

Video Article

The Bovine Lung in Biomedical Research: Visually Guided Bronchoscopy, Intrabronchial Inoculation and *In Vivo* Sampling Techniques

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Abstract

There is an ongoing search for alternative animal models in research of respiratory medicine. Depending on the goal of the research, large animals as models of pulmonary disease often resemble the situation of the human lung much better than mice do. Working with large animals also offers the opportunity to sample the same animal repeatedly over a certain course of time, which allows long-term studies without sacrificing the animals.

The aim was to establish *in vivo* sampling methods for the use in a bovine model of a respiratory *Chlamydia psittaci* infection. Sampling should be performed at various time points in each animal during the study, and the samples should be suitable to study the host response, as well as the pathogen under experimental conditions.

Bronchoscopy is a valuable diagnostic tool in human and veterinary medicine. It is a safe and minimally invasive procedure. This article describes the intrabronchial inoculation of calves as well as sampling methods for the lower respiratory tract. Videoendoscopic, intrabronchial inoculation leads to very consistent clinical and pathological findings in all inoculated animals and is, therefore, well-suited for use in models of infectious lung disease. The sampling methods described are bronchoalveolar lavage, bronchial brushing and transbronchial lung biopsy. All of these are valuable diagnostic tools in human medicine and could be adapted for experimental purposes to calves aged 6-8 weeks. The samples obtained were suitable for both pathogen detection and characterization of the severity of lung inflammation in the host.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51557/>

Introduction

The Values of Large Animal Models in Biomedical Research

In modern interdisciplinary biomedical research, animal models are still indispensable to elucidate complex interactions — related to health or a disease status — within mammalian organisms. Despite 17 Nobel prizes being awarded to scientists that studied cattle, horses, sheep, or poultry as models for biomedical research¹, nowadays the vast majority of animal experiments are undertaken with rodents, while less than 1% of the studies are working with domestic animals or livestock.

Small animals offer many practical advantages (*i.e.* low cost, genetic malleability, high throughput, availability of numerous genetic, and immunologic tools and kits), and genetically modified murine models are generally accepted to perform mechanistic studies discovering particular molecular pathways. In biomedical research of complex systems, the biological relevance and clinical usefulness of mice models is becoming more and more questionable. They could be misleading and bear the risk of oversimplification of biological complexity²⁻⁹.

Due to inter-species peculiarities, no single animal species will completely mirror the human situation, and the use of more than one model seems to be beneficial in an interdisciplinary biomedical research approach. In the context of translational medicine, large animals offer the opportunity to serve as comparative models providing results with high biological relevance of dual use for both human and animal health¹. Remarkably, the human genome is more closely resembled by the bovine genome than by the genome of laboratory rodents. It also has been confirmed recently that, compared to other taxa, the genome of mice is much more rearranged¹⁰⁻¹².

In a complex study design, use of livestock offers the unique opportunity of intra-individual, long-term studies by repeated collection of a variety of samples *in vivo* from one-and-the-same individual without sacrificing the animal. Therefore, functional, inflammatory and morphological changes can be monitored in the same subject over a certain period of time¹³.

The Bovine Lung as a Suitable Respiratory Model

Due to the high number of significant differences in lung anatomy, respiratory physiology, and pulmonary immunology, mice do not reproduce many important pathophysiological aspects of human pulmonary disease. This must be taken into account when using them as animal models of respiratory disease^{2,9,14-16}. Although peculiarities of anatomy and structure do exist for each mammalian lung, functional characteristics (*i.e.* lung volumes, airflows and respiratory mechanics) are better comparable between adult humans and calves due to similar body weights (50-100 kg).

The species-specific characteristics of the bovine lung are summarized as follows: the left lung consists of two lobes (*lobus cranialis*, which is divided into two segments, and *lobus caudalis*), while the right lung consists of four lobes (*lobus cranialis*, *lobus medius*, *lobus caudalis*, and *lobus accessorius*). Unlike the lung anatomy of most other mammals, the bronchus of the right cranial lobe branches directly from the right lateral side of the trachea. With respect to subgross anatomy, the bovine lung presents a high degree of lobulation and a high percentage of interstitial tissue^{17,18} leading to a relatively low specific lung compliance and a higher pulmonic tissue resistance¹⁹. Therefore, the required breathing activity is rather high compared to other species^{20,21}. The high degree of lobulation leads to strong independence of the segments. Thus, inflammatory processes are limited by connective tissue septa, and diseased and healthy segments often lie within the same lobe. Due to the lack of collateral airways, the bovine lung is particularly suited to mirror obstructive pulmonary dysfunctions¹³. Regarding the vasculature in the bovine lung, the small pulmonary arteries show very prominent smooth muscle layers. Therefore, the calf may also serve as a well-established animal model of pulmonary hypertension or vascular remodeling²²⁻²⁴.

With respect to respiratory infections, naturally occurring diseases exist in livestock that share many similarities with the comparable disease in man. Typical examples are bovine tuberculosis²⁵, respiratory syncytial virus (RSV) infections in calves²⁶⁻²⁸, or naturally acquired *Chlamydia* infections²⁹. Thus, large animal models do closely resemble the situation in the natural host. Therefore, they are most useful for studying host-pathogen interactions and the complex pathophysiology of the corresponding disease in human beings^{30,31}.

As a biologically relevant model of respiratory *Chlamydia psittaci* infection, calves were chosen since bovines represent natural hosts for this pathogen³²⁻³⁵. Information obtained from this model, with respect to pathogenesis of the disease or possible transmission routes between animals and humans, will help to broaden our knowledge with impact for both cattle and man. The model can also help to verify generally accepted and alternative therapeutic options for the elimination of pulmonary *C. psittaci* infections, which is, again, of interest in both veterinary and human medicine.

Techniques Applied to and Specimens Obtainable from the Bovine Respiratory System

This paper describes and illustrates the techniques and diagnostic methods applicable to the bovine lung and used in our model to evaluate both the effects of the pathogen on the mammalian lung and the efficacy of therapeutic intervention.

Bronchoscopy has been performed in human medicine since the 1960s and is considered a safe procedure³⁶. In calves, experimental bronchoscopy was described in 1968 for the first time³⁷. The intrabronchial application of pathogens was suggested by Potgieter *et al.* as a reliable method to produce lower respiratory tract disease in calves³⁸ and is now a widespread method in bovine research^{34,39,40}. Intrabronchial inoculation of a defined amount of the pathogen under videoendoscopic control allows for selective placement of the infectious agent in the lung. This leads to consistent clinical and pathological findings in all animals³⁴ and allows targeted sampling of lung regions that are expected to be altered due to pathogen exposure.

Bronchoalveolar lavage fluid (BALF) is a well-described indicator for the presence and severity of lung inflammation. The bronchoalveolar lavage (BAL) is a standard procedure in human medicine for the diagnosis of a variety of respiratory diseases⁴¹. In live cattle, BAL was introduced by Wilkie and Markham in the late seventies of the last century⁴². It was considered a safe and repeatable technique to study the lower respiratory tract of cattle. Due to the lack of sufficient data on BALF parameters in healthy animals, in 1988 Pringle *et al.* performed BAL on healthy calves with a flexible fiberoptic bronchoscope. The authors also pointed out the need to standardize BAL protocols under experimental conditions to acquire comparable results⁴³. BAL is still used as an *in vivo* sampling method in calves⁴⁴⁻⁴⁶.

Bronchial brushing is commonly used in human medicine as a diagnostic tool to sample neoplastic lesions or for microbiological analysis³⁶. For research purposes, primary cell cultures of epithelial cells harvested by cytological brushing can be obtained⁴⁷. In cattle, the use of bronchial brushings for microbiological analysis has been described to characterize the microbial environment of the lung⁴³.

Transbronchial lung biopsy provides lung tissue samples and is a valuable diagnostic tool for diffuse lung diseases in humans. Iatrogenic pneumothorax and procedure-related hemorrhage are complications associated with this technique. Their incidence is reported to be less than one percent in human patients⁴⁸. Transbronchial lung biopsy is not a common method for the use in cattle, due to the high cost of the equipment required and the time needed to obtain biopsies. Instead, transcutaneous lung biopsies are more convenient under field conditions⁴⁹⁻⁵¹.

Protocol

Ethics Statement

This study was carried out in strict accordance with European and National Law for the Care and Use of Animals. The protocol was approved by the Committee on the Ethics of Animal Experiments and the Protection of Animals in the State of Thuringia, Germany (Permit Number: 04-004/11). All experiments were performed under supervision of the authorized institutional Agent for Animal Protection. Bronchoscopy was strictly performed under general anesthesia. During the study, every effort was made to minimize discomfort or suffering.

General remarks

The described techniques have been developed for calves of approximately 6-8 weeks of age, weighing about 60-80 kg. For the use in other large animal species or calves different in age and bodyweight, the techniques must be adapted to fit the size and weight and take into account the lung anatomy of the particular animal species. All equipment used must be sterile. *Chlamydia psittaci* is a zoonotic bacterium which can cause respiratory and general disease in humans. The non-avian *C. psittaci* strain DC15 used in the present protocol must be handled under biosafety level 2. All work with the pathogen and with infected animals must be performed wearing personal protective equipment, such as

a respirator, a splash water proof jumpsuit, rubber boots and gloves. Wearing a suitable respirator is of great importance, since the natural route of infection for *C. psittaci* is aerogen. Packaging and labeling of samples must be disinfectant proof since all things must be treated with a disinfectant effective against chlamydiae according to the manufacturer's instructions before leaving the animal housing unit.

1. Preparing the Animal for Bronchoscopy

1. Determine the weight of the calf for dosage of anesthetics.
2. Place an intravenous (IV) access in the left jugular vein.
3. First, slowly inject xylazine (0.2 mg/kg bodyweight) over approximately 30 sec, then, after sedation occurs, inject ketamine (2.0 mg/kg bodyweight).
4. Lift the animal onto the table and place it in right lateral recumbency. Once the animal is adequately positioned on the table, check if the IV access is still in place and readjust it if necessary. During anesthesia, regularly check the eyelid reflex to determine the depth of anesthesia.
5. Once the animal is breathing steadily, have someone pull the tongue out and stretch the neck. Place a metal tube speculum in the animal's mouth, using slight rotating movements. Push the speculum forward under sight control, using a flashlight, until the larynx is visible.
6. Maintain anesthesia throughout the whole endoscopic procedure by injecting a bolus of 7 mg xylazine and 70 mg ketamine as needed.

2. Inoculation (Inoculation Sites: Figure 1)

1. Prepare 3 syringes with inoculum, containing 1, 2, and 5 ml of the inoculum.
2. Insert a Teflon tube into the endoscope's working channel. The tube should not protrude from the endoscope's tip.
3. Insert the endoscope through the metal speculum. Slight readjustments of the speculum might be necessary to enable the passing of the larynx. The *Bronchus trachealis*, which branches off to the right side, helps to align the picture on the monitor.
4. Attach the syringe with 5 ml inoculum to the end of the Teflon tube. Navigate the tube into the branches where the inoculum shall be deposited and apply the desired amount (Right lung: *Lobus medius*: 0.5 ml, *Lobus accessorius*: 0.5 ml, *Lobus caudalis*: 0.5 ml and 1.0 ml; Left lung: *Lobus cranialis*, *Pars cranialis*: 0.5 ml, *Pars caudalis*: 0.5 ml, *Lobus caudalis*: 1.5 ml). Attach the syringe with 1 ml inoculum to the tube, then navigate to the *Bronchus trachealis* and deposit the inoculum (*Lobus cranialis*, *Pars caudalis*: 1.0 ml). It is helpful to always approach the localizations in the same order.
5. Remove the endoscope and the speculum.
6. Spray 1 ml of the inoculum into each nostril with an actuator.
7. Bring the animal back to the stable and place it in prone position for waking up. Do not leave the animal unattended or in the company of other animals until it has regained sufficient consciousness to maintain sternal recumbency. The recovery stable should be air-conditioned, since the animal's ability for thermoregulation is decreased under general anesthesia.

NOTE: First clinical signs should occur about 24-36 hr after inoculation, depending on the pathogen used.

3. Sampling Procedures (Sampling Sites: Figure 2)

1. Prepare the animal as described in steps 1.1-1.6.
2. Bronchoalveolar lavage
 1. Place 5 syringes each containing 20 ml of sterile isotonic saline, in a water-bath and allow them to warm up to approximately 38 °C.
 2. Insert a lavage catheter into the endoscope's working channel, then insert the endoscope into the metal speculum and navigate forward into the main bronchus of the left lung until the "wedge position" is reached where the endoscope cannot be pushed any further ahead.
 3. One after another, attach the syringes with the warm NaCl solution to the lavage catheter, instill the fluid and aspirate it directly. The bronchoalveolar lavage fluid must be stored in siliconized glass bottles and put on ice immediately after recovery to prevent the alveolar macrophages from attaching to the glass surface. Note both the amount of instilled saline and the amount of recovered fluid.
 4. Remove the lavage catheter from the working channel.
3. Bronchial brushing
 1. Navigate the endoscope to the desired sampling location, in the described protocol this is the *Bifurcatio tracheae*.
 2. Cover the brush with the tube before inserting it into the endoscope's working channel until the brush's tip appears on the monitor.
 3. Push the brush with the plastic tube forward about 5 cm and uncover it from the plastic tube by pushing the handle, then navigate it to the location that is to be brushed.
 4. Rub off epithelial cells by gently pushing and pulling the brush back and forth while navigating the endoscope to ensure contact between the brush and the wall of the bronchus. Stop rubbing when bleeding occurs. Cover the brush with the tube before pulling it out of the working channel.
 5. Prepare up to five smears on microscope slides by gently rolling the brush over the slide. Fixate the smears in cold methanol for 10 min, air dry and store at -20 °C.
 6. The brush can be rinsed in various media, depending on the purpose of the sampled cells. If taking multiple brushings with the same brush, be sure to only rinse it in media which do not irritate the mucous membrane.
4. Transbronchial lung biopsy
 1. Navigate the endoscope to the desired sampling location, in the described protocol this is the *Pars caudalis* of the *Lobus cranialis*. Before inserting the biopsy forceps into the working channel open and close it a couple of times to ensure that it is working smoothly.
 2. Push the biopsy forceps into the caudal branch of the *Bronchus trachealis* until a slight resistance occurs. Pull back 2-3 cm, open the forceps, push forward about 2 cm, close the forceps, pull back and remove the forceps from the working channel. This requires some practice.

3. Carefully remove the tissue from the biopsy forceps, using a needle or small forceps. Depending on the further use of the tissue, store it in liquid nitrogen or a suitable fixation medium. This should happen right after removal to prevent autolytic processes.
5. Post-procedural treatment
 1. Bring the animal back to the stable and place it in prone position for waking up. Do not leave the animal unattended or in the company of other animals until it has regained sufficient consciousness to maintain sternal recumbency. The recovery stable should be air-conditioned, since the animal's ability for thermoregulation is decreased under general anesthesia.
 2. Monitor the animal closely for signs of pneumothorax for the next 24 hr. Provide feed and fresh water when the animal has regained full consciousness.

Representative Results

Course of Disease

The effect of the pathogen on the animals' health can be assessed by clinical examination. In our respiratory infection models, animals were examined twice daily and clinical observations were recorded using a scoring system. Additional information was captured by performing other *in vivo* sampling methods, e.g. collection of blood and swabs or lung function measurement. Pathological examinations were carried out at different time points after inoculation to describe the progress of the infection³²⁻³⁴.

BALF Recovery Rate

The recovery rate of the instilled fluid was $83.05 \pm 4.58\%$ (mean \pm SD).

Pathogen Detection

Recultivation of the pathogen can be performed from bronchial brushings. Also, PCR screening of various samples is possible to detect the pathogen, e.g. tissue biopsy, cytology brush sample, BALF-cells⁵² or pharyngeal swab. Visualization of the pathogen is possible by performing immunohistochemistry of frozen sections of the lung biopsies and sedimentation preparations of the BALF-cells (**Figure 3**). In previous experiments, PCR of blood samples and swabs (conjunctival, fecal, nasal) were performed to characterize the spreading and shedding of the pathogen³².

Markers of Local Inflammation of Lung Tissue

In the BALF, various parameters of lung inflammation can be studied. The total cell count and the proportion of neutrophils usually increase when lung inflammation is present. For cell differentiation, sedimentation preparations of BALF-cells can be stained according to Giemsa and differentiated using oil immersion (**Figure 4**). Cellular and liquid proportions of the BALF are separated by centrifugation (300 x g; 20 min). The BALF-supernatant contains various markers that change during inflammatory processes in the lung and can be studied under experimental conditions. Examples are total protein and eicosanoids^{29,34}.

A schematic overview of the potential further use of the described samples is shown in **Figure 5**.

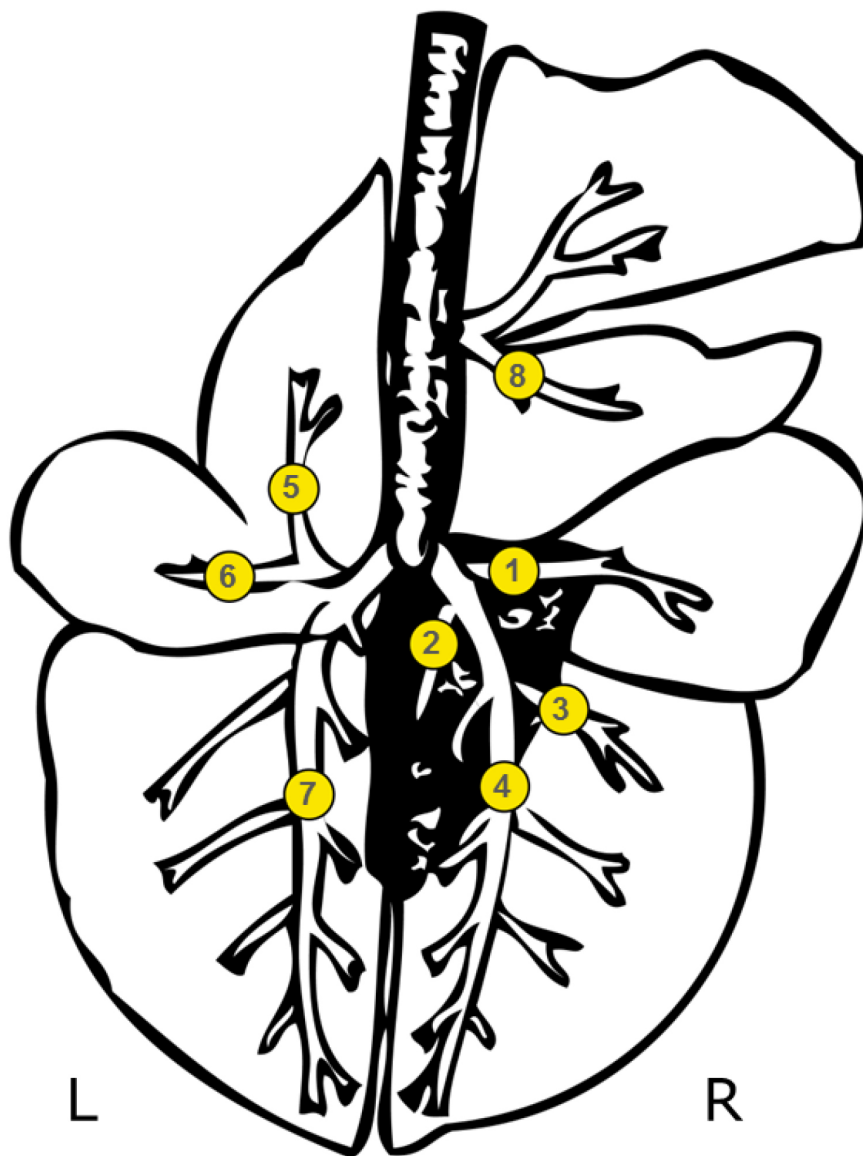


Figure 1. Scheme of the bovine lung with inoculation sites (yellow). The numbers indicate the order in which the inoculum is administered into the different bronchi. R: right; L: left. Right lung: **1** *Lobus medius*: 0.5 ml, **2** *Lobus accessorius*: 0.5 ml, **3** *Lobus caudalis*: 0.5 ml and **4** 1.0 ml; Left lung: *Lobus cranialis*, **5** *Pars cranialis*: 0.5 ml, **6** *Pars caudalis*: 0.5 ml, **7** *Lobus caudalis*: 1.5 ml, **8** *Lobus cranialis, Pars caudalis*: 1.0 ml.

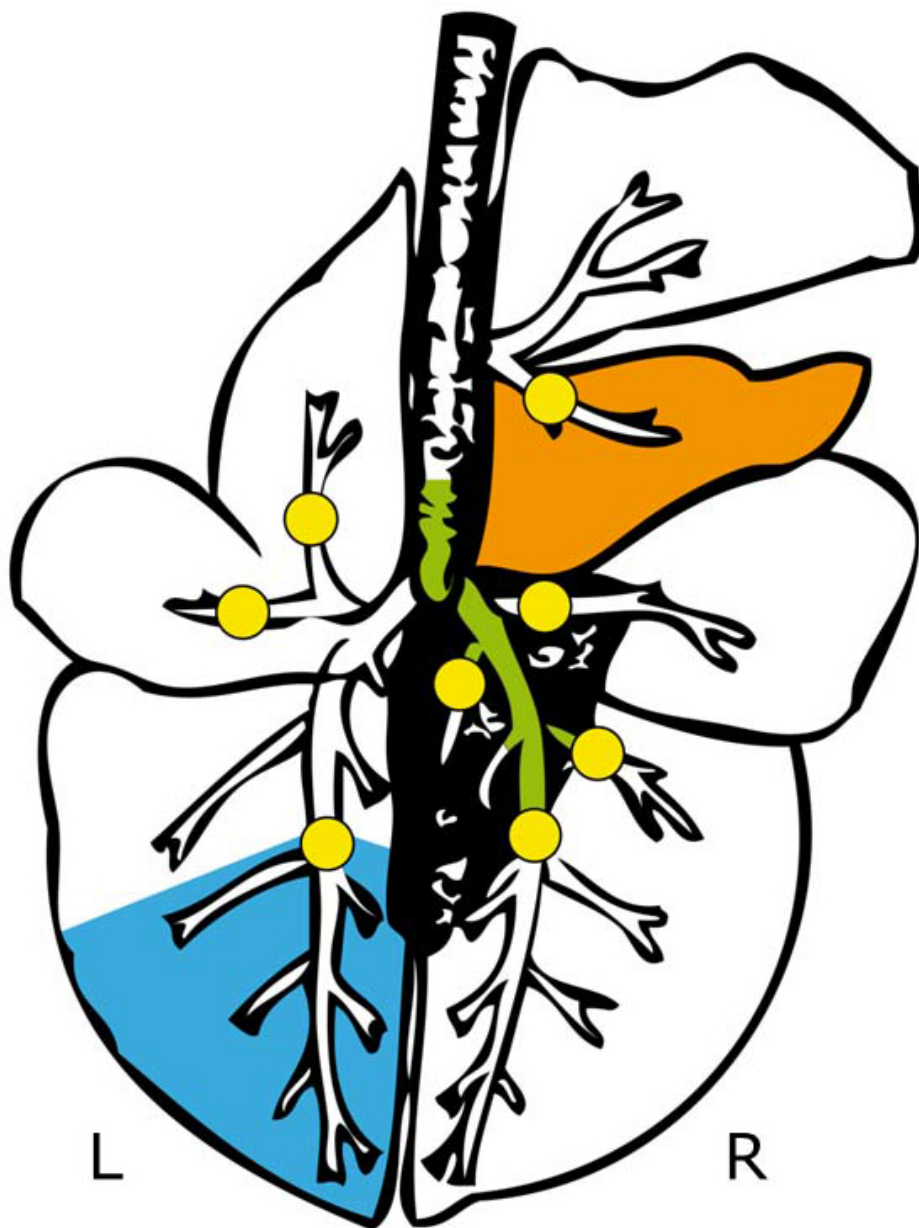


Figure 2. Scheme of the bovine lung with inoculation sites (yellow) and sampling sites: bronchoalveolar lavage (blue), bronchial brushing (green), and lung biopsy (orange). Note that all samples are obtained from regions where the pathogen was deposited before. R: right, L: left.

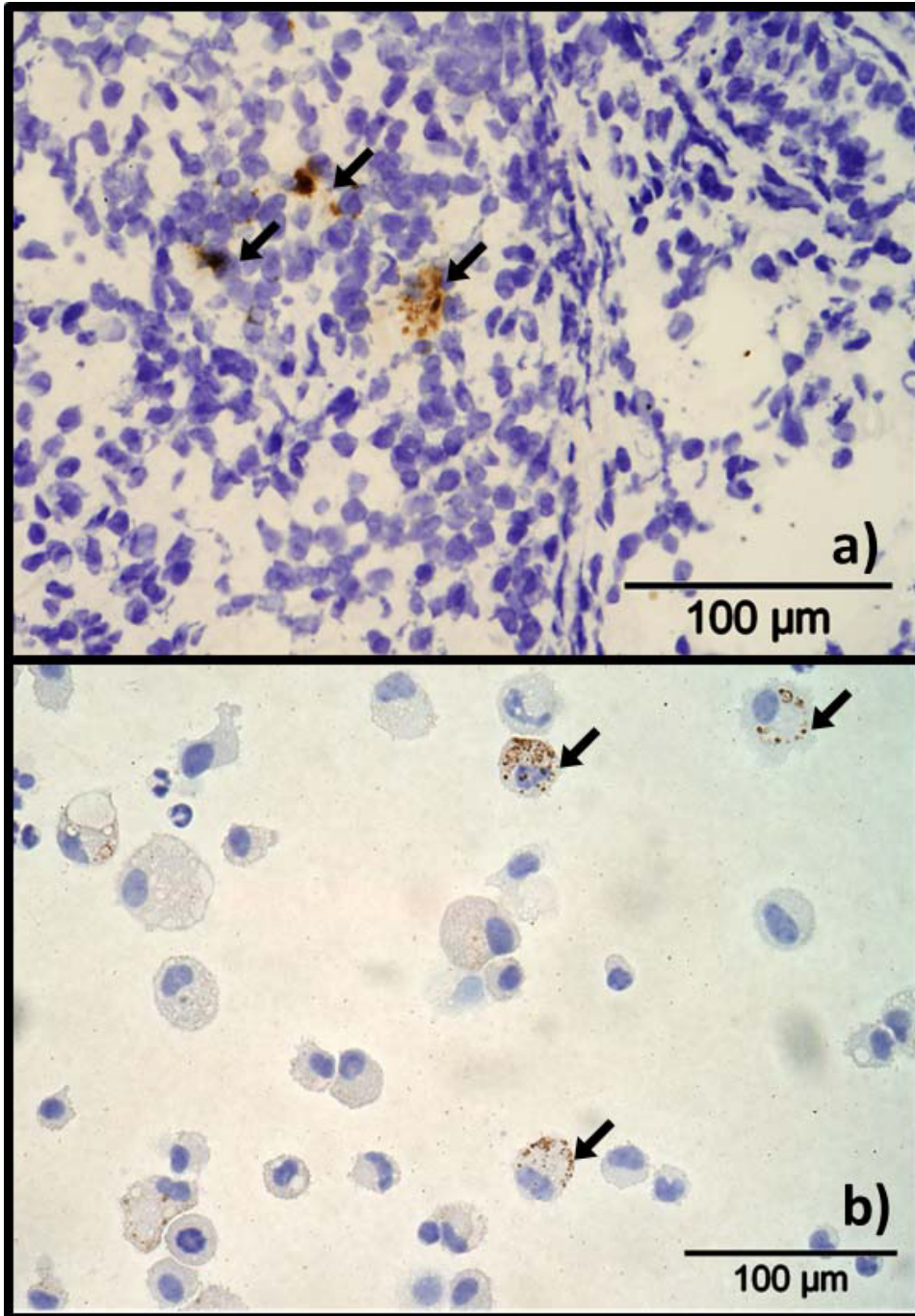


Figure 3. a) Lung biopsy from a calf inoculated with *Chlamydia psittaci* 4 days after inoculation (dpi), b) cellular sediment of BALF from a calf inoculated with *C. psittaci* 9 dpi. Immunohistochemical labeling for chlamydiae. Chlamydial inclusions (arrows) are present in the lung (a) and in alveolar macrophages (b). Hematoxylin counterstain.

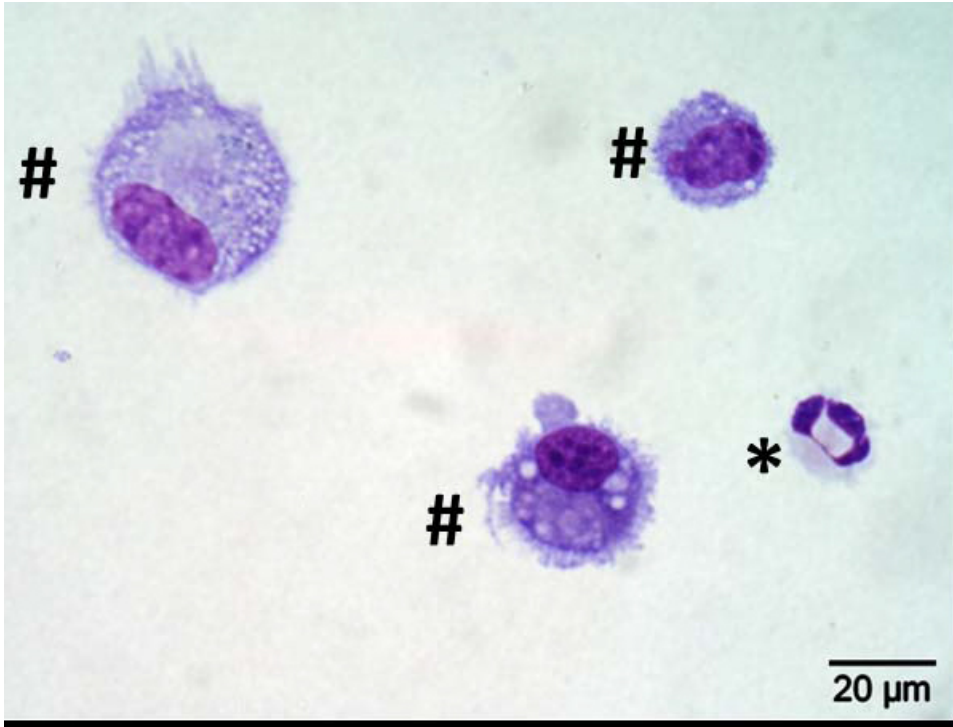


Figure 4. Cellular sediment of BALF from a calf inoculated with *C. psittaci* 9 dpi. Alveolar macrophages (#) are the predominant cell type in the BALF. The amount of neutrophil granulocytes (*) increases when inflammatory processes are present. Modified Pappenheim staining.

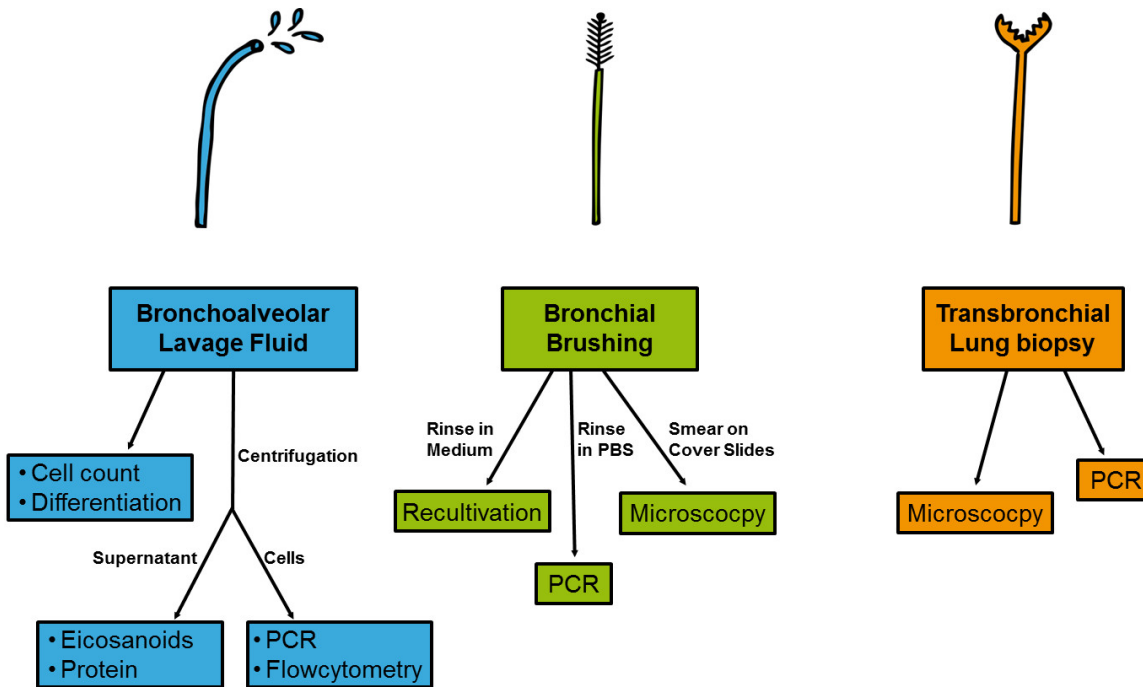


Figure 5. Possible methods for sample preparation. [Please click here to view a larger version of this figure.](#)

Discussion

A bronchoscopic method of inoculation was developed and various bronchoscopic sampling methods were adapted to be used in large animals under experimental conditions. The described techniques are easy to learn, even for examiners with little experience in endoscopy. The process of bronchoscopy is minimally invasive and no adverse effects associated with the methods of the inoculation, as well as the described sampling methods (BAL, transbronchial lung biopsy, bronchial brushing), were ever seen in any of the animals. The complications associated with transbronchial lung biopsies in humans are bleeding and pneumothorax⁴⁸, none of these were seen in the calves that underwent this procedure.

The transbronchial lung biopsy is more time consuming and requires more equipment than the transcutaneous method, but it is less invasive and does not bear the risk of wound infection.

The visually controlled, endoscopic method of inoculation allows the deposition of a defined amount of pathogen at specific sites of the lung. Thus, it results in very consistent clinical and pathological findings in all inoculated animals³²⁻³⁴. However, it does not resemble all features of natural infection in calves. In a model of a respiratory *C. psittaci* infection, the described technique of inoculation led to lung lesions associated with the sites of pathogen placement³⁴, whereas, in naturally acquired infections calves usually develop pneumonia of the apical lobes. This fact has to be taken into account when interpreting the relevance of experimental findings in the context of natural acquired lung infections in bovines.

Videoscopic BAL allows sampling a defined area of the lung. For experimental purposes, this is an advantage compared to the use of a nasal catheter under blind conditions. Due to the anatomy of the bovine lung, the blindly inserted catheter would be pushed to the right diaphragmatic lobe in most cases^{53,54} and the examiner has no influence on the area of the lung that is lavaged. Another advantage of the endoscopic BAL in anesthetized calves in lateral recumbency is the high average recovery rate of instilled fluid of more than 80%. A comparison with other studies reveals that, in standing, sedated calves, a recovery of 133.3 ± 1.6 ml⁴⁶ and 127.13 ± 3.53 ml⁴⁵ after instillation of 240 ml fluid into the caudal lobe is reported. In sedated calves in sternal recumbency 51% of the instilled fluid could be recovered from the cranial lobe and 62% from the caudal lobe⁴³. This means that approximately half of the instilled fluid could be recovered in upright position of the calf. Depending on the amount of BALF needed for further sample preparation, this might not leave enough material to carry out all desired experiments. BAL in cattle has been used by many research groups and many different parameters have been examined under various conditions. Most authors performed lavage of the basal lobes^{43,45,46}, but the amount of fluid used for lavage differs between the research groups. This leads to inconsistency in dilution of the recovered cells, proteins and other substances, making it difficult to compare the findings from different publications. Therefore, for the use in cattle it is recommended to lavage with five fractions of 20 ml (*i.e.* 100 ml in total) body warm, isotonic saline, which are recovered immediately after instillation. When using a lavage catheter with a large diameter (*i.e.* >2 mm), the volume of each fraction needs to be slightly increased, depending on the amount of fluid that will remain in the catheter.

The highly segmented anatomy of the bovine lung leads to a methodical limitation; results obtained from one part of the lung may not be true for the rest of the lung. Since there is no sight control of the whole lung area probed by transbronchial biopsy and lavage, the examiner cannot know whether the sampled areas were healthy or diseased. Therefore, it is very important to sample locations where the pathogen was inoculated before in order to have a higher recovery rate of the pathogen and to have a higher possibility of sampling diseased lung areas. Another limitation is the increased anesthetic risk in animals of poor clinical condition. The described methods should only be used in models of mild to moderate disease to keep the burden for the animals as low as possible. General anesthesia in ruminants should always be kept as short as possible, as the gas development in the rumen increases the anesthetic risk in these species. Animals must be placed in prone position immediately after bronchoscopy to allow the efflux of the developed gas and must be monitored closely until they are completely recovered from anesthesia. Also, the described techniques are not suitable for sampling intervals of less than 24 hr.

The described protocol can be adapted to other infectious agents. Endoscopic inoculation of various pathogens has been described, such as *C. psittaci*³²⁻³⁴, *Pasteurella haemolytica*^{38-40,42}, *Haemophilus somni*⁵⁵, and bovine viral diarrhoea virus⁴⁴. Also, the sites of pathogen deposit in the lung can be adapted to the desired model. When choosing the sampling sites, some important facts have to be taken into account: (i) Sampling sites should be chosen based on the locations of inoculation and on the expected pathological findings. (ii) When necropsy is to be performed care must be taken to leave enough unsampled lung areas for *ex vivo* sampling. (iii) Sampling site locations must be chosen so they can be reached with the equipment. Especially for transbronchial lung biopsy, there are limitations due to the length of biopsy forceps. (iv) The order of sampling is important, bronchial brushing and transbronchial lung biopsy might lead to minor bleeding, which would contaminate the BALF. Therefore, BALF must always be obtained first. When using the protocol in other species, the species-specific lung anatomy must be taken into account.

Disclosures

The authors have nothing to disclose.

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