoFisher (cat no: 14060-040), but it does not specify the amount of calcium present in the buffer. We have provided JoVE with a materials list and include the catalogue number to help the reader.

7. Line 113 50ul of insulin-transferin-sodium-slenite in how much amount of media.

This is for one 500ml bottle of IMDM. We have addressed this in the text.

8. Line 140 Check spelling of "gentile"

This has been addressed.

9. Line 142 Correct the sentence that "do no not"

This has been addressed.

10. Line 147 Explain "ensure any missing"

'Ensure any mixing' refers to the fact that the cell suspension should be layered on top of the histopaque. We have removed this from the manuscript to avoid confusion.

11. Line 163 Mention concentration of CD40L

This has been addressed.

12. Line 172 Explain properly which type of cells are resuspended

This has been addressed.

13. Line 174 Mention appropriate MOI

The appropriate MOI will depend on the experiment in question. If a multi-step growth curve is required, a low MOI is recommended. If a single step growth cure is required, a high MOI is recommended.

Vineeta Bajaj <vineeta.bajaj@jove.com>

re: RE: Response Requested: JoVE Submission JoVE58489R1 - [EMID:daf4b1b4b76fa005]

Andrew Broadbent <andrew.broadbent@pirbright.ac.uk> Tue, Jul 24, 2018 at 1:15 PM
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Editorial Office

Journal Of General Virology

From: Andrew Broadbent <andrew.broadbent@pirbright.ac.uk>
Sent: 12 May 2018 18:35
To: JGV
Subject: permisison for use of figures in another manuscript

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Please could you let me know how to proceed?

Many thanks,

Best wishes,

Andrew

--

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