# final report

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# A Pilot Study of the Primary Causative Agents of Pneumonia in Australian Sheep

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# Abstract

Pneumonia in sheep is a complex disease involving the interaction of pathogen, host and environmental factors.

The objectives of the project were to conduct a pilot study of abattoir sheep to investigate the primary causative agents of pneumonia in Australian sheep and provide preliminary information on the ovine respiratory pathogens circulating in sheep flocks in south eastern Australia.

The pilot study included three abattoirs, including one in South Australia and two in New South Wales.

More than 200 lung samples, including 80 matched tissue and swab samples, and 100 serum samples were collected during five abattoir visits. Lungs with typical signs of broncho-pneumonia (cranio-ventral consolidation with or without pleural involvement) were targeted for sample collection. Of the samples collected, 171 samples were tested for *Mycoplasma ovipneumoniae* by culture and/or qPCR, with 113 (66%) yielding a positive result.

# Executive summary

Pneumonia in sheep is a complex disease involving the interaction of pathogen, host and environmental factors.

The National Sheep Health Monitoring Program data indicates that up to 50 per cent of Australian sheep flocks have endemic pneumonia.

The objectives of the project were to conduct a pilot study of abattoir sheep to investigate the primary causative agents of pneumonia in Australian sheep and provide preliminary information on the ovine respiratory pathogens circulating in sheep flocks in south eastern Australia.

The pilot study included three abattoirs, including one in South Australia and two in New South Wales. All three of the abattoirs were Export Abattoirs, although at the time of the study, one was processing lambs for the domestic Australian market. Samples were collected between July 2018 and May 2019.

More than 200 lung samples, including 80 matched tissue and swab samples, and 100 serum samples were collected during five abattoir visits. Lungs with typical signs of broncho-pneumonia (cranio-ventral consolidation with or without pleural involvement) were targeted for sample collection. Of the samples collected, 171 samples were tested for *Mycoplasma ovipneumoniae* by culture and/or qPCR, with 113 (66%) yielding a positive result.

Testing for respiratory viruses was not carried out.

Recommendations for future research include:

1. A prevalence survey of pneumonia in Australian abattoir sheep that includes:
    a. Four sampling periods – spring, summer, autumn, winter
    b. Four abattoirs around Australia, to provide regional spread of sheep source
    c. Recording of line information, i.e. sheep breed, age and source/region
    d. Lung scoring
    e. Collection of swabs for PCR/culture
    f. Collection of lung tissue for histopathology

    This will provide a comprehensive understanding of the aetiological agents involved in pneumonia in Australian sheep and help underpin the development of control measures.

2. Trialling of a non-steroidal anti-inflammatory drug in combination with antibiotics in therapeutic protocols for bacterial pneumonia in sheep

    Anti-inflammatory drugs are commonly used in the treatment of respiratory disease in other species, including humans and cattle. However, these drugs are rarely used in the treatment of respiratory disease in Australian sheep.

    The non-steroidal anti-inflammatory drug meloxicam in registered for use is sheep in Australia with a general claim for pain and inflammation (Metacam 20 mg/mL). Research to investigate clinical improvement in sheep with pneumonia with combined treatment with

appropriate antibiotics and meloxicam, as well as reduction in lung lesions, pleural adhesions and carcase trimming is warranted (Politis *et al*. 2019).

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# 1 Background

Pneumonia in sheep is a complex disease involving the interaction of pathogen, host and environmental factors.

The National Sheep Health Monitoring Program data indicates that up to 50 per cent of Australian sheep flocks have endemic pneumonia.

The level of ovine pneumonia observed in the National Sheep Health Monitoring Program is consistent with a previous study of the prevalence of pneumonia in South Australian sheep flocks, which also reported that 50 per cent of sheep flocks in that State are affected by pneumonia/pleurisy based on analysis of data from the South Australian Enhanced Abattoir Surveillance Program (Meyer, 2013).

Unlike the parasitic infections such as hydatids, liver fluke and sheep measles, it isn't possible to determine the primary aetiological agent causing ovine pneumonia/pleurisy based solely on necropsy findings. Further testing is usually required.

In cattle, there is good understanding of the aetiological causes of Bovine Respiratory Disease through research conducted internationally and in Australia. A range of vaccine and antimicrobial agents are available to producers to manage the disease. The same is not true of sheep, in which research and control options are limited.

*Mycoplasma ovipneumoniae* is a respiratory pathogen of sheep first isolated from a sheep flock in Australia in the 1950s (St. George *et al.,* 1971; Sullivan *et al.,* 1973a; Sullivan *et al.*, 1973b). It has subsequently been found in most sheep raising countries internationally. *M. ovipneumoniae* can cause both primary pneumonia and predispose to secondary infections with other bacteria. Infected sheep develop nasal discharge, ear infections (ear droop), hacking cough and reduced exercise tolerance due to pneumonia. Chronic infections are common, with bacteria persisting in the nasal cavities, pharynx, inner ear and lower airways/lungs. Infection passes from the ewe to the lamb soon after birth.

Three viruses have been associated with lamb pneumonia: parainfluenza virus type 3, adenovirus and respiratory syncytial virus (Caswell and Williams, 2007). Viral infection can also predispose to secondary infections with bacteria.

The first phase of pneumonia in sheep is usually a silent or sub-clinical pneumonia that is caused by viruses or mycoplasma acting independently or together (Caswell and Williams, 2007). In this phase, the lung lesions are generally small, usually located in the antero-ventral portion of the lung and consist mainly of varying degrees of collapse and consolidation. Pleurisy will also be present in some cases. The sub-clinical phase may resolve, remain in a chronic form or proceed to clinical pneumonia. Some affected lambs will reach slaughter weight without a measurable set-back. Lambs may have subclinical lesions as early as 45 days of age. The prevalence can be quite high (20-90%) and may vary between regions. Disease is discovered only at necropsy or in the abattoir. Bacteria and viruses may be present in nasal cavities, pharynx, inner ear, trachea and abnormal lung tissue.

The second phase of the disease is clinical enzootic pneumonia (Caswell and Williams, 2007). Clinical enzootic pneumonia is generally considered to be the result of a secondary bacterial infection superimposed upon the initial subclinical condition. *Pasteurella multocida* and/or *Mannhaemia haemolytica* are two bacteria commonly incriminated in clinical ovine pneumonia. *Truperella pyogenes* and *Bibersteinia trehalosi* may also be involved.

The trigger factors that predispose sheep to progress from the subclinical to the clinical phase of this disease are not well understood. Environmental stress is commonly described as a prime factor when the disease does progress from one phase to the other (Goodwin-Ray 2006) and recently Spanish researchers have linked clinical pneumonia in sheep caused by *M. ovipneumoniae* to high environmental temperature and low relative humidity (Fernández *et al.* 2016).

In Australia, *M. haemolytica* is largely considered to be the most significant secondary bacterial infection involved in ovine pneumonia. In contrast, recent research from Spain indicates that *M. haemolytica* may play a less important role, with *P. multocida, M. ovipneumoniae* and *T. pyogenes* more commonly isolated from lungs with lesions collected during post-mortem examination of culled sheep (Lacasta *et al.*, 2019).

Pneumonia in lambs has been associated with mortalities, carcase condemnations, reduced liveweight gain, increased time to reach slaughter weight and reduced carcase quality (i.e. thinner, smaller carcases) (Lacasta *et al.,* 2019). In New Zealand the cost of pneumonia in lambs has been estimated as 1.36 NZD per lamb (not taking into account associated mortalities), whereas in Spain it is estimated as 7% of final lamb value (taking into account carcass condemnation, mortalities, treatments, decreased average daily gain and reduced lamb quality (Lacasta *et al.* 2019).

During MLA Project B.AHE.0238 pleurisy trim data were collected on 101 lamb carcasses. Approximately half of the carcases (49 of 101, 48.5 per cent) had half the rib cage removed due to pleurisy and six had three-quarters (one carcase) or the full ribcage (five carcasses) removed.

The average trim weight when ribs were removed was 1.0 kg (one-quarter of the rib cage 0.50 kg, one-half the rib cage 1.0 kg, three-quarter to the full rib cage 1.9 kg). Based on these weights, trimming for pleurisy is estimated to result in a $6 penalty per carcase to producers and an $8-10 loss per kilo in high value cuts (i.e. 'frenched' racks) to the processing sector. In addition will be the financial penalty to some producers from the trimmed carcase no longer being within specification and the on-floor costs incurred by the abattoir in handling affected carcases.

Currently sheep producers receive feedback from the South Australian Enhanced Abattoir Surveillance Program or the National Sheep Health Monitoring Project that their sheep have pneumonia/pleurisy.[1] However, as it is not possible to determine the primary aetiological agent indicated, producers have no way of knowing how to control or limit the effects of the disease. This project aimed to provide preliminary information to help overcome this deficit.

[1] The South Australian Enhanced Abattoir Surveillance Program applies to sheep slaughtered at one abattoir in South Australia. The National Sheep Health Monitoring Project is an intermittent sampling abattoir sampling program that cannot be relied on for accurate national disease prevalence.

Enhanced understanding of the pathology of this disease will provide the opportunity for future development of new diagnostic and control tools/methods which would in turn minimise associated productivity losses.

# 3 Project objectives

The objectives of the project were to:

- Conduct a pilot study of abattoir sheep to investigate the primary causative agents of pneumonia in Australian sheep.
- Collect at least 200 lung and blood samples and perform culture and/or serology on at least 75%. Depending on the target pathogen(s), the assay methods were to include culture, PCR, antigen ELISA, antibody ELISA, immunocytochemistry or immunohistochemistry.
- Provide preliminary information on the ovine respiratory pathogens circulating in sheep flocks in south eastern Australia.

# 4 Methodology

## 4.1 Pilot study of abattoir sheep

The pilot study was initially planned to take place at the Thomas Foods International abattoir at Murray Bridge in South Australia. However, after this abattoir was destroyed by fire, an alternative location for the project was sought.

As a result, the pilot study included three abattoirs:

- Thomas Foods International, Lobethal South Australia
- Southern Meats, Goulburn New South Wales
- Gundagai Meat Processors, Gundagai New South Wales

All three of the abattoirs were Export Abattoirs, although at the time of the study, Gundagai Meat Processors was processing lambs for the domestic Australian market. The other two abattoirs were processing a mix of lambs and mutton sheep for international and domestic markets.

One sampling visit was made to the Lobethal plant (July 2018), two to Goulburn plant (September and December 2018) and two to the Gundagai plant (February and May 2019).

## 4.2 Protocol for collecting matched lung and heart blood samples

The protocol for collecting matched lung and heart blood samples was established during the visit to the Lobethal plant in July 2018.

During the evisceration of an ovine carcase, the lungs and heart are removed as one and placed into the red offal tray, along with the kidneys and liver. (The gastrointestinal tract is placed into a separate green offal tray.) We were able to take advantage of these in-plant procedures to develop a protocol to collect matched lung and heart blood samples.

Initially lung tissue samples were collected directly from the offal trays by asking an inspector to cut off the affected portions of the lung. As time progressed, we found it easier to remove the affected lungs from the offal tray, remove the lungs from the slaughter floor for subsequent scoring and sample collection (tissue or swab). The speed of the chain impedes sample collection in many plants.

Blood samples can be collected by cutting through the major vessels to separate the heart from the lungs and squeezing blood from the heart directly into a centrifuge tube, or by using a sterile plastic transfer pipette to aspirate blood from the red offal tray.

Diseased lungs with extensive pleural adhesions that prevent removal during evisceration can be sampled on the pathology rail. In this instance, the heart is usually adherent to the lungs.

## 4.3 Collection of samples

The samples collected were an opportunistic snapshot based on the animals being processed on the day of the abattoir visit, including mutton sheep and lambs. Line information was not recorded.

Lungs with gross pathology consistent with bronchopneumonia were targeted for sample collection. Because this was a pilot study aimed at isolating respiratory pathogens and method development, normal lungs were not collected.

Samples were collected from organs in the offal trays only, rather than at the pathology rail because this provided a greater number of affected lungs and a greater range of lung pathology.

The lung tissue collected included cranioventral sections of the lungs, the apical lobe, one-half the lung or the whole lung, depending on the location of the plant and speed of the chain. Lung tissue was collected into sterile sample bags, held on wet ice for up to six hours after collection and then stored frozen at -20 $^{o}$C until processing.

When possible, heart blood was collected by inverting the heart over a sterile 15 mL centrifuge tube and gently squeezing blood into the tube. Alternatively, blood was collected from the red offal tray using a sterile 3 mL plastic transfer pipette. Once the blood had clotted the sample was centrifuged at 3000 x g for 15 minutes and the serum removed and stored frozen at -20 $^{0}$C.

Swab samples were collecting using a FLOQSwab™ and UTM™ [2] for the Collection and Preservation of Virus, Chlamydia, Mycoplasma and Ureaplasma (Copan, Italy). The whole lung was removed from the offal tray and placed on a cutting board, dorsal surface uppermost. The dorsal surface of the trachea was cut between the tracheal rings using a sterile scalpel blade. The bronchi of each lung lobe were sampled with the same FLOQSWAB, starting with the right apical lobe and moving clockwise around the lung lobes. The swabs in UTM were placed onto wet ice immediately after sample collection for transport off the slaughter floor and then held at 4 $^{0}$C until processing, within 36 hours.

More than 200 lung samples, including 80 matched tissue and swab samples, and 100 serum samples were collected during the five abattoir visits (Table 1).

[2] Flocked swab with Universal Transport Media https://www.copanusa.com/sample-collection-transport-processing/floqswabs/

**Table 1. Samples collected**

| Abattoir | Date | Number of lung samples | Number of lung swabs | Number of serum samples |
|---|---|---|---|---|
| Thomas Foods International, Lobethal | July 2018 | 59 | - | 23 |
| Southern Meats, Goulburn | September 2018 | 32 | - | - |
| Southern Meats, Goulburn | December 2018 | 26 | - | - |
| Gundagai Meat Processors | February 2019 | 25 | - | - |
| Gundagai Meat Processors | May 2019 | 80 | 80 | |

## 4.4 Culture of *Mycoplasma ovipneumoniae*

A culture method for *Mycoplasma ovipneumoniae* was established, based on published methods (Alley *et al.* 1999; Carmichael *et al.* 1972; Ionas *et al.* 1991; Nicholas and Baker 1998; Taylor 1998).

Broths are cultured at 37 $^0$C aerobically and agar plates at 37 $^0$C in 5% $CO_2$.

## 4.5 PCR

A qPCR method for *Mycoplasma ovipneumoniae* was developed, based on the method published by Yang *et al.* 2014, modified for a RotorGene Q real-time PCR cycler.

DNA extraction prior to qPCR used the DNeasy Blood and Tissue Kit (Qiagen Pty Ltd) for tissue samples and culture, and QIAmp MinElute Media Kit (Qiagen Pty Ltd) for swab samples.

# Results

## 4.6 Number of samples tested

The number of samples tested by culture and PCR is shown in Table 2.

Many of the samples collected during the Lobethal visit were not appropriate for testing. The aim of this visit was to establish the sampling protocol and the appropriate section of the lungs wasn't always sampled by abattoir personnel.

The serum samples have not been tested and are stored at -20 $^{o}$C until appropriate test methods are available.

**Table 2. Number of samples tested**

| Abattoir | Date | Number of lung samples | Number of lung samples tested | Number of lung swabs | Number of lung swabs tested |
|---|---|---|---|---|---|
| Thomas Foods International, Lobethal | July 2018 | 59 | 6* | - | - |
| Southern Meats, Goulburn | September 2018 | 32 | 23** | - | - |
| Southern Meats, Goulburn | December 2018 | 27 | 27 | - | - |
| Gundagai Meat Processors | February 2019 | 25 | 21 | - | - |
| Gundagai Meat Processors | May 2019 | 80 | 43 | 80 | 80 |
| **Total** | | **223** | **114** | **80** | **80** |

* *Mycoplasma* cultures contaminated with mould and not tested further

** The majority of the samples collected were from mutton carcases in which the lungs were over-inflated and rubbery, with patchy dorsal-ventral haemorrhage. These samples were collected at the request of the plant.

## 4.7 Number of samples positive for *Mycoplasma ovipneumoniae*

The number of samples positive for *Mycoplasma ovipneumoniae* on culture and/or qPCR is shown in Table 3.

**Table 3. Number of samples positive for *Mycoplasma ovipneumoniae* on culture and/or qPCR**

| Abattoir | Date | Number of lung samples tested | Number of lung samples positive | Number of lung swabs tested | Number of lung swabs positive |
|---|---|---|---|---|---|
| Southern Meats, Goulburn | December 2018 | 27 | 11 | - | - |
| Gundagai Meat Processors | February 2019 | 21 | 19 | - | - |
| Gundagai Meat Processors | May 2019 | 43 | 24 | 80 | 59 |

Based on the large number of samples that tested positive for *Mycoplasma*, a decision was made in consultation with Meat & Livestock Australia not to test for the respiratory viruses or *Chlamydia.*

### 4.7.1 Comparison of swabs and lung tissue for culture of *Mycoplasma ovipneumoniae*

As described in section 3.3, lung tissue samples were collected onto wet ice and then stored at -20 $^{o}$C until processing. This required adequate freezer space. The samples were also messy to process in the lab.

Lung tissue samples and the FLOQSwab™ with UTM™ for the Collection and Preservation of Virus, Chlamydia, Mycoplasma and Ureaplasma (Copan, Italy) were compared for the detection of *Mycoplasma ovipneumoniae* using 43 of the samples collected at Gundagai Meat Processors in May 2019.

Results are shown in Table 4. Swabs returned more positive results than lung tissue ($X^2$ (1, 43) = 12.35, $P$ <0.001).

**Table 4. Comparison of lung samples and swabs for culture of *Mycoplasma ovipneumoniae***

| | | Lung tissue | | |
|---|---|---|---|---|
| | | **Positive** | **Negative** | **Total** |
| **Swab** | **Positive** | 22 | 8 | 30 |
| | **Negative** | 2 | 11 | 13 |
| | **Total** | 24 | 19 | 43 |

### 4.7.2 Comparison of culture and qPCR for detection of *Mycoplasma ovipneumoniae* from swab samples

When culturing *Mycoplasma ovipneumoniae,* broths are left for three weeks to allow a colour change in the pH indicator. When a colour change occurs, DNA is extracted to allow confirmation by qPCR.

If a colour change does not occur within three weeks, the sample is not confirmed negative until DNA is extracted and qPCR conducted.

This process is time-consuming and laborious.

Using the 80 swabs samples collected at Gundagai Meat Processors in May 2019, qPCR and culture were compared for detection of *Mycoplasma ovipneumoniae*. Fifty-nine of the 80 swab samples were positive on culture and 48 were positive on qPCR.

Options to improve the sensitivity of the qPCR including centrifuging the UTM prior to DNA extraction (Besser *et al.* 2019) or concentrating the sample using an AMICON Ultra Centrifugal (Merck Pty Ltd.) These options will be investigated before future sample collection.

# 6 Discussion

The project achieved its objectives, despite the need to find alternative abattoirs for the study after the fire at the Murray Bridge abattoir, as well as laboratory space. This had financial implications for the project, because it had been planned to conduct the study at just one abattoir, in which laboratory space was available.

## 6.1 Conduct a pilot study of abattoir sheep

The pilot study included three abattoirs, Thomas Foods International, Lobethal South Australia; Southern Meats, Goulburn New South Wales; and Gundagai Meat Processors, Gundagai New South Wales.

One sampling visit was made to the Lobethal plant (July 2018), two to Goulburn plant (September and December 2018) and two to the Gundagai plant (February and May 2019).

### 6.1.1 Collect at least 200 lung and blood samples

More than 200 lung samples, including 80 matched tissue and swab samples, and 100 serum samples were collected during the five abattoir visits. More than 75% (78.2%) of the samples were tested for *Mycoplasma ovipneumoniae* by culture and/or qPCR.

## 6.2 Provide preliminary information on the ovine respiratory pathogens circulating in sheep flocks in south eastern Australia

The results for the project have provided preliminary information that suggests *Mycoplasma* infection may be more widespread in sheep flocks in south eastern Australia than previously recognized.

In addition, the project has developed culture and PCR methods that will enable more widespread, structured surveys of *Mycoplasma* infection and respiratory disease in Australian sheep.

# 8 Conclusions/recommendations

## 8.1 Recommendations for future research

1. A prevalence survey of pneumonia in Australian abattoir sheep that includes:
    a. Four sampling periods – spring, summer, autumn, winter
    b. Four abattoirs around Australia, to provide regional spread of sheep source
    c. The recording of line information, i.e. sheep breed, age and source/region
    d. Lung scoring
    e. The collection of swabs for PCR/culture
    f. The collection of lung tissue for histopathology

    This will provide a comprehensive understanding of the aetiological agents involved in pneumonia in Australian sheep and help underpin the development of control measures.

2. Trialling of a non-steroidal anti-inflammatory drug in combination with antibiotics in therapeutic protocols for bacterial pneumonia in sheep

    Anti-inflammatory drugs are commonly used in the treatment of respiratory disease in other species, including humans and cattle. However, these drugs are rarely used in the treatment of respiratory disease in Australian sheep.

    The non-steroidal anti-inflammatory drug meloxicam is registered for use in sheep in Australia with a general claim for pain and inflammation (Metacam 20 mg/mL). Research to investigate clinical improvement in sheep with pneumonia with combined treatment with appropriate antibiotics and meloxicam, as well as reduction in lung lesions, pleural adhesions and carcase trimming is warranted (Politis *et al*. 2019).

# 9 Key messages

The project was a pilot study and has not resulted in key messages that can be disseminated to producers and processors at this time. Instead, the project will help to inform future research into pneumonia in Australian sheep.

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