ORIGINAL RESEARCH ARTICLE

Effect of glycerol on preservation of *Mycoplasma capricolum* subsp. *capripneumonia* (Mccp) in frozen lung Samples

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ABSTRACT

Mycoplasma capricolum subspecies *capripneumoniae* (Mccp) is the causative agent of contagious caprine pleuropneumonia (CCPP), a disease that affects the respiratory system of goats. Diagnosis of CCPP encounters challenges since Mccp is fastidious making it difficult to isolate or revive from stored samples. This study assessed the effect of glycerol on success of isolating Mccp from stored lung tissues. Experimentally infected goats developed fever and respiratory both consistent with CCPP and about 83% of the infected goats showed a variety of gross lesions including red hepatization, hyperemia, fibrinous pleural deposits and fibrinous adhesions. Three pieces of lung samples were collected from the same site of each of the 24 experimentally infected goats immediately after euthanasia. One piece was placed in media prepared with 8 weeks old serum immediately after collection for cultivation. The other two pieces were stored with and without glycerol at- 40℃. The stored samples were retrieved and cultured after 21, 34 and 39 months and the samples where Mccp was isolated re-cultured after six months. Mccp was not isolated in fresh lung samples. There was 50% (4/8) success in isolation of Mccp for lung samples stored in glycerol for 21 months compared to 37.5% (3/8) for those stored for 34 months. Out of the four lung samples which yielded Mccp at 21 months, Mccp was isolated in all four at 27 months, 3 samples at 34 months, and in no samples after 40 months of storage. At all-time points, samples stored without glycerol did not show growth. The findings demonstrate the effect of glycerol in preserving Mccp viability in longer duration periods storage. Further studies are needed to assess success of isolating Mccp using a single batch of quality-controlled serum, aliquoted and stored at different time intervals.

Keywords: CCPP, Glycerol, Lung samples, Mccp isolation, Storage duration.

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1.0 Introduction

Contagious caprine pleuropneumonia (CCPP) is a lung disease of goats associated with accumulation of lung exudate in thorax cavity, fibrous adhesion of lungs to the chest wall, froth in the trachea, enlarged respiratory (mediastinal) lymph nodes, lung with areas of pneumonia and lung exudate containing large clots of fibrin (Teshome *et al.*, 2019). The causative agent of CCPP is *Mycoplasma capricolum subspecies capripneumoniae* (Mccp). The disease occurs in the Middle East, Eastern Africa and some countries in Asia. Mccp was first isolated in Kenya in the year 1976 (Ahaduzzam et al., 2021). CCPP causes huge economic losses, associated with high morbidity, sometimes of up to 100% and mortality between 60 and 100% (Rehman *et al.*, 2022). Three hundred million goats are at risk of contracting the disease in 30 African countries as well as in Asia (Code *et al.*, 2021). Approximately USD 507 million is lost yearly in these countries where the disease is endemic. The losses are due to lowered production, greatly affecting sub-Saharan Africa, Middle East and Asia where most people depend on livestock as a source of livelihood (Gitau *et al.*, 2023).

Documentation on the prevalence of Contagious Caprine Pleuropneumonia (CCPP) in Kenya remains limited, despite the nation's involvement in traditional and extensive goat husbandry practices. Kenya has implemented a structured vaccination regimen, either through systematic programs or vaccination protocols informed by risk assessments, aimed at mitigating incidences of the disease to either prevent its occurrence or manage its spread. The primary vaccine utilized to combat CCPP is CAPRIVAX™, an inactivated vaccine derived from Mccp, originally identified as the F38 biotype. Despite regular vaccination efforts targeting CCPP, recurrent outbreaks persist, particularly within the arid and semiarid regions of the country (Lugonzo et al., 2023).

Mccp belongs to a group of five closely related ruminant mycoplasmas that share similar biochemical features, with serological cross reactions that at times cause erroneous diagnosis of the disease (Manso-Silván *et al.*, 2011). Initial signs and symptoms include lethargy, anorexia and high fever of up to 41- 43℃, followed by coughing after 2-3 days (Code *et al.*, 2021). In the final stages, goats become very weak and are unable to stand or move. The neck also stiffens and there is continuous discharge of saliva and abortion in pregnant goats (Caprine, 2015). The goats usually die within 7 to10 days after infection. Post-mortem examination reveals fibrinous pleuropneumonia, with massive lung hepatization and pleurisy, often accompanied by accumulation of straw-colored pleural fluid (Code *et al.*, 2021).

Diagnosis of CCPP is through clinical signs, isolation, polymerase chain reaction (PCR) and serological test of Mccp (OIE, 2021). However, isolation of the strains is difficult because Mccp is fragile and dies off fast, making it difficult to isolate from goat lung samples or revive cultures (OIE, 2021). It is for this reason that sodium pyruvate is added to the media used for isolating Mccp (Khan *et al.*, 2019). But even with this modification, the success of isolation is still not satisfactory, especially from samples stored for a long duration (Yatoo *et al.*, 2019). Freezing and thawing cycles due to power cuts and/or frequent opening and closing of freezers also reduces the chances of recovering Mccp (Gille *et al.*, 2018). Mycoplasmas lack peptidoglycan cell wall,

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which makes them highly sensitive to ice crystal formed during freezing and thawing and easily die in cold storage (Al-Farha *et al.*, 2018).

There are no reports on structured studies of isolation of Mccp following storage for a long duration. However, information exists on the use of glycerol for storage of E. *coli* cultures used in molecular genetic studies and *Mycoplasma hyorhinis* strains (Sunarno et al., 2021), (Pérez et al., 2016). Glycerol is suspected to help enhance the viability of microorganisms in addition, components of media used for Mccp isolation include serum, a key factor for consideration as a source of sterols, proteins, amino acids, carbohydrates, vitamins, lipids, trace elements, growth factors, and minerals (Pérez et al., 2016).

Serum deteriorates due to different reasons, including frequent freeze thaw of serum. Observations indicate that using serum that is more than 8 weeks old after storage at-40℃ under our laboratory conditions supports little or no growth at all hence the need to consider the age of the serum in media while studying the effect of glycerol on preservation of Mccp viability. This study reports on findings on the effect of storing at-40℃, Mccp infected goat lung tissue samples in glycerol (Pérez et al., 2016).

2.0 Materials and methods

2.1 Study animals

The study used samples of a larger study conducted in 2018, 2019 and 2020 that assessed the use of Mccp Baringo isolate from Kenya as challenge material in the selection of a suitable CCPP infection model. Post mortem samples collected in the three years were used to investigate the effect of glycerol on the storage of Mccp. A total of 86 male, castrated small East African breed of goats (SEAGs) aged between 2 and 4 years were used in a study consisting of 4 animal trials. The goats were sourced from Western Kenya a known CCPP free region and transported to the Kenya Agricultural Research Organization (KALRO), Veterinary Science Research Institute Muguga.

Before infection, the goats were randomly grouped, housed in different pens. The goats were provided with hay, mineral salt lick and water *ad libitum* at night and allowed to feed on the same but from outside the housing pen during the day. The goats were monitored daily and a record kept on their general body condition, rectal temperature, appetite, presence of cough and other symptoms suggestive of respiratory infection.

2.2 Infection by trans-tracheal inoculation

Baringo culture isolate for Mccp from Kenya was inoculated in tryptose media broth in tubes of 15 ml to make dilutions of ten in one to ten in twelve. Twenty microliters of the titrations were drop plated on tryptose agar. Broth cultures in the tubes and agar plates were placed in the incubator set at 37oC, humid and anaerobic condition overnight. Observations and recorded findings for the agar plates and broth cultures were done for 7 days and when confirmed to be at 10^7colour changing units (CCU) the culture was upscaled to 1liter using tryptose broth in a flask and grown until it achieved an orange color change. 30ml was drawn from the flask with

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1 liter culture into a 50ml syringe and 18 G needle, introduced into the 86 goats through the trans-tracheal route. The culture was followed with 15ml agar (1.5%) with a melting point of 40℃ and flushed down with 10 ml sterile PBS. This was carried out by qualified veterinary doctor in the mornings before the goats were released to feed.

Confirming that the syringe was in the tracheal was observed by having air flowing in the syringe and no vacuum or resistance on inoculation. An indication of successful delivery of the culture into the trachea was confirmed by the goat coughing immediately after the procedure. The goats were observed for adverse reaction at 1, 4 and 6 hours after the infection procedure.

2.3 Sample collection and storage

Infected goats were observed for clinical signs and at post mortem examination for lesions. Goats were followed up for 28 days unless the criteria to euthanize for animal welfare was reached. The criteria included elevated temperature > 39.5^{o}C for 3 or more days, anorexia or recumbence for two days.

At post mortem all goats were killed regardless of whether they had clinical signs for CCPP or not. For purposes of this study, eight lung samples were collected in 2018, 2019 and 2020 representing storage durations 21, 34 and 39 months. The lung samples were collected from each goat at the point of intersection between healthy and pneumonic lung lesion (Table 1).

The lung pieces were divided into three pieces with the first piece immediately suspended in tryptose broth media prepared with 8 weeks old serum for cultivation of Mccp, the second piece suspended in glycerol and stored frozen while the third was stored frozen without glycerol at-40^{o}C. Glycerol was used at a ratio of 4 to 1 (80%: glycerol to piece of 20 % lung) by volume as recommended in Perez(Pérez, 2016 C:\Users\Guest\Downloads\Pérez, - PérezC:\Users\Guest\Downloads\Pérez, - PérezC:\Users\Guest\Downloads\Pérez, - PérezC:\Users\Guest\Downloads\Pérez, - PérezC:\Users\Guest\Downloads\Pérez, - Pérez). Samples yielding Mccp were re-cultured after 6 months at 27 and 40 months of storage. Culturing was done by growing in broth with serum stored for both 2 and 8 weeks.

Table 1. Duration of storage, use of glycerol and age of pig serum used to make media for isolation of Mccp from lung samples

Duration of lung sample storage	Glycerol (n=8)	Pig serum age (weeks)
21 months (2020)	With	2
	Without	
	With	8
	Without	
34 months (2019)	With	2
	Without	
	With	8
	Without	
39 months (2018)	With	2
	Without	
	With	8

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Without

3.0 Results

3.1 Clinical and postmortem lesions

Some of the twenty goats exhibited clinical signs consistent with CCPP and included labored breathing and fever. After the goats were humanely killed, a variety of lesions as listed in Table 2. About four fifth (83%) of all the infected in the three years (2018, 2019 and 2020) developed post mortem lesions with the remainder 17% consisting of the 50% (4/8) out of the eight experimental goats in 2018 which did not show any gross lesions on postmortem examination.

Generally, goats, which had an acute form of CCPP had lesions such as red hepatization, red and grey hepatization, hyperemia and fibrinous pleural deposits while those for the subacute form included gray hepatization. Lesions in the chronic form of CCPP included sequestra, necrotic lesions formation and fibrous adhesions, which were often very extensive even in goats that did not manifest fever.

Table 2. Days of fever and post mortem lesions for the twenty-(24) four goats from where Mccp was isolated.

Lung sample ID	Year of trial	Days of fever/ day killed	Post mortem findings
G279	2020	1	Red hepatization
G281	2020	1	Grey hepatization
G280	2020	7	Grey hepatization
G291	2020	0	Extensive Fibrous adhesions
G286	2020	0	Sequestra
G268	2020	4	Red hepatization
G274	2020	0	Sequestra
G278	2020	4	Fibrous adhesions
G119	P2/2019/2	0	Early red hepatization
G101	P1/2019/2	1	Hypereamia
G108	P1/2019/2	0	Hypereamia
G104	P1/2019/2	1	Hypereamia
G102	P1/2019//2	3	Red & Grey hepatization
G103	P1/2019/2	4	Red & Grey hepatization
G105	P2/2019/2	0	Hypereamia
G118	P2/2019/2	0	Red hepatization
G73	P4/2018	0	Nil
G81	P3/2018	0	Nil
G70	P2/2018	0	Red hepatization
G49	P2/2018	0	Nil
G57	P2/2018	0	Red hepatization
G38	P2/2018	1	Nil
G46	P1/2018	0	Nil

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G31	P1/2019	0	Remission

3.2 Mccp culture results

All samples cultured collected immediately (1hour) after postmortem (fresh samples) did not result in isolation of Mccp. The fresh samples were cultured in media prepared using pig serum stored for a duration of 8 weeks. However, after modification to include storage of lung samples in glycerol, there was 50% success in isolation of Mccp for lung samples stored for 21 months compared to 37.5% for those stored for 34 months for 21 month and 34 months, respectively (Table 3). This was achieved where pig serum aged 2 weeks was used to make media for isolation. The same was not possible pig serum was collected 8 weeks before it was used.

Samples stored under similar conditions (with glycerol) but grown in media with pig serum collected 8 weeks before use did not show Mccp growth for both 21 and 34 months time points of storage

Table 3. Mccp isolations from lung samples stored using glycerol until 21 and 34 months storage time points

Duration of storage	Lung sample ID	Pig serum age (weeks)	Glycerol storage	% Mccp isolation
21 Months (2020)	G279	2	With	100 (1/1)
	G281			0 (0/1)
	G280			0 (0/1)
	G291			0 (0/1)
	G286			100 (1/1)
	G268			0 (0/1)
	G274			100 (1/1)
	G278			100 (1/1)
34 months (2019)	G119	2	With	100 (1/1)
	G101			0 (0/1)
	G108			0 (0/1)
	G104			0 (0/1)
	G102			100 (1/1)
	G103			100 (1/1)
	G105			0 (0/1)
	G118			0 (0/1)

3.3 Second recovery of Mccp

The four lungs samples from which Mccp was isolated at the 21 months storage duration time point still produced Mccp (4/4) six months later at the 27 months storage duration time point when isolation was done using media made from pig serum collected 2 weeks before use (Table 4). However, the colony changing units (CCUs) for all the four positive lung samples had reduced from 10^7 at 21 months storage time point to 10^6 at 27 months storage time point.

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Table 4. Colony Changing Units (CCU) for isolation done on positive colonies after 6 months

Lung sample ID	Positive at 21 months	Positive at 27 months	CCU for isolation done at 21 months	CCU for isolation done at 27 months
G279	1	1	10^7	10^6
G286	1	1	10^7	10^6
G274	1	1	10^7	10^6
G278	1	1	10^7	10^6
Lung sample ID	Positive at 34 months	Negative at 40 months	CCU for isolation done at 34 months	CCU for isolation done at 40 months
G119	Positive	Negative	10^6	10^4
G102	Positive	Negative	10^6	10^3
G103	Positive	Negative	10^6	10^2

On the contrary, the three samples which were positive at 34 months duration storage time point were all negative (0/3) for Mccp at 40 months storage time point (Table 4). The repeat culture after 6 months was also done using media made from pig serum collected 2 weeks before use. The CCU for two lung samples had reduced from 10^6 at 34 months storage time point to 10^4 at 40 months storage point while one lung sample had reduced from the 10^6 at the 34 months storage time point to 10^3 at the 40 months storage time point.

4.0 Discussion

Infection of goats by artificial infection with Baringo strain Mccp strain resulted in clinical and postmortem lesions consistent with CCPP in some goats. The infected goats developed fever and respiratory signs like labored breathing associated with CCPP in goats. This finding was consistent with an experimental study which also established that goats infected artificially with Mccp developed clinical signs including respiratory signs like labored breathing and fever (Zhang *et al.*, 2022).

The study has established that storing Mccp in glycerol at-40℃ while improving on the media to utilize freshly collected pig serum (two weeks following collection) has a positive effect on recovery of Mccp after storage for a long duration. Glycerol makes Mycoplasma cells to stay alive and viable for a longer duration because as it is a great source of carbon and energy (Pérez et al., 2016). Findings of this study support this argument since media without glycerol did not support growth of Mccp in samples that were grown in media with freshly collected serum; suggesting that freshly collected serum alone was not responsible for the observed growth.

On re-culture, Mccp isolation was possible from lung samples, which were 27 months in storage when using media made from pig serum collected before 2 weeks. The color changing units for the broth also showed evidence that at 10^6 and 10^7 the Mccp cells were still viable and 10^3 and 10^4 suggested that the Mccp cells had started losing their viability at this period. The findings suggested that at-40℃, the cryopreservative property of glycerol could support viability for

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Mccp for a duration not exceeding 34 months. This is much longer than the finding of Pérez et al., (2016) who assessed the viability of *M. hyorhinis* strains preserved by freezing using glycerol and dimethyl sulfoxide as cryopreserving agents. *M. hyorhinis* samples frozen at-20ºC and-70ºC remained viable for 90 days, while those without cryopreservatives did not grow in both storage temperatures. This was attributed to the fact that cryopreserving agents such as 5% glycerol or 2.5% dimethylsulfoxide preserved the viability *M. hyorhinis* during freezing and subsequent storage conditions.

It is worth noting that glycerol keeps the cells hydrated at-40℃ and thus increases the chance of reviving Mycoplasmas, because it is a highly hydrophilic molecule and thus easily enters the cell through water channels on cell membrane (Al-Farha *et al.*, 2018). While inside the cells, glycerol molecules maintain intracellular water, which consequently decreases the freezing-point. This phenomenon is suspected to protect cells from damage during cooling processes by precluding excessive dehydration, inhibiting osmotic shock and preventing the formation of large ice crystals within the cell (Zhang *et al.*, 2022).

Media prepared from two-week-old pig serum stored at -20°C supported growth, as the nutrients remained intact. Conversely, media made from serum aged eight weeks did not sustain growth due to nutrient degradation. Decreases in storage temperatures, often resulting from power failures, contribute to this nutrient loss in serum, thereby hindering the growth support for mycoplasma (Freshney and Amanda, 2021).

The success of isolating Mccp was also dependent on the duration of storage of pig serum used, since growth was not observed when glycerol was used in the absence of freshly collected pig serum. It is evidently clear in this study that media prepared with pig serum collected two weeks prior to use has a better chance of supporting Mccp growth compared to media prepared from pig serum collected eight weeks prior to use . However, this study did not establish at what point in time the serum loses the ability to support growth. Serum is known to be a complex mix of albumins, growth factors and growth inhibitors. It is one of the most important components of cell culture media and serves as a source of amino acids, proteins, vitamins (particularly fat-soluble vitamins such as A, D, E, and K), carbohydrates, lipids, hormones, growth factors, minerals, and trace elements. All these nutrients help to support growth of Mccp cells (Freshney and Amanda 2021). Although it is not known which components of the media affected the growth, the findings give us an indication of the length of storage of serum at-40^{o}C, after which the batch of serum should be discarded. At the same time, there is a need to investigate further on duration of storage as well as alternative temperatures at which the serum should be stored to prolong its use.

The study shows that after a certain duration of storage at-40^{o}C, even a combination of glycerol and freshly collected serum will not support growth. This suggests that isolation was possible at 34 months, beyond this period it was not possible to isolate Mccp.

5.0 Conclusion and recommendation

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Lung tissues stored in glycerol enhances chances of isolating Mccp even at storage duration of 34 months at -40°C for a period not exceeding 34 months. Serum quality and temperature during storage condition is also key in Mccp isolation. It is recommended to collect goat lung samples suspected of CCPP in glycerol to enhance viability of Mccp in lung specimens under storage. More is required to establish optimum storage duration of lung tissues to prolong viability of Mccp in stored lung specimens for successful recovery of Mccp and as well as determining best quality of serum

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6.2 General acknowledgement

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6.3 Conflict of interest

None.

6.4 Data availability

All data are available within the manuscript, and additional data are available from the corresponding authors upon request.

6.5 Ethical approvals

The study was performed after obtaining institutional ethical approval from the Institutional Animal Care and Use Committee (IACUC) of KALRO-Muguga, reference number KALRO-VSRI/IACUC015/15112017.

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