Bovine Endometrial Epithelial and Stromal Cells

Standard Operating Procedures for Isolation and Culture of Primary Bovine Endometrial Epithelial and Stromal Cells

J G Cronin[1*], G D Healey*, S B P MacKintosh*, M L Turner, L L Healy, I M Sheldon

Institute of Life Science, College of Medicine, Swansea University, Singleton Park, Swansea, SA2 8PP, United Kingdom

* These authors contributed equally to the SOP.

[1] E-mail: J.cronin@swansea.ac.uk

1. Introduction

1.1 Scope

This standard operating procedure describes how we carry out the isolation and culture of primary bovine endometrial cells.

When describing this SOP, please cite the **following primary reference**:

Turner ML, Cronin J.G, Healey GD, Sheldon IM, 2014 Epithelial and stromal cells of bovine endometrium have roles in innate immunity and initiate inflammatory responses to bacterial lipopeptides in vitro via Toll-like receptors TLR2, TLR1 and TLR6 Endocrinology, 155:1453-1465

1.2 Background

The isolation and culture of bovine endometrial cells has been described for over fifty years. Founding techniques for the present Standard Operating Procedure developed at Swansea University were:

A. Fortier MA, Guilbault LA, Grasso F 1988 Specific properties of epithelial and stromal cells from the endometrium of cows. J Reprod Fertil 83:239–248

B. Cheng Z, Elmes M, Abayasekera DRE, Wathes DC 2003 Effects of conjugated linoleic acid on prostaglandins produced by cells isolated from maternal intercotyledonary endometrium, fetal allantochorion and amnion in late pregnant ewes. Biochim Biophys Acta 1633:170–178

C. Herath S, Fischer DP, Werling D, Williams EJ, Lilly ST, Dobson H, Bryant CE, Sheldon IM, 2006 Expression and function of Toll-like receptor 4 in the endometrial cells of the uterus. Endocrinology 147:562-570

Broadly speaking the process of isolating endometrial cells involves the mechanical dissection of the endometrium followed by enzymatic digestion of the tissue to yield a homogeneous mixed cell population. The mixed cell population is then 'cleaned' to remove extracellular material before the cells are placed into culture, whereby they can either be kept as a mixed population or separated into pure populations based upon the differential adhesive properties of epithelial and stromal cells.

The techniques described in this SOP have been adapted from isolation and culture techniques refined within Prof Sheldon's lab over the past nine years. The benefits of these techniques are to routinely and consistently produce pure endometrial cell populations for experimental uses. The use of pure cell populations is important for the evaluation of innate immune responses as it enables the influence of effector molecules within a specific cell population to be ascertained.

1.3 Limitations and alternative procedures

All procedures were carried out in compliance with the Animals (Scientific Procedures) Act 1986, and experimental protocols were approved by the local ethics review committee. Bovine uteri were collected at a local abattoir from postpubertal nonpregnant animals with no evidence of genital disease or microbial infection, and the uteri were kept on ice until further processing in the laboratory. Postpartum cattle were not used because experiments would be confounded by ubiquitous bacterial contamination of the uterus, existing endometrial inflammation [25], disruption of the epithelium, and the presence of damage-associated molecular patterns. The stage of the reproductive cycle was determined by observation of ovarian morphology as previously described. Genital tracts were selected for endometrial culture from animals on Days 1–4 of the estrous cycle with an ovarian stage I corpus luteum containing a recently ruptured follicle because in these animals, similar to postpartum cows, peripheral plasma progesterone concentrations are basal.

The limitations of this approach are that the isolation procedure necessarily removes extracellular material present in the endometrium. Pure cell populations will also not be subject to the normal paracrine, autocrine and endocrine interactions between cells that are fundamental to normal function and dependent on the correct spatial arrangement of cells and architecture of tissues. [1, 2, 3, 4].

Usage: This protocol has been used to isolate primary bovine endometrial epithelial and stromal cells for several papers [5-13]. These papers, and others from the group, should be consulted for details of cell purity analyses and cell responses.

Disclaimer: All details were correct at the time of writing this protocol, but need to be checked by the operator. We make no warranty for the procedures. Local approvals for the work need to be sought.

2. Equipment Requirement

Equipment	Company	Cat #
37°C Incubator with 5% CO_2 internal atmosphere	-	-
37°C Shaking water bath	-	-
Sterilin culture tubes (13ml)	SLS	TIS5404
Sterile scissors and forceps	-	-
Pipette Boy	-	-
10 ml Serological pipettes	StarLab	E4860-1011
5 ml Serological pipettes	StarLab	E4860-0511
Pipettes (P1000, P200 & P20)	-	-
1000 µl pipette tips	StarLab	S1126-7810
200 µl pipette tips	StarLab	S1120-8810
20 µl pipette tips	StarLab	S1120-1810
Sterile 3 ml Pasteur pipettes	SLS	PIP4210
60 ml sterile container	SLS	SLS-SLS7540
Skirted 50 ml centrifuge tubes	SLS	TUB0208
Parafilm	SLS	FIL1022
250 ml Vacuum-pack filter unit	Helena Biosciences	9925OT
70 µm cell strainers	VWR	734-0003
40 µm cell strainers	VWR	734-0002
24-well tissue culture plates	Helena Biosciences	92024T
75 cm^2 filter-cap tissue culture flasks	Greiner Bio-one	658975
Haemacytometer	ISL	BVS100
Class II biological safety cabinet	-	-
Aspirator	-	-
Microscope	-	-
Centrifuge	-	-

3. Reagent Requirement

Equipment	Company	Cat #
RPMI 1640	Sigma	R8758
Foetal bovine serum, heat inactivated (FBS)	Biosera	S1810
Penicillin / Streptomycin solution (Pen/Strep)	Sigma	P4458
Amphotericin B	Sigma	A2942
70% Ethanol	-	-
Sterile water	Sigma	W3500
Hanks balanced salt solution (HBSS)	Sigma	H6648
Dulbecco's Phosphate buffered saline (D-PBS)	Sigma	D8537
Collagenase II	Sigma	C6885
Bovine serum albumin (BSA)	Sigma	A7906
4% DNAse I	Sigma	DN25
Trypsin EDTA	Sigma	T4049
Trypan blue	Sigma	T8154
Virkon	VWR	148-0200
Medis	NVS	MD307
Accutase	PAA laboratories	L11-007

4. Reagent Preparation

Reagents that are supplied in bulk (e.g. 500 ml) should be divided into aliquots of the required volume so that freeze-thawing is avoided. Once reagents have been aliquoted they should be frozen and stored at -20°C until required. Care should be taken to ensure that reagents are aliquoted into vessels that have sufficient room for liquid expansion upon freezing. Ensure FBS is completely thawed and is mixed thoroughly prior to aliquoting.

Reagent	Constituents
Complete Media	• 500 ml RPMI 1640 • 50 ml FBS • 5 ml Pen / Strep • 5 ml Amphotericin B
Endo Wash	• 1000 ml D-PBS • 10 ml Pen / Strep • 10 ml Amphotericin B
Endo Strip Wash	• 500 ml HBSS • 5 ml Pen / Strep • 5 ml Amphotericin B
Digestive Solution [note 1]	• 50 ml HBSS • 25 mg Collagenase II • 50 mg BSA • 125 µl 4% DNAse I • 375 BAEE units Trypsin EDTA
Stop Solution	• 500 ml HBSS • 50 ml FBS
Note 1	To prepare 4% DNAse I, add 2.5 ml of DEPC treated water to 100 mg of DNAse I. Filter sterilise before aliquotting into sterile vials and storing at -20°C. Prepare the digestive solution (omitting the Collagenase II and DNAse I no more than 24 h in advance and store at 4°C. Prior to use, add the Collagenase II, filter sterilise, and then add the DNAse I.

5. Procedure

5.1 Preparation

1. Ensure all solutions are prepared and ready for use and required equipment is available.

2. All procedures should be performed using good aseptic and tissue culture technique to prevent contamination of cultured cells.

3. Clean a suitable work surface with 70% ethanol.

4. Secure aluminium foil over the work surface, spray with 70% ethanol and wipe down.

5. For each uterus, prepare two 60 ml pots, one containing 25ml of **endo strip wash** and the other 25 ml HBSS; and a 50 ml centrifuge tube containing 25 ml HBSS and label.

6. Half fill a 200 ml beaker with 70% ethanol for use during dissection.

5.2 Staging

1. Identify the stage of oestrus cycle by examining the ovaries as described. During the luteal phase the CL has a purple colouration and is vascularised with a protruding red spot representing the site of the most recent ovulation [4].
2. Different stages can be used for evaluation, and this needs to be stated in the paper [5-13].

5.3 Tissue dissection (see section 7 for images of procedures)

1. Maintain the sterility of the instruments (forceps and scissors) throughout the procedure by swirling in the 70% ethanol and shaking off any excess before continuing with the dissection.

2. Wash the uterus, specifically the ipsilateral horn, with 70% ethanol.

3. Make a transverse cut across the horn, immediately above the intercornual ligament.

4. Open up the ipsilateral horn by making an incomplete transverse cut into the centre of the horn, directly above the intercornual ligament. Then feed the scissors into the horn's exposed lumen and cut longitudinally towards the ovary.

5. Open out the horn and clean the endometrium with **endo wash**.

6. Using tissue forceps to keep the endometrial tissue taut dissect the endometrium into thin strips and place directly into the 60 ml pot containing **endo strip wash**.

7. Once all the endometrium strips have been placed into the 60 ml pot, secure the lid and swirl the contents.

8. Using sterile forceps gently drag the dissected endometrial strips across the rim of the pot and transfer the tissue pieces into a 60 ml pot containing **HBSS**. Again, secure the lid and swirl the contents.

9. Using sterile forceps gently drag the dissected endometrial strips across the rim of the pot and dissect the tissue into ~3-5 mm^3 pieces before placing in the 50 ml centrifuge tube containing **HBSS**. Ensure that the pieces are not cut too small as this will make the filtration step more difficult. Once all the endometrial strips have been cut into pieces and are in the centrifuge tube, secure the lid and swirl the contents.

10. Place the centrifuge tube contain the endometrial pieces and **HBSS** into an incubator or water bath and incubate at 37°C for 10 min. Ensure the outside surfaces of the centrifuge tube are thoroughly cleaned using 70% ethanol or Medis before and after incubation.

5.4 Tissue digestion and cell isolation

1. Within a class II biological safety cabinet (tissue culture hood), decant (note 2) off the supernatant and add digestive solution to the tube containing the pieces of endometrial tissue until a total volume of 40 ml is reached. (note 3) Secure the lid, seal with parafilm and then swirl the contents.

    Note 2: Some residual supernatant after decanting is acceptable since not decanting all the supernatant ensures that none of the tissue is lost.
    Note 3: If a large amount of endometrial tissue has been harvested, it can be split into two equal volumes and the digest step carried out in two tubes.

2. Incubate the centrifuge tube(s) containing the **digestive solution** and chopped endometrial strips in a shaking water bath at 37°C for 1 h.

3. Following incubation, clean the external surfaces of the centrifuge tubes with 70% ethanol.

4. Within a class II biological safety cabinet, carefully filter the digested supernatant / tissue through a 70 µm cell strainer into a second 50 ml centrifuge tube containing 5 ml of warm (37°C) **stop solution**. Secure the lid and vortex. This filtration step removes large cell debris and can be aided by holding the cell strainer at an angle of 45°, which will prevent the formation of a vacuum between the cell strainer and the interior surface of the centrifuge tube. (note 5)

Then pass the resultant solution through a 40 µm cell strainer in the same manner to isolate mainly stromal cells in the centrifuge tube. The larger epithelial cells remain principally in the 40 µm cell strainer, and these can be collected by flipping the strainer over, and back-flushing the strainer with 10 ml **stop solution** into a third 50 ml centrifuge tube containing 5 ml of warm (37°C) **stop solution**.

Note 5: A plastic Pasteur pipette should be used to gently swirl the contents of the cell strainer and aid filtration. After filtration the filtrate may appear to contain a tissue-like substance; these are fats / lipids that have passed through the strainer, which is normal.

5. Centrifuge the filtrates at 700 × g for 7 min at 25°C.

6. Within a class II biological safety cabinet, decant off the supernatant into a liquid waste vessel containing virkon. Add 5 ml of warm (37°C) **sterile water** (note 6) to the cell pellet and gently mixing for 30 s.

Note 6: The addition of sterile water lyses eryhtrocytes and improves the purity of the culture.

7. Following the lysis step, **immediately** add 45 ml of **stop solution** (note 7) to the cell suspension. Secure the cap and mix the contents thoroughly by swirling and inverting.

Note 7: The addition of stop solution alters the osmotic potential of the cell suspension from hypotonic to isotonic thereby preventing any further cell lysis.

8. Centrifuge the cell suspension at 700 × g for 7 min at 25°C.

9. Check the colour of the cell pellet to ensure all the erythrocytes have been lysed, (note 8) if not then repeat *5.4 steps 6 – 8* before proceeding.

Note 8: If the pellet is red in colour, there are still erythrocytes present

10. Within a class II biological safety cabinet, decant off the supernatant into a liquid waste vessel containing virkon. Add 5 ml of warm (37°C) **complete medium** to the cell pellet, (note 9) secure the lid and homogenise the solution by vortexing for 30 s.

Note 9: If the tissue was split during *5.4 step 1*, endometrial cells derived from each fraction can now be pooled.

11. Determine the cell count by mixing 10 µl of the cell suspension with 90 µl of sterile filtered trypan blue (**Dilution factor** = 10). Pipette 10 µl of the mixture onto a haemocytometer and count the number of viable (not blue) cells present in **four major** grids (each with 4 x 4 minor lines) of the ten major grids for each of the 10 chambers on the FastRead102 plastic slides of the haemocytometer. The number of cells per ml of solution is then determined by the following equation:

$$Number\ of\ cells = \frac{Total\ count}{4} \times\ dilution\ factor\ \times\ 1 \times 10^4$$

12. Adjust the cell density to 1×10^5 cells ml^{-1} in warm (37°C) **complete medium** and seed the cells into either 24-well flat bottom tissue culture plates (1 ml/well) or 75 cm^2 filter capped tissue culture flasks (30 ml).

13. Incubate the plates or flasks in an incubator at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

14. After an initial incubation period of 18 h, the stromal cells will have adhered to the interior surface of the culture vessel. Using a Pasteur pipette, remove the culture supernatant (containing contaminating epithelial cells) from each well or flask and discard or transfer to a correspondingly labelled 24-well plate or flask. Add fresh **complete medium** to the stromal cell cultures. Adherence of the epithelial cells to fresh culture plates or flasks will take approximately 48 h.

15. Incubate the plates or flasks in an incubator at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

16. Culture medium should be changed every 48 h after the initial cell separation (5.4 - 14).

17. Primary cultures should be examined using an inverted microscope 4 days after the initial separation (5.4 - 14). If the differential adhesive properties of epithelial and stromal cells have not resulted in pure populations further processing will be required (see section 5.5).

18. Cell confluence should be monitored daily and not allowed to reach 100%. If maintaining cells in 75 cm^2 filter capped tissue culture flasks, they should be split once they have reached 80% confluent (see section 5.6). If cells are in 24-well plates, once 80% confluence is reached, cells are ready for experimental use.

5.5 Differential endometrial cell splitting

If the epithelial and stromal cells have not been successfully isolated from each other during the differential sieving and primary culture, further purification can be achieved using differential cell splitting

1. Within a class II biological safety cabinet partially fill a 50 ml centrifuge tube with 10 ml sterile, warm (≥ 22°C) **D-PBS**. Prepare one tube per flask / plate to be split.

2. Place the required amount of accutase in a water bath at 37°C.

3. Aspirate the **complete medium** from the flask / plate and discard in a liquid waste vessel containing virkon.

4. Add the sterile, warm **D-PBS** (either 0.5 ml per well for plates or 20 ml for a flask) and gently swirl to wash the cells.

5. Aspirate the **D-PBS** and repeat the wash step. (note 10)

   Note 10: Correct washing ensures that any residual FBS, Mg or Ca from the **complete medium**, which would interfere with the activity of **accutase**, is removed.

6. Add warm (37°C) **accutase** to the cells (150 µl per well or 10 ml per flask), secure the lid and gently swirl.

7. Incubate the plate / flask in an incubator at 37°C.

8. Incubation with **accutase** will cause stromal cells to detach from the culture surface first (after approximately 2-5 min), whilst epithelial cells will take 15-20 min to detach. Cells should be monitored closely using an inverted microscope to determine when the stromal cells have detached. (note 11)

   Note 11: Detached cells will have a pin-head appearance once they have lifted away from the culture surface. Detachment can be aided by gently tapping the side of the plate / flask with the palm of a hand.

9. Once the stromal cells have detached, pipette the **accutase**/stromal cell mix into the 10 ml sterile **D-PBS** prepared earlier (5.5 – 1). Seal the neck of the tube with parafilm and place in a water bath at 37°C.

10. Gently wash the cells (1×) still attached to the culture surface as previously described and then add the required amount of **complete medium** to the plate / flask before returning to culture in an incubator at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

11. Remove the **Accutase**/stromal cell mix from the water bath and clean the external surfaces with **70% ethanol**. Centrifuge at 700 × g for 7 min at 25°C.

12. Within a class II biological safety cabinet, decant off the supernatant and resuspend the cell pellet in 5 ml fresh, warm (37°C) **complete medium**.

13. Count the cells as described in 5.4 – 11.

14. Adjust the cell density to 1×10^5 cells ml^{-1} in warm (37°C) **complete medium** and seed the cells into either 24-well flat bottom tissue culture plates (1 ml) or 75 cm^2 filter capped tissue culture flasks (30 ml).

15. Return the cells to culture in an incubator at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

5.6 Endometrial cell passage

Endometrial cell passage is undertaken for pure cell populations being maintained with 75 cm^2 flasks to prevent them exceeding 80% confluence. This usually applies to stromal rather than epithelial cells. However, passaging cells is best avoided as this tends to change their phenotype.

1. Within a class II biological safety cabinet partially fill a 50 ml centrifuge tube with 10 ml sterile, warm (≥ 22°C) **D-PBS**. Prepare one tube per flask / plate to be split.

2. Place the required amount of accutase in a water bath at 37°C.

3. Aspirate the **complete medium** from the flask and discard in a liquid waste vessel containing virkon.

4. Add the sterile, warm **D-PBS** (20 ml) and gently swirl to wash the cells.

5. Aspirate the **D-PBS** and repeat the wash step. (note 10)

6. Add warm (37°C) **accutase** to the cells (10 ml per flask), secure the lid and gently swirl.

7. Incubate the plate / flask in an incubator at 37°C.

8. Incubation with **accutase** will cause stromal cells to detach from the culture surface first (after approximately 2-5 min), whilst epithelial cells will take 15-20 min to detach. Cells should be monitored closely using an inverted microscope to determine when the cells have detached. (note 11)

9. Once detached, pipette the **accutase**/cell mix into the sterile **D-PBS** prepared earlier and centrifuge at 700 × g for 7 min at 25°C.

10. Within a class II biological safety cabinet, decant off the supernatant and resuspend the cell pellet in 5 ml fresh, warm (37°C) **complete medium**.

11. Count the cells as described in 5.4 – 11.

12. Adjust the cell density to 1×10^5 cells ml^{-1} in warm (37°C) **complete medium** and seed the cells into 75 cm^2 filter capped tissue culture flasks (30 ml).

13. Return the cells to culture in an incubator at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

6. References

1 Herath S, Lilly ST, Fischer DP, Williams EJ, Dobson H, Bryant CE, Sheldon IM: Bacterial lipopolysaccharide induces an endocrine switch from prostaglandin F2alpha to prostaglandin E2 in bovine endometrium. *Endocrinology* 2009;**150**:1912-1920.

2 Vogel V, Sheetz M: Local force and geometry sensing regulate cell functions. *Nature Reviews Molecular Cell Biology* 2006;**7**:265-275.

3 Cukierman E, Pankov R, Stevens DR, Yamada KM: Taking Cell-Matrix Adhesions to the Third Dimension. *Science* 2001;**294**:1708-1712.

4 Ireland JJ, Murphee RL, Coulson PB: Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. *J Dairy Sci* 1980;**63**:155-160.

5 Cronin JG, Turner ML, Goetze L, Bryant CE, Sheldon IM, 2012 Toll-Like Receptor 4 and MYD88-Dependent Signaling Mechanisms of the Innate Immune System are Essential for the Response to Lipopolysaccharide by Epithelial and Stromal Cells of the Bovine Endometrium. Biology of Reproduction 86: 51

6 MacKintosh SBP, Schuberth HJ, Healy LL, Sheldon IM 2013 Polarised bovine endometrial epithelial cells vectorially secrete prostaglandins and chemotactic factors under physiological and pathological conditions Reproduction 145: 57-72.

7 Jacca S, Franceschi V, Colagiorgi A, Sheldon M, and Donofrio G 2013 Bovine Endometrial Stromal Cells Support Tumor Necrosis Factor Alpha-Induced Bovine Herpesvirus Type 4 Enhanced Replication Biology of Reproduction 88:135.

8 Healy LL, Cronin JG, Sheldon IM 2014 Endometrial cells sense and react to tissue damage during infection of the bovine endometrium via interleukin 1. Scientific Reports, 4: 7060, 1-9

9 Saut JPE, Healey GD, Borges AM, Sheldon IM 2014 Ovarian steroids do not impact bovine endometrial cytokine/chemokine responses to E.coli or LPS in vitro. Reproduction,148:593-606

10 Carvalho AV, Reinaud P, Forde N, Healey GD, Eozenou C, Giraud-Delville C, Mansouri-Attia N, Gall L, Richard C, Lonergan P, Sheldon IM, Lea RG, Sandra O. 2014 SOCS genes expression during physiological and perturbed implantation in bovine endometrium. Reproduction. 148: 545-557

11 Carvalho AV, Eozenou C, Healey GD, Forde N, Reinaud P, Chebrout M, Gall L, Rodde N, Padilla AL, Delville CG, Leveugle M, Richard C, Sheldon IM, Lonergan P, Jolivet G, Sandra O 2014 Analysis of STAT1 expression and biological activity reveals interferon-tau-dependent STAT1-regulated SOCS genes in the bovine endometrium Reproduction, Fertility and Development 28(4) 459-47410

12 Amos MR, Healey GD, Goldstone RJ, Mahan SM, Düvel A, Schuberth HJ, Sandra O, Zieger P, Dieuzy-Labaye I, Smith DGE, Sheldon IM 2014 Differential EndometrialCell Sensitivity to a Cholesterol-Dependent Cytolysin Links Trueperella pyogenes to Uterine Disease in Cattle. Biology of Reproduction 90, 54, 1-13

13 Turner ML, Cronin J.G, Healey GD, Sheldon IM, 2014 Epithelial and stromal cells of bovine endometrium have roles in innate immunity and initiate inflammatory responses to bacterial lipopeptides in vitro via Toll-like receptors TLR2, TLR1 and TLR6 Endocrinology, 155:1453-1465

7. Dissection of Bovine Endometrium

1. Each uterus is processed separately through-out the whole process of dissection and cell isolation. Uteri are selected according to stage of cycle as determined by the corpus luteum (CL) (Appendix 1).

2. The external surface of the uteri were rinsed with 70% ethanol. The reproductive tract was then laid out as shown in Fig A. Endometrial tissue was collected from the horn ipsilateral to the CL.

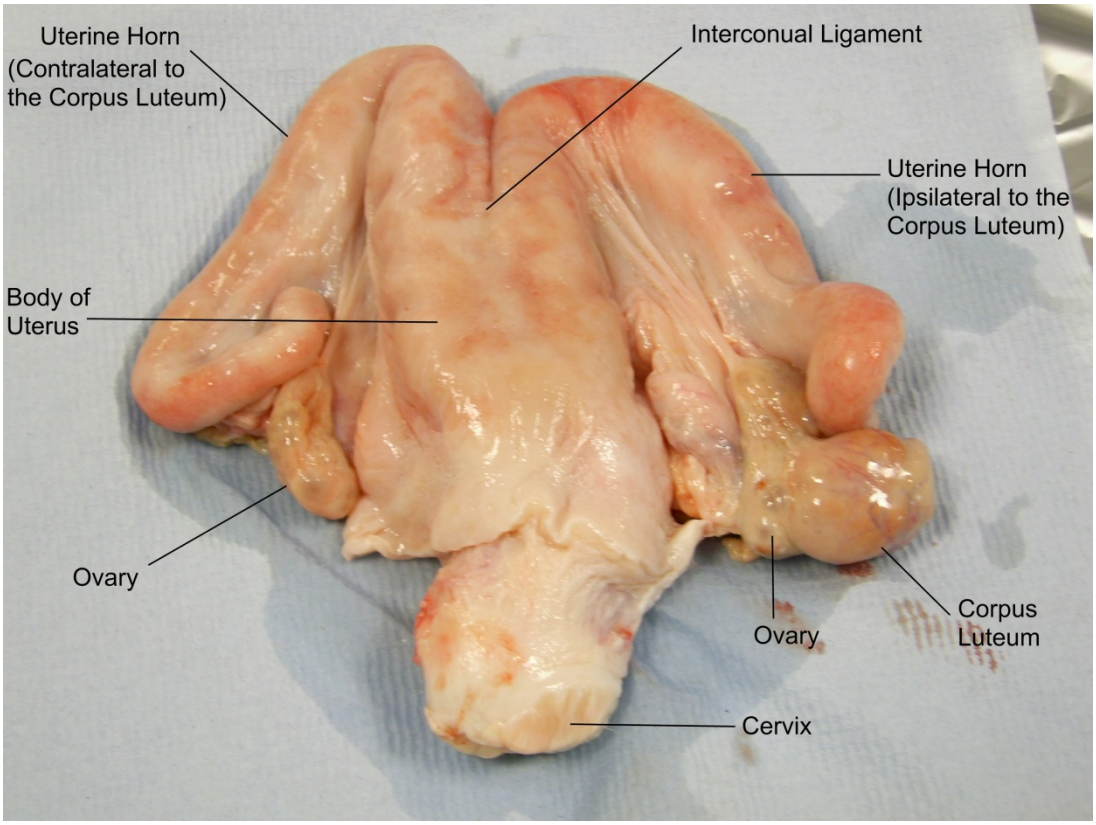

Figure A. The Bovine Reproductive Tract.

3. A transverse cut was made through the uterine horn ipsilateral to the CL, just above the intercornual ligament (Fig B).

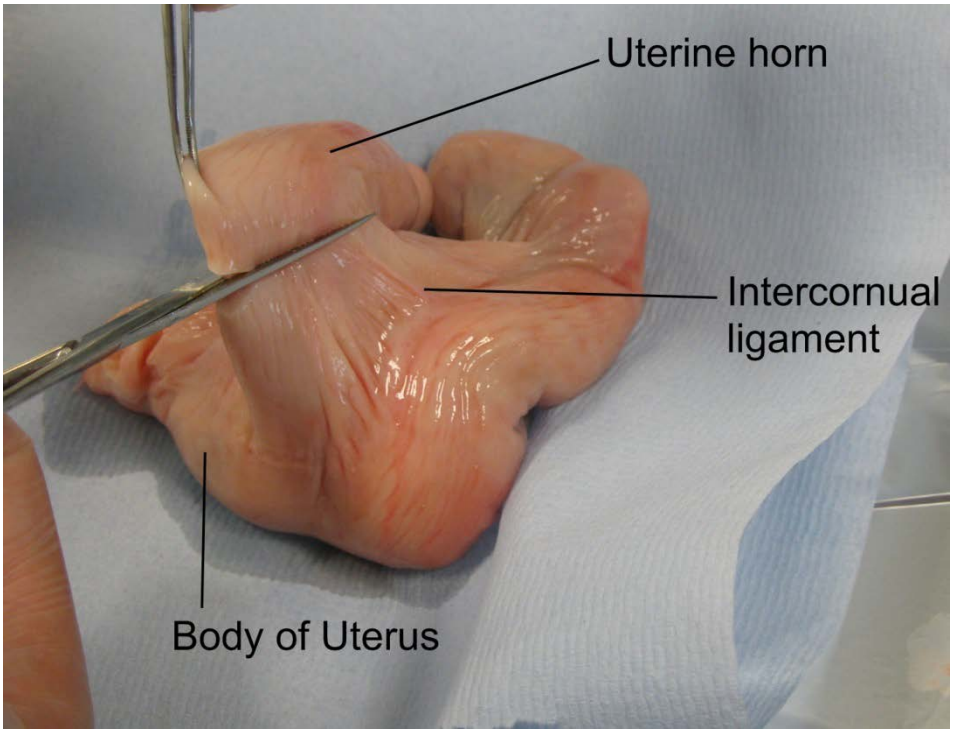

Figure B. Transverse cut across the uterine horn

4. A longitudinal cut was then made through the uterine horn to reveal the endometrium (Fig C).

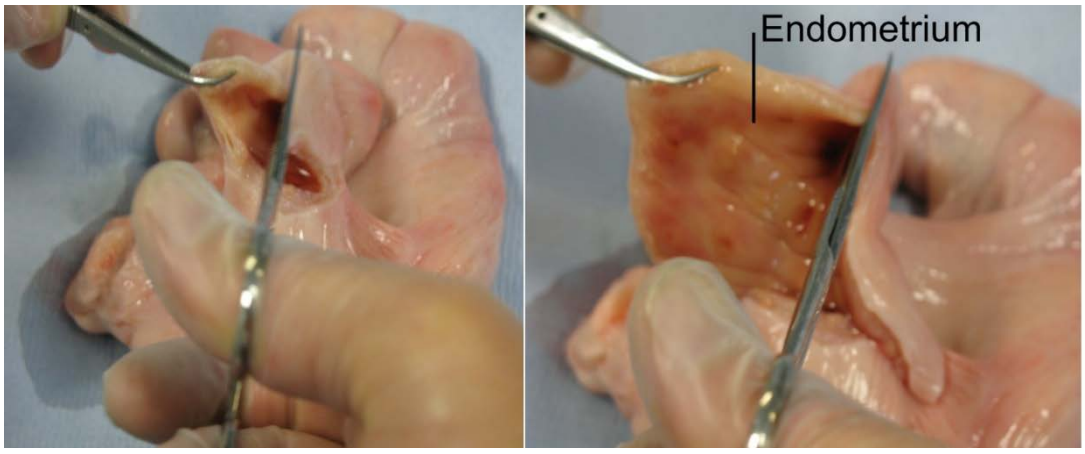

Figure C. Longitudinal cut along the uterine horn.

5. The luminal surface of the horn was rinsed with phosphate buffered saline (PBS, Sigma) containing 50 IU/ml penicillin, 50 µg/ml streptomycin and 2.5 µg/ml amphotericin B (Sigma). The endometrial tissue was then be dissected off. This was achieved by pinching the endometrial folds and pulling gently with sterile forceps to create a 'tenting' of endometrial tissue. The tissue can then be cut with sterile surgical scissors, which are held parallel to the uterine horn. Dissected tissue should be thin enough that it is practically transparent. (Fig D).

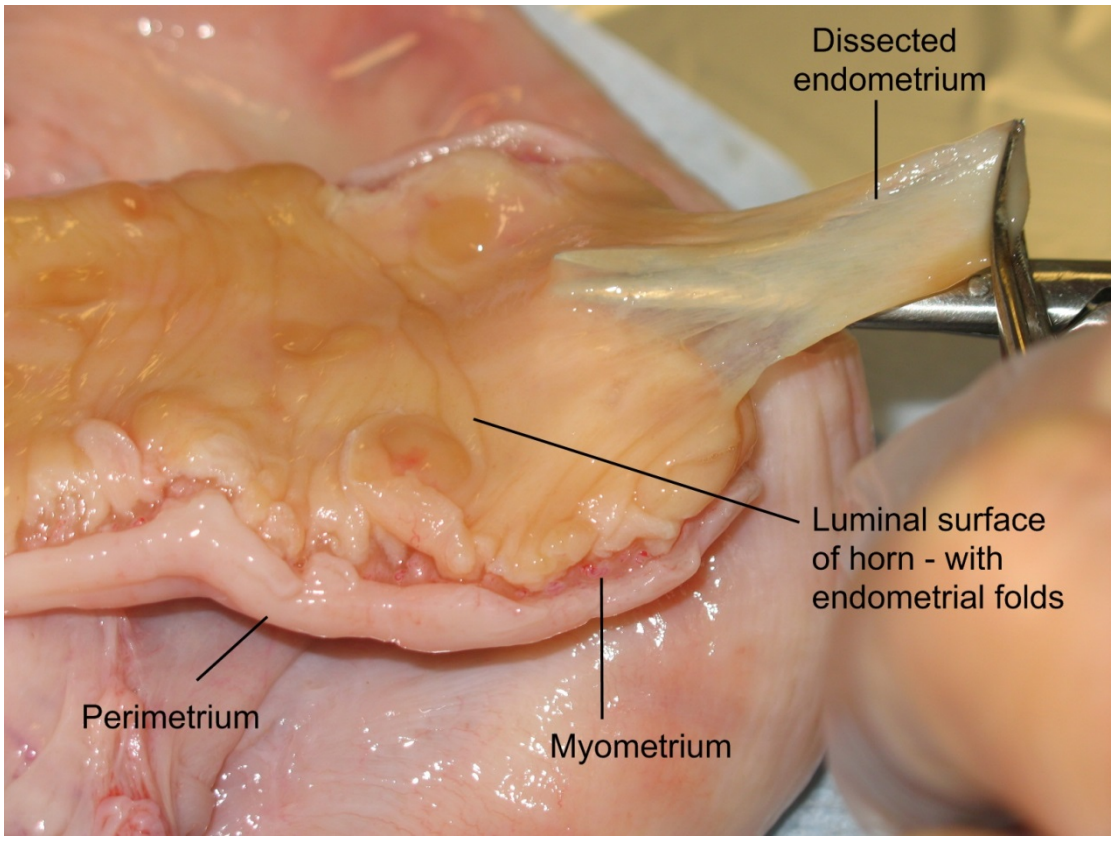

Figure D. Dissection of endometrium, showing the various layers of the uterus.

6. Dissected tissue is cut into strips and placed in PBS containing 50 IU/ml penicillin, 50 µg/ml streptomycin, and 2.5 µg/ml amphotericin B (Sigma). These strips are then transferred to hanks buffered saline solution (HBSS, Sigma). The strips are then cut into 3 to 5 mm^3 pieces into fresh HBSS, and incubated at 25ºC for 10 min before being placed in digest solution.