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A ligated intestinal loop model in anesthetized specific pathogen free chickens to study Clostridium perfringens virulence.

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Journal of Visualized Experiments

Dear Editorial Board,

On behalf of all co-authors, please find enclosed the manuscript titled "A ligated intestinal loop model in anesthetized specific pathogen free chickens to study *Clostridium perfringens* virulence". The content is original and has not been submitted to another journal. We believe that the manuscript meets all guidelines described in the "Manuscript Instruction for Authors (rev. October 2013)" as found on your website.

This work describes in detail the protocol, list of materials and representative results of an *in vivo* infection model called "intestinal ligated loop model" to study necrotic enteritis, caused by the bacterium *Clostridium perfringens*, in chickens. This technique has the advantage of using a small number of animals compared to other *in vivo* infection models commonly used to study necrotic enteritis in chickens. Moreover, chickens used in our experiments are anesthetized during the infection, in contrast with other infection models where pain is poorly managed and mortality can be high. We consider that this model reduces animal use and pain to a maximum when studying necrotic enteritis. The procedures involve high precision and technical skills and the sole description of the methods in a text format would most likely result in a poor reproducibility of the method by other researchers interested in replicating the intestinal ligated loop model. It is our wish to share this method and allow researchers from around the globe to have a unique and precious visual access to this animal model. This is what motivated us to submit this manuscript to the Journal of Visualized Experiments. We strongly believe that the uniqueness of JoVE format using both video and text descriptions is a must in order to detail the methodology used and avoid future potential problems in live animals caused by a poor execution.

Drs. Boulianne and Parent developed the model. Drs. Boulianne and Parent performed the surgeries. Dr. Burns assisted in developing the anesthesia protocol. Dr. Parent edited the video and wrote the manuscript. Drs Burns, Desrochers and Boulianne edited the manuscript.

We would like to thank Ms. Nandita Singh from the JoVE editorial team for providing assistance in the submission process.

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Thank you for your consideration of our manuscript. We look forward to hearing from you.

Best regards,

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TITLE:

A Ligated Intestinal Loop Model in Anesthetized Specific Pathogen Free Chickens to Study *Clostridium perfringens* Virulence.

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KEYWORDS:

Chicken, intestinal ligated loop, necrotic enteritis, *Clostridium perfringens*, small intestine, pathogenesis, *in vivo*.

SHORT ABSTRACT:

Here we present a protocol to surgically create 'intestinal ligated loops' in chicken small intestines. This procedure allows for the comparison of multiple *Clostridium perfringens* strains' virulence *in situ* in a single host. This method markedly decreases the number of chickens usually necessary for similar *in vivo* experiments.

LONG ABSTRACT:

Necrotic enteritis was studied in chickens using various *in vivo* infection models. Most of these use a combination of predisposing factors, such as coccidiosis and diet, with gavage or administration via the feed using *Clostridium perfringens*. In these models, the comparison of multiple *C. perfringens* strains for virulence studies requires a large number of hosts to obtain significant results. Mortality during the course of the study can be high depending on the experimental model, hence raising ethical concerns regarding animal welfare in research. The development of new infection models requiring fewer animals to study pathogenesis, yet providing statistically significant and valid results, is important in reducing animal use in research. Intestinal ligated loop models have been used to study clostridial infections in various species such as mice, rabbits and calves. Following surgical procedures to create ligated loop segments, *C. perfringens* strains are injected directly into the loops to establish a close contact between the bacteria and the intestinal mucosa. Samples of the small intestine and luminal contents are taken

at the termination of the procedures after a few hours. Multiple bacterial strains can be inoculated in each animal, hence reducing the number of required subjects in the experiments. Also, procedures are performed under general anesthesia to reduce animal pain. In chickens, this model would be more appropriate than oral administration to compare *C. perfringens* strain pathogenicity because fewer animals are needed, no predisposing factors are required to induce the disease, and pain is controlled by analgesics. The intestinal ligated loop model is poorly described in chickens and standardization is essential for its optimal use. This manuscript provides all the necessary steps to create numerous intestinal ligated loops in chickens and brings information on the critical points to obtain valid results.

INTRODUCTION:

The use of animal models to study infectious diseases affecting humans and animals is a central tool in assessing virulence factors regulating the pathogenesis of a specific condition as well as to elaborate strategies to prevent or cure diseases[1]. Despite numerous advantages of using animal models to study various diseases, ethical concerns regarding this practice are arising. Researchers need to minimize animal use while obtaining significant and valid results. The concept of the 3 Rs principles (Replace, Reduce and Refine) was elaborated to ensure that welfare issues were addressed in such trials. In chicken necrotic enteritis studies, *in vivo* chicken models are used to investigate the etiology, prevention, and treatments of this condition[2-5]. Pathogenic strains of *C. perfringens* carrying specific virulence factors, such as the NetB toxin[6], are administered to cause necrotic enteritis in chickens[7]. The replacement principle is, therefore, difficult to achieve in such case as these virulence factors may not be as critical in other animal species. Most models for necrotic enteritis in chickens use a combination of risk factors, such as coccidiosis and altered diet to induce necrotic enteritis followed by gavage of broth culture containing large numbers of *C. perfringens*[2]. These models will induce the disease in a large number of chickens, and mortality can be important in such trials[8], thus raising concerns about the reduction and refinement principles in animal research.

Intestinal ligated loops models are a desirable alternative for the study of diseases induced by intestinal pathogens with respect to the principle reduction, and refinement. In these models, intestinal segments called 'loops' are created by placing ligatures along the intestinal tract to form independent and hermetic compartments where pathogens can be injected alone[9] or with other molecules, such as vaccine candidates[10,11]. The pathogens of interest are placed in close contact with the intestinal cells and after a few hours of infection time, intestinal samples can be recovered for further analysis. This allows the use of multiple treatment and control groups in the same animal. Statistical analysis can be performed with repeated measures models, which increases the power of discrimination between groups and reduces the number of necessary chickens compared to oral gavage trials. Also, surgical procedures and subsequent infection times are performed under continuous general anesthesia and analgesia, hence minimizing the animal pain. Closed loop ligations are an ideal template for reducing host numbers and creating a more humane system in animal research.

Intestinal ligated loops models are well described in various species, such as calves, rabbits and mice[2,9], but poorly described in chickens[7]. For an optimal use of this surgical model, proper

technique and execution are essential for the creation of ligated intestinal loops to avoid damages to the intestinal integrity. The goal of this manuscript is to describe a step by step method in the creation of multiple intestinal loops in a chicken model. This technique is limited by the surgeon's skill and experience, as accurate procedures are essential for the success of the project.

PROTOCOL:

All procedures with live animal use were authorized by the Ethical Committee of the Faculty of Veterinary Medicine, Université de Montréal (CÉUA, 'Comité d'éthique de l'utilisation des Animaux').

1. Considerations Before Surgery

1.1. Select 10 weeks old specific pathogen free (SPF) leghorn chickens for the surgery.

Note: Their weight must be between 1.0 and 1.2 kg.

1.2. Withdraw the feed 12 h prior to the procedures to empty the intestinal tract.

1.3. Prior to surgery, place the chickens under general anesthesia following a protocol verified by an anesthesiologist. Premedicate each chicken 15 min prior to the surgery with an intramuscular (IM) injection of midazolam 1 mg/kg and butorphanol 4 mg/kg. Repeat, every 4 h, the injection of butorphanol 4 mg/kg IM or when the breathing pattern changes and frequency increases during anesthesia. For the general anesthesia, during all procedures, administer isoflurane at a concentration between 1.5 and 2.5% with an intratracheal tube.

Note: The use of premedication for sedation and analgesia is not mandatory, but will significantly improve the depth of anesthesia, reduce the quantity of anesthetics necessary during all procedures, as well as enable researchers to control pain levels.

1.4. Monitor the anesthesia by recording body temperature, heart rate and electrocardiogram (ECG), respiratory rate and rhythm, oxygen saturation, expired carbon dioxide (CO_2) and neurologic reflexes.

Note: A person must be assigned to monitor these parameters throughout all the procedures.

1.5. Follow sterility principles throughout the surgical procedures. Ensure that the surgeon and the assistant surgeon dress with sterile gowns, gloves, a mask and protective goggles according to institutional preoperative protocols. Ensure that the surgeon scrubs the hands with a chlorhexidine gluconate impregnated sterile brush up to the elbow with emphasis on fingernails and each finger surface.

1.6. Sterilize the surgical instruments with a sterilizer.

2. Surgical Site Preparation

2.1. Firstly, manually remove the feathers from the abdomen with a gentle traction.

2.2. Scrub the surgical site with a chlorhexidine gluconate detergent impregnated sterile brush. Gently scrub the skin from the center to the outside for a total contact time of 5 min without coming back to the center.

2.3. Perform 3 alternative passages on the surgical site with a chlorhexidine gluconate solution and isopropyl alcohol using sterile gauzes.

Note: The passage must start from the middle of the surgical site to the borders to avoid contamination from the non-disinfected areas to the surgical site.

2.4. Place the sterile surgical drape on the chicken.

3. Extraction of the Small Intestines from the Abdominal Cavity

3.1. Incise the surgical drape with scissors to expose only the skin of the surgical site.

3.2. Incise the skin by performing an ''L'' shape low-midline incision with a scalpel blade #3.

3.2.1. Start the first incision 1 cm caudal to the sternum and end it at 1 cm cranial to the cloaca.

3.2.2. Perform the second incision perpendicular to the first incision. Start from the caudal end of the first incision and continue 5 cm to the left side of the abdomen by following the pelvis line.

Note: This opening will create a flap that will allow an easier extraction of the intestines.

3.3. With surgery scissors, cut the peritoneum and abdominal muscles with the same ''L'' shape pattern to open the abdominal cavity.

Note: This will expose the air sacs. Intestines are located underneath these structures. Air sacs must be humidified with gauzes saturated with sterile saline 0.85 % to avoid desiccation and possible rupture throughout the procedure.

3.4. Insert a Snook spay hook in the abdominal cavity by following the left abdominal wall and work the way around the abdominal air sacs.

3.5. Gently exteriorize the intestines and spread these out on the surgical field.

Note: Keep intestines humidified by frequently spraying sterile saline 0.9% throughout the procedure. Also, gauzes humidified with sterile saline can be placed on the intestines to avoid desiccation.

3.6. Grab the intestines using a dry sterile gauze and gently pull out the small intestines by manual traction to expose the jejunum.

Note: Mesenteric vessels are fragile and excessive tension may rupture them which may lead to intestinal ischemic lesions and greatly compromise the outcome of the procedure.

4. Fabrication of Intestinal Loops

4.1. Creation of loop no. 1.

4.1.1. Place a simple ligature with a polyglactin multifilament synthetic absorbable material in the proximal jejunum by avoiding the ligature of major mesenteric vessels.

4.1.2. Place a distal ligature 2 cm away from the proximal ligature.

Note: Simple ligatures made with multifilament suture material are placed along the intestinal tract to create hermetic segments. A schema (**Figure 1**) describes the location of all ligatures. The loops consist of a proximal and distal ligature separated by 2 cm.

4.2. Creation of interloop no. 1.

4.2.1. Place a simple ligature 0.5 cm aborally to the distal ligature of loop no.1.

Note: Between successive loops, there are interloops of 1 cm, *i.e.* segments making a physical separation between the loops. To likely decrease the possible risk of cross-contamination from one loop to another, a simple ligature is placed in the middle of the interloop (0.5 cm from the distal and proximal ligature of 2 adjacent loops). This reduces the risk of a possible leaked contaminant from a loop to reach the ligature of an adjacent loop. These segments are not mandatory but will decrease the risk of cross-contamination.

4.3. Creation of subsequent loops and interloops.

4.3.1. Repeat steps 4.1 and 4.2 to obtain the number of desired loops.

Note: In this experiment, 9 loops and 8 interloops were created. Total length amounted to 26 cm. The number of loops can be adjusted depending on the study.

5. Injection of *Clostridium perfringens* Strains Into the Loops

5.1. Inject with a sterile syringe and needle 26 G the pathogen in loop no.1. Gently insert the needle on the antimesenteric side of the intestine at a 45° angle.

Note: In this experiment, 0.2 mL of brain heart infusion (BHI) containing 1 x 10^6 CFU (colony

forming units) of *C. perfringens* in the mid-log growth phase was injected in each loop. 5 different strains were injected in 5 different loops and the 4 remaining loops were injected with a negative control containing no bacteria (sterile BHI), alternating pathogens and sterile BHI loops.

5.2. Repeat step 5.1 to inject all loops.

5.3. Gently replace the intestinal tract in the dorsal area of the abdominal cavity, underneath the air sacs.

6. Closure of the Abdominal Cavity

6.1. The suture of the peritoneum and abdominal muscles.

6.1.1. Suture the peritoneum and abdominal muscles along the pelvis (made in step 3.3) with a simple continuous suture pattern using a polyglactin multifilament synthetic absorbable material.

6.1.2. Suture the peritoneum and abdominal muscles from the sternum to the cloaca (made in step 3.3. with a simple continuous suture pattern using a polyglactin multifilament synthetic absorbable material.

6.2. The suture of the skin.

6.2.1. Using a polyglactin multifilament synthetic material and a simple continuous suture pattern, close the incision of the skin along the pelvis made in step 3.2.

6.2.2. Using the same multifilament ligature material and a simple continuous suture pattern, close the incision of the skin from the sternum to the cloaca made in step 3.2.

7. Conclusion

7.1. After the surgical procedures, keep the chicken under general anesthesia with proper use of analgesics to minimize animal pain during the infection time. Continue anesthetic monitoring to ensure an adequate anesthesia level.

7.2. After the desired infection time, humanely euthanize the chicken by cervical dislocation under general anesthesia.

Note: For these procedures, the required time to induce microscopic lesions with *C. perfringens* pathogenic strains was 7 h.

7.3. With a scalpel blade #3, cut a 0.5 cm to 1 cm long loop section and place it in 10% formalin for fixation overnight and further histopathological analysis.

7.4. With the same scalpel blade, cut the remaining loop section and place it in a sterile microcentrifuge tube for the isolation of the *C. perfringens* strains in each loop further bacteriologic analysis for their genetic characterization.

7.5. Repeat steps 7.2 and 7.3 for all loops by changing the scalpel blade between every loop.

REPRESENTATIVE RESULTS:

A schematic of the 9 intestinal loops and 8 interloops is shown in **Figure 1**. In this model, a total of 9 loops and 8 interloops are created with simple ligatures. A loop consists of proximal and distal ligatures spaced by 2 cm measured from the proximal ligature. Two adjacent loops are separated by an interloop of 1 cm. To decrease the possible risk of cross-contamination between loops by leakage from a ligature, an interloop ligature is placed mid-way from the distal ligature and proximal ligature of 2 adjacent loops. The number of loops can be adjusted depending on the requirements of the study.

[Place Figure 1 here].

Figure 2A (HPS stain) shows the microscopic appearance of an intestinal loop injected with a control sterile medium. The mucosal brush border is intact and there is no necrosis present in the section. Mild congestion may be present in the intestinal layers (mucosa, submucosa, muscularis, and serosa) of the segments. **Figure 2B** (HPS stain) shows mucosal necrosis 7 hours following the injection of a pathogenic strain of *Clostridium perfringens* recovered from a clinical case of necrotic enteritis on a chicken farm. Villi tips are eroded with necrotic enterocytes surrounding the villi tips. In the necrotic material surrounding the villi, clusters of large rod-shaped bacteria are observed (arrows). These bacteria were further identified as Gram-positive bacteria with a Twort's stain (not shown). This finding, combined with the isolation by bacteriology of large numbers of *Clostridium perfringens*, indicates the lesions were caused by this bacterium. **Figure 2C** (HPS stain) shows histological lesions of an ischemic loop, where necrosis is not related to the injection of pathogenic *C. perfringens* strains but the result of poorly executed procedures. This magnification shows severe coagulation necrosis that could be misinterpreted as lesions caused by *Clostridium perfringens*. However, no bacteria are observed in this section. Also, the blood vessels in the muscular layer are severely dilated by the accumulation of large numbers of degenerating erythrocytes (arrows). This is indicative of vascular congestion and in this case, the congestion was caused by ligated mesenteric blood vessels. Ischemic loops can be easily identified macroscopically during the surgical procedures; following severe vascular congestion, the serosa will be dark blue instead of its normal pink-reddish coloration.

[Place Figure 2 here].

In this study, loops were injected with either a sterile control culture medium (BHI) or one of the five different strains of *Clostridium perfringens* recovered from various antibiotic free chicken flocks affected or not with necrotic enteritis[12]. Ten chickens were used in this study. Strains pathogenicity could be determined based on ability to repeatedly cause lesions compatible with

necrotic enteritis to the mucosa. While strains recovered from clinical cases of necrotic enteritis showed the ability to cause mucosal necrosis 7 h post-infection, strains recovered from clinically healthy chicken farms did not induce mucosal necrosis and the mucosa was similar to the normal findings from the control loops injected with BHI[12]. By using numerous loops in the same animal, five *C. perfringens* strains and four control loops could be used in the same bird.

FIGURE LEGENDS:

Figure 1: **Schematic representation of ligated intestinal loops.**

Figure 2: **Histopathologic findings 7 h post-infection. (A)** There are no necrotic lesions in the mucosa on histopathology following the injection of Brain Heart Infusion (BHI), a sterile culture medium. **(B)** In loops injected with a pathogenic strain of *Clostridium perfringens*, there is marked necrosis of the mucosa with numerous large Gram-positive rod-shaped bacteria covering the mucosa. **(C)** Ischemic lesions can resemble necrotic lesions caused by *C. perfringens*.

DISCUSSION:

Intestinal loops models have been described in numerous species to study host-pathogen interaction and pathogenesis of diseases caused by various intestinal pathogens, such as *Clostridium perfringens*, *Clostridium difficile* and *Salmonella enterica* [7,9,13-15]. It has also been used to analyze the mucosal immune response[16], the efficacy of antibodies to neutralize bacterial toxins excreted by intestinal pathogens[11] and to evaluate vaccine candidates to prevent intestinal diseases[10]. However, this model using numerous ligated loops in a single individual animal is poorly described in chickens and standardization of this technique is mandatory to obtain valid results as inadequate manipulations may lead to false results and misinterpretations. This manuscript shows the creation of 9 successive ligated loops located in the jejunum and ileum of the chicken gastro-intestinal tract. This study used Specific Pathogen Free (SPF) leghorn male chickens weighing between 1.0 and 1.2 kg. The maximum number of loops allowed by this breed at this weight was 9, since 26 cm of small intestines could be easily exteriorized from the coelomic cavity. It is not excluded that by using another breed and/or a heavier leghorn male chicken, the total number of loops created may be different, even higher than 9. Intestinal loops length could also be modified, but the 2-cm length was considered optimal to allow sample collection for histopathology and bacteriology while using the maximum capacity of the available intestinal length.

The intestinal ligated loop technique described in this article can be a valuable alternative to the current models available to study necrotic enteritis in chickens caused by *Clostridium perfringens*. According to the results described in a previous article[12], the evaluation of 5 *C. perfringens* strains with 10 chickens was sufficient to differentiate highly virulent and commensal strains of *C. perfringens*. In comparison, other infection models using *per os* inoculation to reproduce necrotic enteritis with *C. perfringens* will require a large number of individuals to obtain the same results. In the literature, the number of birds needed to evaluate the virulence of *C. perfringens* strains ranges from 10 to 30 chickens per group[17-19] to a hundred hosts per group[20]. In regard to the refinement principle for animal use in research, it would be advised to use the technique requiring the lowest number of hosts to obtain significant results. The intestinal ligated loop

model described in this article is most likely the model requiring the lowest number of chickens to compare the virulence of *C. perfringens* strains.

Since cross-contamination by leakage from a ligature could be evaluated only after *C. perfringens* culture and characterization by pulsed field gel electrophoresis (PFGE) many days after the procedures, it was of the utmost importance to minimize this risk by including 1 cm interloops containing an interloop ligature mid-way between 2 adjacent loops. By taking these precautions, it was possible to avoid a possible cross-contamination. Indeed, while pathogenic strains caused microscopic necrotic lesions concordant with necrotic enteritis, there were no significant microscopic lesions in adjacent loops injected with non-pathogenic *C. perfringens* strains or the BHI culture medium negative control. This was indicative that different strains could be safely injected in adjacent intestinal loops of the same chicken.

One of the most challenging issues with this model was the exteriorization of the small intestines without compromising ventilation and intestinal vascularization. Birds have a different respiratory system than mammals. The diaphragm is absent, and ventilation is controlled by airsacs, thin-walled structures in the abdominal cavity. There are 7 of these in chickens and they are divided in the cervical (1), anterior thoracic (2), posterior thoracic (2) and abdominal (2) air sacs. These balloon-like structures are directly connected to the lungs and allow for the movement of air in the respiratory tract. Upon opening the abdominal cavity between the sternum and cloaca, abdominal airsacs are prominent and tend to be easily ruptured, especially if dry. For this reason, constant humidification with gauzes damped with sterile saline is important to ease proper ventilation and anesthesia during procedures. Also, it can be difficult to avoid small punctures in the abdominal airsacs while exteriorizing the intestines out of the abdominal cavity. It is important to monitor anesthetic parameters, such as respiratory rate and pattern, palpebral reflexes, heart rate and chicken's movements, to make sure the chicken stays under an adequate plane of anesthesia. Small air leakage is less likely to affect the quality of anesthesia, but this leakage must be minimized. It is important to hermetically close the peritoneum after the procedures. This will reestablish the internal air pressure and ventilation will return to normal after this step. After the closure of this structure, there should be no air leakage from the abdomen. This can be verified by spraying a small amount of sterile saline on the sutures and look for bubble formation indicating air coming from the abdominal cavity. If this happens, additional simple skin sutures can be placed at the air leakage site.

Another technically challenging aspect is related to the risk of ischemic lesions developing in the small intestine due to poorly executed surgical procedures. Ischemia happens when there is a restriction of blood supply to tissues, causing a shortage of oxygen and other molecules needed for cellular metabolism. In this case, the intestine will rapidly become macroscopically dark blue instead of pink-reddish and the intestinal mucosal cells will degenerate quickly. This will cause ischemic lesions unrelated to the injected pathogen and it will interfere with the histopathological interpretation. Two problematic situations can lead to ischemia. First, ligature of mesenteric vessels by inadvertence will cause this condition. For this reason, it is crucial to look carefully at the placement of the ligatures along the intestinal tract to avoid including the mesenteric vessels. Second, ischemia can happen if hemorrhages of the mesenteric vessels occur

during the procedures. These structures are thin walled and fragile. Excessive tension can lead to their rupture and while manually exteriorizing the intestines, attention should be given to the tension on the mesenteric vessels to ensure their integrity during this step. Also, mesenteric vessels hemorrhage can occur if these are punctured with a surgical suture needle. The surgeon must look carefully at the location pierced through the mesentery with the needle. Bleeding can be stopped in both situations, but the coagulation process will stop or reduce significantly the blood flow to these intestinal segments, hence causing irreversible lesions and compromising the validity of the results.

This model is useful for the study of host-pathogen interactions and pathogenesis of intestinal diseases caused by infectious agents. The model proposed can be adapted depending on the study by changing the number of loops created or by modifying the length of each loop. Before implementing such changes, it is recommended to standardize the method to ensure significant results. Limitations of this technique are skill-oriented and only properly trained personnel should perform this surgery on live animals.

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Disclosures:

The authors have nothing to disclose.

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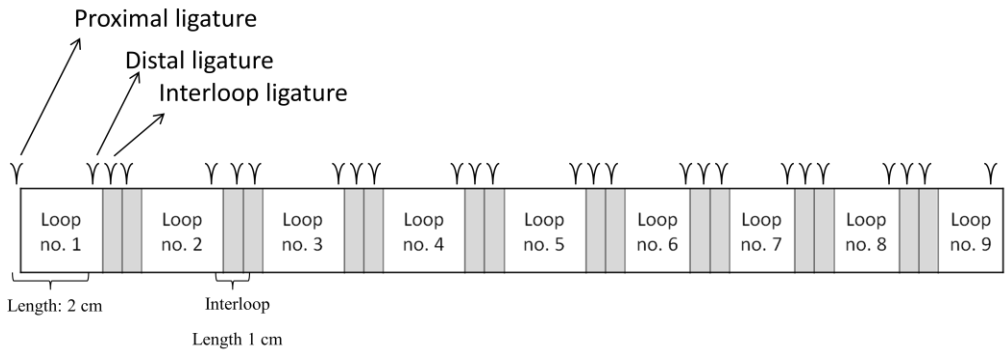

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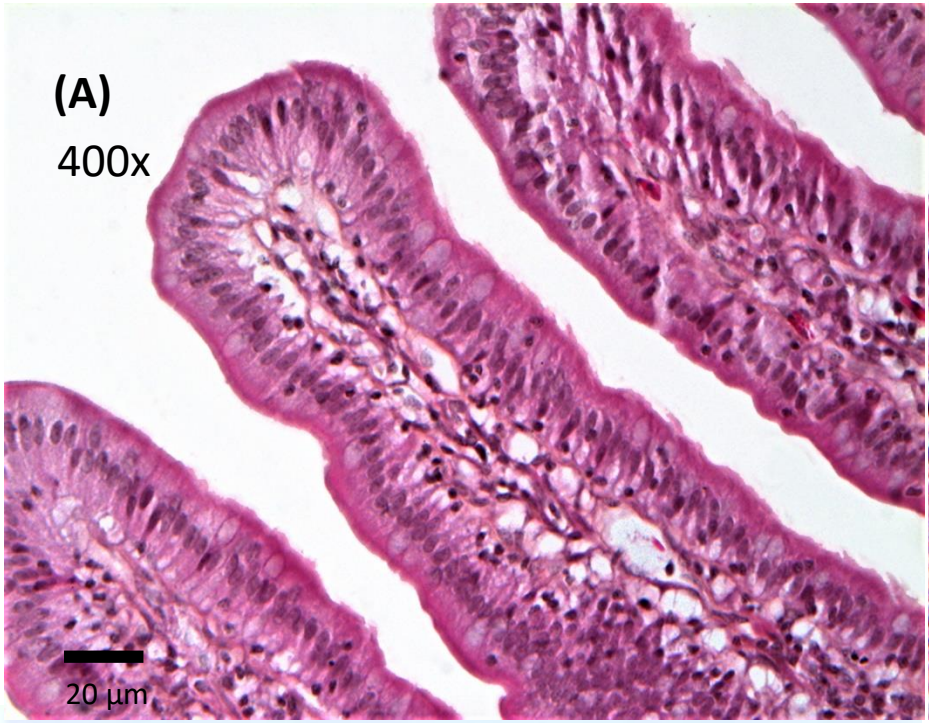

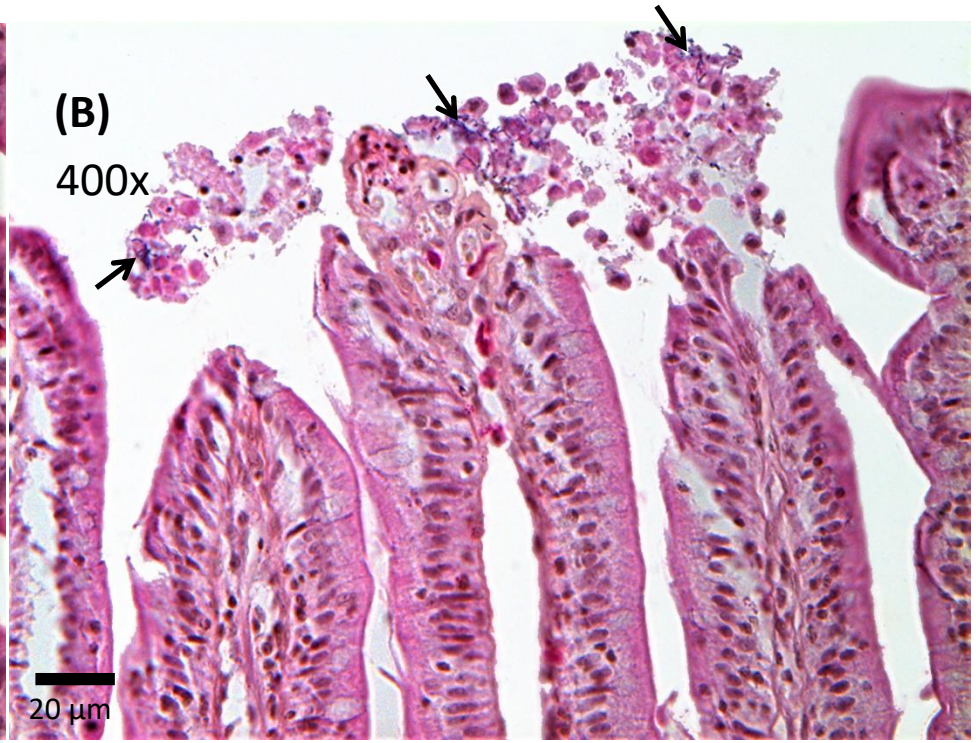

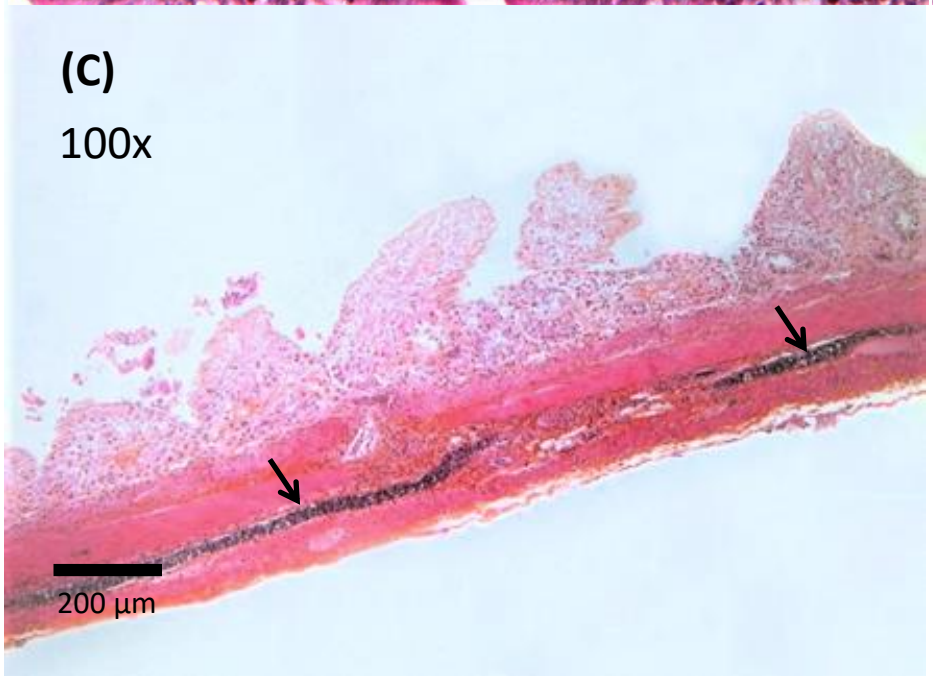

Materials Table

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
EZ-Scrub 747	Becton Dickinson and Company		4% chlorhexidine gluconate detergent impregnated sterile brush. No catalog number available. Web address: https://www.bd.com/en-us/offerings/capabilities/infection-prevention/surgical-hand-scrubs/ez-scrub-preoperative-surgical-scrub-brushes
Isopropyl alcohol 70% USP 4 L	Commercial isopropyl alcohol	P016IP70	Web address: http://www.comalc.com/products/
Chlorexidine	Sigma-Aldrich	282227-1G	Chlorhexidine gluconate solution, must be diluted to 4%. Web address: http://www.sigmaaldrich.com/catalog/product/aldrich/282227?lang=fr&region=CA&gclid=EAIaIQobChMI8vHr4_OY1wIV3rrACh2ZWQKuEAAYAiAAEgLKx_D_BwE
Surgical Drape Small (27 inch x 24 inch) with 3 x 6 inch Fenestration	Veterinary Specialty Products	32724	Web address: http://www.vetspecialtyproducts.com/index.cfm?fuseaction=ecommercecatalog.detail&productgroup_id=15
Scalpel blades #3	Swann-Morton	301	Web address: https://www.swann-morton.com/product/16.php

Sterile saline NaCl 0.9%	Sigma-Aldrich	S8776-100ML	Web address: http://www.sigmaaldrich.com/catalog/product/sigma/s8776?lang=fr&region=CA
Vicryl 3-0	Ethicon	D9003	Polyglactin multifilament absorbable suture material. Web address: http://www.ethicon.com/healthcare-professionals/products/wound-closure/absorbable-sutures/coated-vicryl-polyglactin-910-suture
Syringe 1 ml with 26G needle	Becton Dickinson and Company	329652	Web address: https://www.bd.com/en-us/offerings/capabilities/diabetes-care/insulin-syringes/bd-1-ml-conventional-insulin-syringes
Brain Heart Infusion	Sigma-Aldrich	1104930500	Web address: http://www.sigmaaldrich.com/catalog/product/mm/110493?lang=fr&region=CA&cm_sp=Insite-_-prodRecCold_xorders-_-prodRecCold2-1
Eppendorf tube	Sigma-Aldrich	T9661-500EA	Microcentrifuge tube. Web address: http://www.sigmaaldrich.com/catalog/product/sigma/t9661?lang=fr&region=CA&gclid=EAIaIQobChMI-YizzfiY1wIVRkCGCh30SQjuEAAYAiAAEgLK5fD_BwE

Formalin solution, buffered neutral, 10%	Sigma-Aldrich	HT501128-4L	Ratio tissue : formalin of 1: 10 for adequate fixation. Web address: http://www.sigmaaldrich.com/catalog/product/sigma/ht501128?lang=fr&region=CA
Clostridium perfringens strains	Université de Montréal, Chaire en recherche avicole	N/A	Specific to each laboratory, available upon request to correspondant author

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Title of Article: A ligated intestinal loop model in anesthetized specific pathogen free chickens to study Clostridium perfringens virulence.

Author(s): Eric Parent, Patrick Burns, André Desrochers, and Martine Boulianne

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Department: Department of Clinical Sciences, Faculty of Veterinary Medicine,

Institution: Université de Montréal

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For the comment on the Gram-positive rods, this stain (HPS), very similar to the HE stain, do not identify them. We used a Twort's staining to ensure these large rods where Gram-positive.

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