

Full Length Research Paper

An alternative caprine infection model for *Mycoplasma mycoides*

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Ruminant infection models for *Mycoplasma mycoides* are invasive and laborious factors that may interfere with the experiment outcomes. This brief review presents an alternative method that may minimize on the mentioned challenges earlier mentioned. *M. mycoides* subsp. *capri* GM12 and *M. mycoides* subsp. *capri* YCP1.1 Δ 68 strains were used; the former, causing a septicemia in goats. An intra-tracheal inoculation technique was employed after aseptic preparation as an alternative to the common intubation method. The former is cheaper, less laborious to handlers, with minimal stress to goats and more time-efficient. In conclusion, this may be used as an alternative to the intubation method. The described technique here will be useful in contagious bovine pleuropneumonia and contagious caprine pleuropneumonia research with modifications, diseases for cattle and small ruminants, respectively.

Key words: *Mycoplasma mycoides*, animal infection model, ruminants.

INTRODUCTION

The *Mycoplasma mycoides* cluster, comprising ruminant pathogens existed under a common ancestor (Fischer et al., 2012). These cause notifiable diseases in sub-Saharan Africa and parts of Asia that include contagious bovine pleuropneumonia (CBPP) a disease of cattle caused by *M. mycoides* subsp *mycoides* (*Mmm*) (Food and Agricultural Organization-FAO, 2003) and contagious caprine pleuropneumonia (CCPP) in goats caused by *Mycoplasma capricolum* subsp *capripneumoniae* (*Mccp*) (Thiaucourt and Bölske, 1996). CBPP has been reported in 27 African countries, with losses estimated at 2 billion

United States dollars annually (Otte et al., 2004).

The recommended control measures for CBPP are massive vaccination, quarantine and stamping out policy (March, 2004). However, the efficacy of these interventions has not been well studied in Africa even when it is known that some present immediate challenges. For example, the existing vaccine strain T1/44 is of low efficacy, presents with side effects (Hubschle et al., 2002) and causes clinical disease on experimental infection in cattle (Mbulu et al., 2004). Therefore, there is need for more studies keen on the

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biology of *M. mycoides* subsp *mycoides*, and corresponding host-pathogen interactions. Related *Mycoplasma* strains of the *M. mycoides* cluster would provide valuable understanding in such studies.

However, in these studies, we need to experimentally infect animal subjects. The intra tracheal intubation method commonly used presents with challenges of stress to the animals. In small ruminants, it calls for the usage of sedatives and anesthetics. These drugs have a narrow safety margin with potential to cause ruminal stasis (Taylor, 1991). Consequently, a caprine infection model for *M. mycoides* is prerequisite for systematic investigations with regard to host-pathogen interactions (Jores et al., 2013), taking into consideration animal welfare. This study presents an alternative infection model in goats for *Mycoplasma* research.

MATERIALS AND METHODS

Study design and protocol

Details and major outcomes of this work are well elucidated in the work of Jores et al. (2019). The study was carried out following approval by the ILRI Institutional Animal Care and Use Committee IACUC with a reference number 2011.09.10.

Description of the *Mycoplasma* isolates used

M. mycoides subsp. *capri* GM12 (*Mmc*-GM12) is a pathogen of goats that causes severe septicaemia. The two *Mycoplasma* strains used in this experiment were *Mmc* GM12 (Wild type strain) and *Mmc* YCP1.1 Δ 68. The latter is a mutant of *Mmc* GM12, whereby a total of 68 genes were deleted using the Tandem Repeat Endonuclease cleavage (TREC) technique. Among the gene loci deleted are *GlpO*, *GlpF*, *GlpK*, *gtsA*, *gtsB*, *gtsC* and *gtsD*. The first three facilitate glycerol utilization whereas the latter four are cell membrane transport proteins for *Mycoplasma* metabolites. These metabolites contribute to the establishment of an infection in a susceptible host.

Growth of *Mycoplasma* cultures

Culture of blood samples for isolation of *Mycoplasma* was carried out at 37°C using pleuropneumonia like organism (PPLO) media (that is agar; Difco™, lot no., 4169667 or broth; Difco™, lot no., 3361447, supplemented with 20% horse serum, 0.5% glucose, 0.03% penicillin G, 20 mg/ml thallium acetate and 0.9 g L⁻¹ yeast extract). In liquid media, phenol red was used as a pH indicator for color change from red to orange or yellow, as a sign for growth. Each sample was cultured in duplicate and color changing units noted after incubation at 37°C for 10 days (Jores et al., 2019). In addition, the liquid cultures were inoculated on PPLO agar plates and characteristic "fried egg" *Mycoplasma* colonies identified a true reflection of *Mycoplasma* bacterial growths.

Preparation of animals and infection procedure

All animals were screened and declared free of any *Mycoplasma* prior to the start of the experiment (Table 1). In addition, the collected blood samples had no other bacterial growths, which

would prejudice the experiment outcomes. A total of eight (8) and six (6) goats received the *Mmc* GM12 and *Mmc* YCP 1.1 Δ 68 strains as inoculum, respectively. Each goat was restrained in a standing position; head elevated to expose the ventral cervical area which was shaved using an electric shaver at about the 5-10th cartilaginous ring position (Figure 1i). The shaved area was approximately 4x10 cm in area. The site was disinfected 3 times using 70% ethanol using a sterile piece of gauze. The skin at the prepared site was displaced laterally, about 0.5 cm and the space between cartilaginous rings located by palpation and on confirmation, a sterile hypodermic 18-gauge needle introduced perpendicularly into the trachea lumen. Once the needle bevel was within the lumen of the trachea, *Mycoplasma* culture (10⁹) colony forming units were introduced into the trachea space and holding the needle in place, the used needle flushed with 10 ml of a 1X phosphate buffered saline (PBS) solution. Thereafter, the needle was removed and the site disinfected. This method took a time range of 2 to 5 min, a commendable duration that would inflict minimal stress on the experiment subjects as a result of the procedure. Details of the procedure are demonstrated in Figure 1i-iii.

Monitoring of clinical signs, sample collection and handling

Clinical parameters

Monitoring for signs of ill health was done for atleast 30 min per session, three times a day through a wide glass screen without interruption of the animals. These included discerning signs of inappetence or anorexia, depression, respiratory distress, coughing, nasal discharge and ocular discharge. Morning daily rectal temperature was measured using a digital thermometer, once a day. The aberrant findings thereafter were recorded in the data collection forms.

Oxygen concentration

The oxygen concentration was measured using the Edan VE-H100B Oximeter. Once the power button was turned on and the screen display showing the oxygen and heart rate parameter icons, the rectal probe was inserted into the rectum after applying KY jelly. Once a stable reading for the oxygen concentration was obtained after a good signal picked as indicated on the screen and manufacturer's instructions, the reading was recorded on the data sheet.

Heart rate

The heart rate was measured daily using a stethoscope placed on the left side of the cranial third of the animal's lateral ventral thoracic area about the 3rd-4th rib position. Once the counts per minute were attained, the value was entered into the data sheet.

Nasal swabs

Once an animal was restrained in a standing position with the head held by the chin and horns, a piece of gauze containing 70% ethanol was wiped around the nasal opening. Thereafter a sterile cotton swab was introduced and moved in a circular motion against the inner most nasal walls. The swab was immediately placed in PPLO broth and incubated at 37°C for 10 days.

Collection of whole blood

An animal was restrained in a standing position and the head

Table 1. Blood culture results pre and post infection.

Mmc strain inoculated in animal	Animal ID	Pre-infection cultures	Post infection cultures (blood)- time point 1 DPI
Mmc GM12 parent strain	CK043	-	+
	CK048	-	+
	CK034	-	+
	CK032	-	+
	CK046	-	+
	CK040	-	+
	CK051	-	+
	CL002	-	+
Mmc YCP 1.1 Δ 68	CK047	-	-
	CK049	-	-
	CK045	-	-
	CK035	-	-
	CL003	-	-
	CL001	-	-

slightly tilted to the lateral side with the head extended. The jugular groove was located and a sterile vacutainer needle and tube used to collect blood for routine red blood and white blood cell profiling. Whole blood was then cultured for any *Mycoplasma* and other bacteria.

Euthanasia

A decision to euthanize (intravenous injection of Lethobarb Euthanasia Injection, Virbac 200 mg/kg body weight) was made once an animal developed severe disease associated with unwarranted moderate to severe suffering (e.g. A fever of 41 °C and above for three consecutive days, an oxygen saturation of 92% or less and a lateral recumbence of atleast a day without ability to pick feed or water).

RESULTS

Isolation of *Mycoplasma* from whole blood

As indicated, all samples from the animals that received the parent strain of *Mmc* GM12 had positive cultures (+) in PPLO broth but no growth in the mutant group, which were all negative (-) (Table 1). As shown, no mycoplasma growths were seen post-infection in both groups but identified for the group that received the wild type strain. In addition, no growths were seen for the mutant group after infection, possibly because this strain was potentially attenuated and failed to establish an infection

The alternative infection model in goats

Figure 1 shows an image with the intra-tracheal infection route of a goat with *Mycoplasma mycoides*. (i) Locating

the trachea and aseptic introduction of a sterile hypodermic needle; (ii) checking that the needle bevel is within the tracheal lumen by aspirating air using an empty syringe attached onto the introduced needle in (i) and (iii) administering of *Mycoplasma mycoides* culture into the tracheal lumen.

Comparative lung pathology of the two groups

Figure 2 shows a lung presenting a classic case of pneumonia among the animal group that received *Mmc* GM12 *Mycoplasma* strain (*left image with an arrow*) as compared to another that received the *Mmc* YCP1.1 Δ 68 strain (*right image*), the latter looks apparently normal on gross examination.

DISCUSSION

Pathogenesis studies call for the best animal infection model, preferably a natural host of the organism under investigation with strict animal welfare observed.

In this communication, we present an alternative infection model (Figure 1) in goats that took an average implementation time of 2 to 5 min. This involved location of trachea, after proper animal restraint, aseptic preparation of the inoculation site and introduction of the infectious agent into the trachea lumen. Earlier *Mycoplasma* infection studies in cattle employed the intra-tracheal intubation technique (Nkando et al., 2011, 2016). Advantages of the alternative method are: possible less stress exerted on the animal during the procedure, reduced execution time as well as zero use of

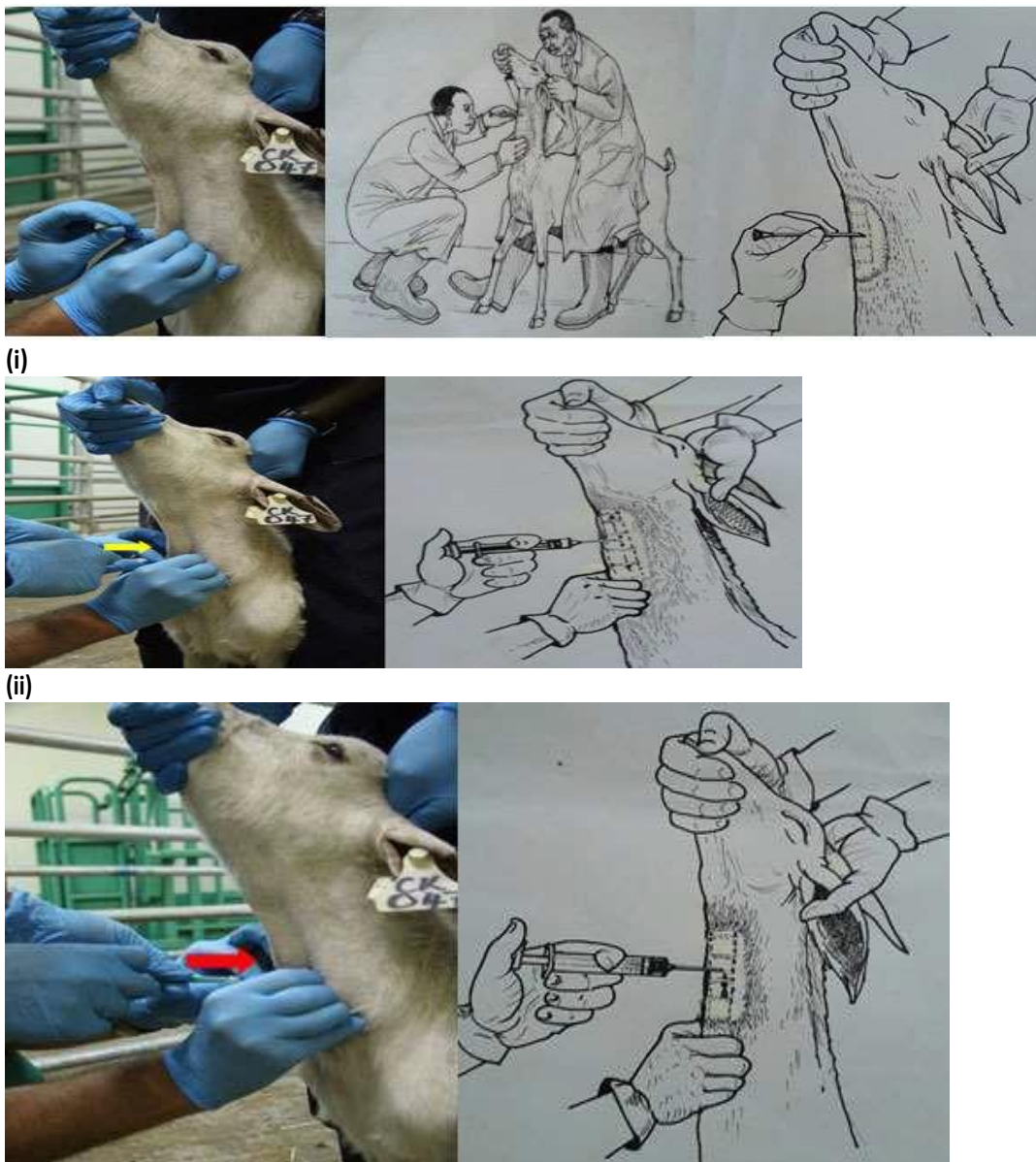


Figure 1. An image showing the intra-tracheal infection route of a goat with *Mycoplasma mycoides*. (i) Locating the trachea and aseptic introduction of a sterile hypodermic needle; (ii) Checking that the needle bevel is within the tracheal lumen by aspirating air using an empty syringe attached onto the introduced needle in (i) above and (iii) Administering of mycoplasma mycoides culture into the tracheal lumen.

pharmacological agents to calm the animal. In addition, the alternative method does not entail introduction of hot *Mycoplasma* agar down the trachea as shown in Nkando et al. (2011) and Nkando et al., (2016). This is desirable when making a choice for an infection model, which is reproducible while mimicking the natural infection scenarios.

Since *Mycoplasma* bacteria was isolated post infection from the wild type animal group (Table 1), this was evidence that the infection model was a success.

Furthermore, much as no *Mycoplasma* growths were identified in the mutant group, this may perhaps be due to the *Mmc* YCP 1.1 Δ 68 strain succumbing to the host defence mechanisms that hindered its proliferation without significant pathology (Figure 2). Whereas carrying out a blood culture would aid in the identification of bacteria in blood (Paisley and Lauer, 1994; Spencer, 1988) as this is a septicaemia causing organism, it was observed that no *Mycoplasma* was harvested from the group that received the attenuated strain, *Mmc* 1.1 delta

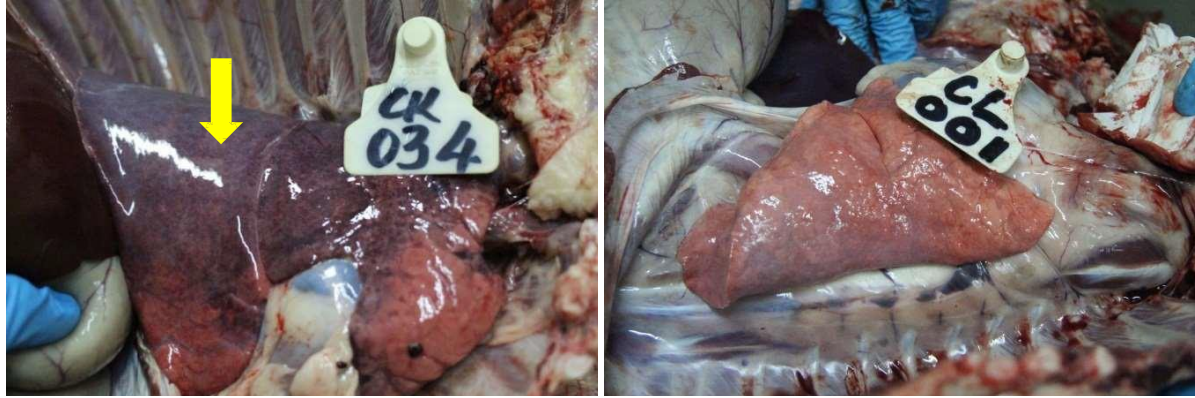


Figure 2. A comparison of the lung pathology from candidates of the two infection groups.

68 post infection. This could have been due to the fact that the host immune system countered establishment of an infection, with complete clearance of the bacteria. Another possibility is that the mutant strain could have been maintained at minimal concentrations in circulation to levels that would not be detected on blood culture, although minimal *Mycoplasma* bacterial amounts detectable on culture to our knowledge are yet to be known, a similar observation by Alexander et al. (1999). In comparison to the group that received the wild type *Mmc* GM12 strain, full blown septicaemia and other related clinical signs could be due to a compromised immune system as exemplified by Monserrat et al. (2013), which was not the case for the mutant group. This supports the suggestion that targeted mutagenesis of potential virulence factors led to partial or complete attenuation for the *Mmc* 1.1 delta 68 strain. It is against this background that this work reports the use of an in-vivo experiment, comparing a wild type (*Mmc* GM12) and *Mmc* 1.1 delta 68 strains to ascertain whether the mutant after target specific edits led to attenuation. This alternative method was adopted with modifications for other ruminant *Mycoplasma in-vivo* work by Jores et al. (2018) and Liljander et al. (2019), with infection success observed.

Limitations

This study did not take into consideration breed and sex variations in addition to different *Mycoplasma* concentrations for inoculation. This was an initial experiment for proof of principle; however, subsequent experiments may consider some other parameters such as age, sex, breed and varying the inoculation doses of the bacteria under investigation.

Conclusion

The infection model presented here was a success for

the *Mycoplasma in-vivo* investigations based on the clear distinction between the results pre and post infection. This could be adopted for other *Mycoplasma* animal infection experiments although with modifications in both large and small ruminants.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

- Alexander H, Marlies B, Silbylle S, Klaus U, Konrad B, Gerd do R (1999). PCR and Blood Culture for Detection of *Escherichia coli* Bacteremia in Rats. *Journal of Clinical Microbiology* 37(8):2479–2482.
- Fischer A, Beth S, Cecilia M, Martin H, Christiane S, Erik B, Edy MV, Joachim F, Joerg J (2012). The Origin of the 'Mycoplasma mycoides Cluster' Coincides with Domestication of Ruminants. *PLoS ONE* 7(4):e36150
- Food and Agriculture Organization of the United Nations (FAO) (2003). Contagious bovine pleuropneumonia. *EMPRESS Transboundary Animal Diseases Bulletin*, 5612-5621.
- Hubschle O, Lelli R, Frey J, Nicholas RAJ (2002). Contagious bovine pleuropneumonia and vaccine strain T1/44. *Veterinary Record* 150(19):615.
- Jores J, Ma L, Ssajjakambwe P, Schieck E, Liljander A, Chandran S, Stoffel MH, Cippa V, Arfi Y, Assad-Garcia N, Falquet L, Sirand-Pugnet P, Blanchard A, Lartigue C, Posthaus H, Labroussaa F, Vashee S (2019). Removal of a Subset of Non-essential Genes Fully Attenuates a Highly Virulent *Mycoplasma* Strain. *Frontiers in*

- Microbiology 10:664. doi: 10.3389/fmicb.2019.00664.
- Jores J, Schieck E, Liljander A, Sacchini F, Posthaus H, Lartigue C, Blanchard A, Labroussaa F, Vashee S (2018). Capsular polysaccharide in *Mycoplasma mycoides* is a virulence factor. *Journal of Infectious Diseases* DOI, 10.1093/infdis/jiy713.
- Jores J, Mariner CJ, Naessens J (2013). Development of an improved vaccine for contagious bovine pleuropneumonia: an African perspective on challenges and proposed actions. *Veterinary Research* 44:122.
- Liljander A, Sacchini F, Stoffel MH, Schieck E, Stokar-Regenscheit, Nadine, Labroussaa F, Heller M, Salt J, Frey J, Falquet L, Goovaerts D, Jores J (2019). Reproduction of contagious caprine pleuropneumonia reveals the ability of convalescent sera to reduce hydrogen-peroxide production in vitro. *Veterinary Research* (50):1-10 <https://doi.org/10.1186/s13567-019-0628-0>.
- March JB (2004). Improved formulations for existing CBPP vaccines — Recommendations for change. *Vaccine* 22(31-32):4358-64
- Mbulu RS, Tjipura-Zaire G, Lelli R, Frey J, Pilo P, Vilei EM, Mettler F, Nicholas RA, Huebschle OJ (2004). Contagious bovine pleuropneumonia (CBPP) caused by vaccine T1/44 *Mycoplasma mycoides* subsp. *mycoides* SC. *Veterinary Microbiology* 98(3-4):229-34.
- Monserat J, de Pablo R, Diaz-Martín D, Rodríguez-Zapata M, de la Hera A, Prieto A, Alvarez-Mon M (2013). Early alterations of B cells in patients with septic shock. *Critical Care* 17(3):R105.
- Nkando I, Perez-Casal J, Mwirigi M, Prysliak T, Townsend H, Berberov E, Kuria J, Mugambi J, Soi R, Liljander A, Jores J, Gerdt V, Potter A, Naessens J, Wesonga H (2016). Recombinant *Mycoplasma mycoides* proteins elicit protective immune responses against contagious bovine pleuropneumonia. *Veterinary Immunology and Immunopathology* 171:103-114.
- Nkando I, Ndinda J, Kuria J, Naessens J, Mbithi F, Schnier C, Gicheru M, McKeever D, Wesonga H (2011). Efficacy of two vaccine formulations against contagious bovine pleuropneumonia (CBPP) in Kenyan indigenous cattle. *Research in Veterinary Science* 93(2):568-73
- Otte MJ, Nugent R, McLeod A (2004). *Transboundary Animal Diseases: Assessment of socio-economic impacts and institutional responses, in Livestock Policy Discussion paper: Food and Agriculture Organisation; Livestock Information and Policy Branch, AGAL, pp.1-46* <http://www.fao.org/3/a-ag273e.pdf>
- Paisley JW, Lauer BA (1994). Pediatric blood cultures. *Clinical Laboratory Medicine* 14(1):17-30
- Spencer RS (1988). Blood cultures: where do we stand? *Journal of Clinica Pathology* 41(6):668-670.
- Taylor MP (1991). Anaesthesia in Sheep and Goats. In *Practice* 13(1):31-36.
- Thiaucourt F, Bölske G (1996). Contagious caprine pleuropneumonia and other pulmonary mycoplasmoses of sheep and goats. *Revue Scientifique et Technique* 15(4):1397-1414.