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RESEARCH ARTICLE

Bronchiolar Epithelium Revealed as a Primary Tropism Site of *Mycoplasma capricolum* subsp. *capripneumoniae* in a Novel Caprine CCPP Model

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ABSTRACT

Contagious caprine pleuropneumonia (CCPP) is a fatal disease of goats, caused by *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp), characterized by high morbidity and mortality. CCPP is listed by WOAH as one of the most severe diseases that affect goats and cause significant economic losses. Despite its serious implications, limited information is available so far about Mccp tissue tropism in the natural host. In this study, novel endemic Mccp strains (n=04) were isolated from local goats and were characterized using conventional and molecular tools. The tissue tropism of local Mccp strains in the natural host was elucidated by a novel caprine infection model (CCPP model). The pathogenesis and tissue tropism of Mccp were comprehensively studied employing hematology, gross and histopathology, immunohistochemistry (IHC), and PCR techniques. Hematological analyses exhibited significant decreases in RBCs count and PCV ($P<0.05$), with a significant increase in MCV, TLC, and DLC. Postmortem examination revealed moderate-to-severe gross lesions in the respiratory passages and lungs that include tracheal hemorrhages, bronchio-alveolar exudation, fibrinous pleurisy, and pulmonary red-hepatization. Histopathology revealed leukocytic infiltration in the bronchiolar mucosa and alveoli, fibrosis of bronchiolar and interalveolar septa, exfoliation of bronchiolar epithelium, and emphysema. Interestingly, IHC revealed reactivity of Mccp (antigens) in bronchiolar epithelium, while tracheal epithelium was non-reactive to anti-Mccp antisera. These outcomes advocate that the bronchiolar epithelium is the primary niche for Mccp in goats during infections, providing significant insights into CCPP pathogenesis in goats and updates for future diagnostic and therapeutic strategies.

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INTRODUCTION

Contagious caprine pleuropneumonia (CCPP) is a severe contagious disease of small ruminants, especially goats. It is known, worldwide, for serious respiratory discomfort, high morbidity (reaching up to 100%), and high mortality (80-100%) in some flocks (Iqbal *et al*., 2019). The primary etiological agent of CCPP is Mccp (Abdollahi *et al*., 2023). Importantly, Mccp belongs to the *Mycoplasma mycoides* cluster (*Mm* cluster). The *Mm* cluster comprises six closely related mycoplasmas, causes similar respiratory distresses in ruminants, that includes three members from the mycoides species, i.e., *Mycoplasma mycoides* subsp. *mycoides* small colony (MmmSC); *Mycoplasma mycoides* subsp. *capri* (Mmc); *Mycoplasma mycoides* subsp. *mycoides* large colony (MmmLC) and three members from *capricolum* species, i.e., Mccp; *Mycoplasma capricolum* subsp. *capricolum* (Mcc) and *Mycoplasma* subsp. *bovine* group 7 (BG7) (Saddique *et al*., 2012; Iqbal *et al*., 2019).

CCPP is accountable for causing substantial animal health problems and economic losses, predominantly in Asia (South Asia), Africa, and the Middle East (Shaheen *et al*., 2024). The financial losses are mainly because of

reduced animal production, costly treatments, poor veterinary services, high fatalities and trade embargos (Rehman *et al*., 2022). These implications of the disease further deteriorate the economic landscape for farmers (poor farmers)-mainly those in arid; semi-arid and mountainous areas; where goats farming is a major source of livelihood and where goats are considered as the poor man's cow (Ahmad *et al*., 2021). Though CCPP is a socio-economically important disease, however it is underrated in several countries of the world including Pakistan. Furthermore, this lack of serious attention leads to major knowledge gaps in the containment of the disease, pathogenesis, and vaccine development (Nicholas and Churchward, 2012; Hayatullah *et al*., 2024).

Despite having a relatively small genome, Mccp can cause severe pleuropneumonia with high mortality rates in susceptible goat populations. Its fastidious growth and largely obscure metabolic capacity further complicate it's *in vitro* investigations (Ahmad *et al*., 2021; Yuan *et al*., 2020). Recently, significant developments have been made in the detection of potential virulence factors of Mccp (including surface lipoproteins, and hemolysin) using genomics, proteomics, and metabolomics; however, the pathways that drive Mccp affinity for specific tissues (lungs and pleura) have not been completely elucidated (Chen *et al*., 2017; Hao *et al*., 2023).

The understanding of CCPP in Pakistan has significantly been improved during the last decade. In the past, limited diagnostic tools and cross-reactions in serological tests often led to confusion between Mccp and Mmc. As a result, locally produced vaccines were primarily developed using Mmc strains. However, with recent advances in molecular techniques such as PCR and sequencing, researchers have confirmed that Mccp is the actual cause of CCPP outbreaks in Pakistan (Awan et al., 2010; Peyraud *et al*., 2014; Banaras *et al*., 2016; Ahmad *et al*., 2021; Rehman *et al*., 2022; Akhtar *et al*., 2022). This creates a challenge, as disease control efforts are still based on a vaccine targeting a different but related member of *Mm* cluster. Although some cross-protection might occur due to shared antigens, it is probably inadequate, which explains why CCPP still appears even in vaccinated herds. Several outbreaks of CCPP caused by Mccp have been confirmed in goats, previously immunized with the local Mmc-based vaccine (Ahmad *et al*., 2021).

The limited use of a natural host infection model (caprine CCPP model) has further delayed research into understanding the Mccp tropism. Several models (lab animal models) have failed to reproduce the natural development of the disease, and therefore, the understanding regarding Mccp tropism remains unclear. Additionally, genoplasticity among Mccp strains could affect the host tissues tropism and clinical implications. However, the association among strains variability and disease severity remains vague so far (Yuan *et al*., 2020).

Thus, there is a dire need for the establishment of an animal disease model (caprine CCPP model) that can precisely depict; (a) natural onset of the disease (b) the disease development, and (c) tissue distribution of the pathogen, in order to improve our understanding regarding the behavior of Mccp in the natural setting. Knowing the molecular interplay (during infection) between the natural host immune system and Mccp is vital for the effective control of the disease (Chen *et al*., 2017; Iqbal *et al*., 2019).

Hence, this study aimed to contribute in filling these knowledge gaps using the endemic Mccp strain (Ahmad *et al*., 2021; Akhtar *et al*., 2022) in goats to determine the distribution of Mccp in the host tissues. The current study revealed that local Mccp strain predominantly colonized bronchiolar epithelia, with minimal involvement of the trachea; a finding that could redefine current paradigm of CCPP pathogenesis.

MATERIALS AND METHODS

Study area and sample size: The study was conducted in Khyber Pakhtunkhwa and Gilgit-Baltistan regions of Pakistan. For systematic sampling, the regions were divided into four geographical zones; central zone (Nowshera, Peshawar, Charsadda and Mardan), northern zone (Gilgit, Chitral, Buner, Swat), tribal zone (Khyber, Mohmand, Bajaur), and southern zone (Lakki, Bannu, D.I Khan and Karak). The animals were humanely restrained as per the guidelines of the ethical committee, Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture, Peshawar (FAHVS, UAP), Pakistan. A total of 1200 samples were collected from CCPP-suspected goats (Table 1). The sample size was determined using Thrusfield's formula (2018) for an expected disease prevalence of 8% (based on previous regional surveillance data), with a confidence level of 95% and an absolute precision of 5%. All collected samples were transported under 4°C to the Pathology Lab, FAHVS, UAP.

Table 1: Collection of samples from goats at different zones of KP, Pakistan.

Zones	Types of samples collected from goats				
	Nasal swab	Tracheal swab	Lung's tissue	Pleural fluid	Total
Northern zone	100	100	50	50	300
Central zone	100	100	50	50	300
Southern zone	100	100	50	50	300
Tribal zone	100	100	50	50	300
Total	400	400	200	200	1200

Detection of the potential pathogen in tissue and fluid samples: Nasal, tracheal and pleural fluid samples were inoculated into 5mL PPLO broth (Difco, USA), supplemented as reported elsewhere (Ahmad *et al*., 2021). Lung samples were homogenized in sterile PBS (1:10w/v) before culturing (Chao *et al*., 2019). All samples were incubated at 37°C in the presence of 5% CO_2 for 5-10 days (Hayatullah *et al.,* 2024). After streaking on PPLO agar, "fried egg" positive colonies were sub-cultured in PPLO broth for purification. PCR was performed for the confirmation of Mccp culture (Woubit *et al*., 2004).

Hematological analysis: Blood samples were collected in EDTA tubes from Mccp-infected goats for the complete blood count (CBC) using a hematology analyzer (Mindray, China). Briefly, hemoglobin concentration (Hb), packed cell volume (PCV), total erythrocyte counts (TEC), total leukocytes count (TLC), and differential leukocytes count (DLC) were recorded as reported elsewhere (Liljander *et al*., 2019).

Experimental challenge model: A total of ten healthy goats were selected and purchased for the experimental CCPP challenge model. Goats between 12 and 24 months of age, weighing 13-27kg (mean ±SD: 20.3 ± 4.1kg) were selected. The selected animals were confirmed to be seronegative for anti-Mccp antibodies through cELISA (IDEX CCPP). The animals were housed in quarantine at the FAHVS, UAP's small ruminants shed for 21 days. The environment for experimental goats was kept at 25°C (ambient temperature), 55% humidity, and 12hr light/dark cycle. A single dose of ivermectin (0.2mg/kg SC) and immunization with the PPR and enterotoxemia vaccines were administered as preventative measures, 14 days before the challenge. The animals were acclimatized for 7 days before an inoculation in the University's challenge facility, where they had *ad libitum* access to water, alfalfa hay (2.5% body weight/day), concentrate (14% CP, 1% body weight BID), and mineral licks. Goats were examined twice a day for casualties, while different parameters including rectal temperature, respiration rate, nasal discharge, and cough, were measured once a week.

Mccp challenge and antibody production: The experimental goats were divided into two groups. Seven goats in group (A) intratracheally received 5mL (1×10^8CFU/mL) culture of Mccp-MRG-g strain, a locally isolated strain by our lab (Akhtar *et al*., 2022), whereas 03 goats in group (B) received 5mL of sterile PPLO broth (Khan *et al*., 2017) for three consecutive days**.** Serum samples were collected at 0, 7th, 14th, 21st and 28th day post-infection (dpi). Nasal as well as tracheal swabs were collected on daily basis from 0-28 dpi and cultured simultaneously. The experimentally challenged dead animals were necropsied after death, whereas the remaining infected animals were euthanized at day 28dpi for postmortem analysis.

Postmortem examinations: The necropsy was systematically performed for the evaluation of gross lesions referring to the WOAH CCPP-scoring system. Significant gross-pathological lesions metrics such as; (i) pleural fluid effusion (volume in mL), (ii) hepatization area (% of lung surface), and (iii) adhesion severity (scored 0-4) were recorded in challenged-goats. Additionally, fresh tissue samples from lungs, trachea, and other affected organs were collected from died animals during experiment and euthanized animals and stored at -86°C for molecular analysis. For histopathological analyses, tissue samples were fixed in 10% buffered formalin (Sigma Aldrich) before processing.

Immunohistochemistry (IHC): For the immunohistochemical analysis, 4µm tissue sections were prepared using a microtome (Sakura, USA). Antigen was retrieved using citrate buffer (pH 6.0) at 95°C for 20min. A peroxidase block was applied using 3% H_2O_2 in methanol. The primary antibody, a pooled sera from Group A (day 28), was diluted 1:200 in PBS (1X) having 1% BSA and incubated overnight at 4°C. Mccp antigens were probed using secondary mouse anti-caprine IgG-HRP antibodies (Abcam) and DAB chromogen (Vector-4100). Hematoxylin was employed as a counterstain. IHC scoring was done based on staining intensity (0-3 scale) and the distribution of Mccp in tissue sections. Intensity was scored as follows; 0=negative, 1=faint, 2=moderate, and 3=strong.

RESULTS

Isolation and molecular confirmation of Mccp strains: The bacterial growth in PPLO broth was indicated by a color change of broth from pink to yellow and turbidity ≥0.8 OD_{600} after 5-10 days (Fig. 1A). PCR amplification of the 16S rRNA gene generated a 316-bp product, confirming the presence of Mccp in the collected samples. Twelve hundred samples obtained from goats suspected of having CCPP, showed a varying geographic prevalence of Mccp by PCR including 9.6% in the Northern zone, 7.3% in the Central zone, 6.3% in the Tribal zone, and 5.6% in the Southern zone, with an overall prevalence of 7.25% (Table 2). No significant inter-zonal differences were found by statistical analysis (χ^2=3.82, p=0.281).

Furthermore, pure and typical colonies of Mccp with a fried-egg morphology (diameter of 150-300µm, central nipple-like elevation) were observed on PPLO agar under microscope (Fig. 1B). Importantly, four novel strains of Mccp (Table 3) from various studied areas (Fig. 2) were successfully isolated, purified and confirmed by species-specific PCR (Fig. 1C).

Table 2: Molecular detection (PCR) of *Mycoplasma capricolum* subsp. *capripneumoniae* in samples collected from CCPP suspected goats.

Zones	No. of sample	Positive sample	Percentage of Mccp	Chi square	p-value
Northern zone	300	29	9.6	7.41	0.061
Central zone	300	22	7.3		
Southern zone	300	17	5.6		
Tribal zone	300	19	6.3		
Total	1200	87	7.25		

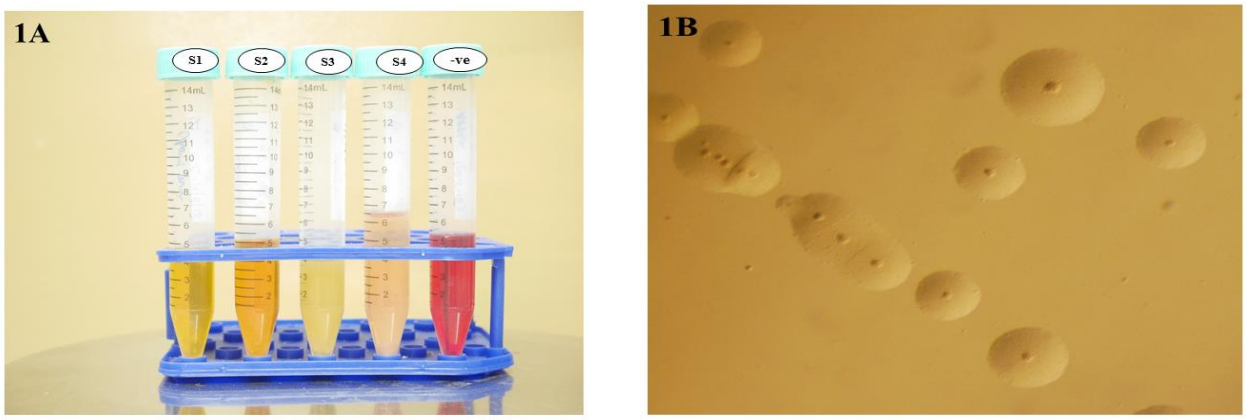
Statistical analyses exhibited insignificant association (P>0.05) between 04 geographical zones and *Mycoplasma* strains.

Table 3: Isolates of Mccp identified and purified from positive samples.

S.No,	Strain ID	Host	Location	Supplier	Sample source
01	Mccp-MRG-g	Capra hircus	Gilgit, Pakistan	UoA-P	Lung tissue
02	Mccp-MRG-s	Capra hircus	Swat, Pakistan	KP, UoA-P	Pleural fluid
03	Mccp-MRG-c	Capra hircus	Chitral, Pakistan	KP, UoA-P	Pleural fluid
04	Mccp-MRG-b	Capra hircus	Bunir, Pakistan	KP, UoA-P	Lung tissue

Hematological alterations in infected goats: Challenged goats in group-A depicted significant changes in CBC compared to the animals in group-B. Total erythrocyte count reduced by 38% and PCV decreased by 42% (P<0.001) indicated erythrocytopenia in experimentally infected goats. Additionally, in erythrocyte indices, an increase in mean corpuscular hemoglobin (MCH) by 29% (P<0.003) and mean corpuscular hemoglobin concentration (MCHC) by 24% (P<0.002) were recorded in challenged goats. Notably, a significant rise in the total WBC count (P<0.001) indicated leukocytosis (Table 4). Additionally, compared to the negative control group-B, infected goats showed significant leukocytosis, lymphocytosis, neutrophilia, and monocytosis (Table 5).

Gross pathological lesions: In infected goats (group-B), gross pathological lesions were observed, particularly in the respiratory system. The trachea of 80% challenged goats depicted petechial hemorrhages coupled with frothy-mucopurulent exudate (3-5mL) (Fig. 3A and 3B). In the



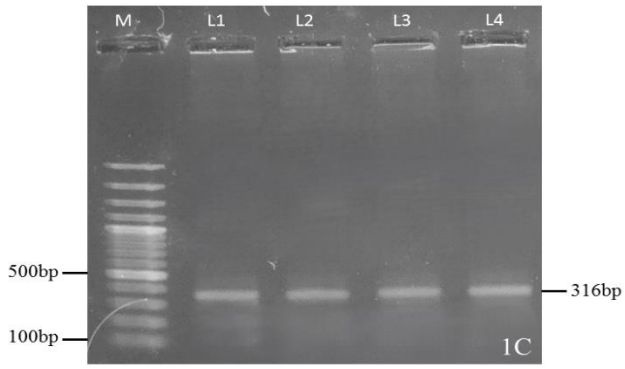


Fig. 1: Identification of Mccp by culture and PCR: (A), Growth of Mycoplasma indicated by color change of PPLO broth (S1-S4) compared to negative control; (A), Mccp specific colonies (Fried egg) on PPLO agar (100x); (C), PCR confirmed growth of Mccp, M- DNA marker, L1- Positive control, L2-L4- samples positive for Mccp generated an amplicon of 316bp.

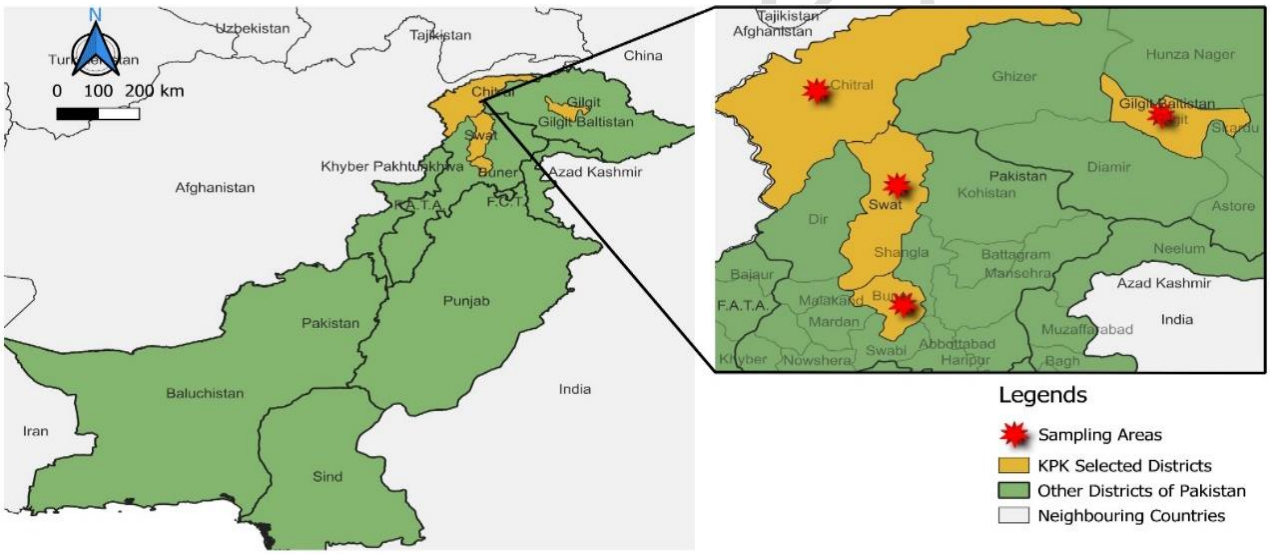


Fig. 2: Map of Khyber Pakhtunkhwa represents the different regions where new Mccp strains are isolated from suspected goats.

Table 4: Erythrocytic count, and indices of goats challenged with the endemic Mccp isolate

DPI	Goats	RBC-10^{12}/L	Hb-g/dl	PCV-%	MCH-pg	MCV-fl	MCHC-g/dl
Day 0	NC	9.5±0.8^{a}	8.3±0.2^{ab}	23.3±0.7^{a}	6.2±0.1^{ab}	18.6±1.0^{f}	30.0±0.1^{a}
	Challenged	9.4±1.0^{a}	8.2±0.4^{ab}	22.2±1.0^{a}	6.2±0.2^{ab}	19.0±0.4^{f}	30.0±0.2^{a}
Day 6	NC	9.3±0.80^{a}	8.3±0.2^{ab}	21.8±0.8^{a}	6.2±0.1^{ab}	18.6±1.0^{f}	30.0±0.1^{a}
	Challenged	9.0±1.0^{a}	8.0±0.2^{ab}	20.0±1.0^{b}	6.2±0.1^{ab}	20.2±2.1^{ef}	30.0±0.2^{a}
Day 12	NC	10.0±0.5^{a}	8.0±0.6^{ab}	24.0±0.4^{a}	6.4±0.1^{ab}	22.0±2.2^{ef}	30.0±0.1^{a}
	Challenged	7.0±0.5^{c}	8.1±1.3^{a}	12.1^{d}±2.0^{c}	6.5±0.3^{ab}	30.1±4.0^{c}	30.0^{ab}±0.1^{a}
Day 18	NC	9.3±0.4^{a}	8.7±0.4^{ab}	24.0±0.5^{a}	6.4± 0.1^{ab}	25.0±0.1^{de}	31.2±0.2^{a}
	Challenged	4.1±0.9^{d}	6.4±0.3^{b}	8.0±1.1^{d}	6.5±0.3^{ab}	39.0±6.1^{b}	30.3±0.1^{a}
Day 24	NC	9.4±0.3^{a}	8.1±0.4^{ab}	24.6±0.5^{a}	6.4±0.1^{ab}	27.1±7.0^{de}	30.2±0.1^{a}
	Challenged	3.0±0.7^{d}	6.2±1.0^{b}	5.7±1.1^{e}	6.7±0.3^{a}	42.0±5.0^{ab}	31.1±2.1^{a}
Day 28	NC	8.4±1.6^{b}	8.0±0.2^{ab}	24.0±0.3^{a}	6.4±0.1^{ab}	27.2±6.5^{cd}	30.2±0.1^{a}
	Challenged	3.1±0.7^{d}	6.1±1.0^{b}	5.8±1.1^{e}	6.7±0.3^{a}	42.2±5.0^{a}	31.0±1.3^{a}

^{a}, ^{b}, ^{c}, ^{d}, ^{e}means with different superscript within column are significantly different at P<0.05. DPI, day post-infection; NC, Negative Control experimental goat; TEC, Total erythrocytes count; Hb, Haemoglobin; PCV, Packed cell volume; MCV, Mean corpuscular volume; MCH, Mean corpuscular haemoglobin; MCHC, Mean corpuscular haemoglobin concentration.

lungs and thoracic cavity of infected animals; (i) straw-colored pleural fluid ranged from 50-100mL, (ii) cranio-ventral lobar consolidation (40–60% surface area) and (iii) fibrin deposition (2-5mm thick) (Fig. 3C and 3D) were observed. Necrotic lesions with multiple coagulative foci (0.5-3cm in diameter) were also recorded in lungs of challenged animals. In contrast, no lesions were observed in the animals of negative control group (Table 6).

Histopathological findings in trachea and lungs of experimental animals: In the trachea, observed histopathological lesions comprised leukocytic infiltration (mainly neutrophils, macrophages, and lymphocytes (40-60 cells per 400X field), and considerable epithelial sloughing (70-90% of the tracheal mucosa exhibited loss of ciliated epithelium). Additionally, submucosal edema was observed coupled-with the submucosal layer thickening from 50±10µm to 200 ± 30µm (P<0.001). Pulmonary histopathological lesions were characterized by; **(a)** 3.5-fold thickening of inter-alveolar septa (25.8 ± 3.2µm vs. 7.3 ± 1.5µm; P<0.001), **(b)** bronchiolar epithelial sloughing affecting 60–80% of terminal bronchioles, and **(c)** emphysema with rupture of alveolar walls in 30–50% of the parenchyma (Tables 7). Furthermore, fibrinopurulent exudate occluded 40–60% of the bronchioles (Fig. 4A-F).

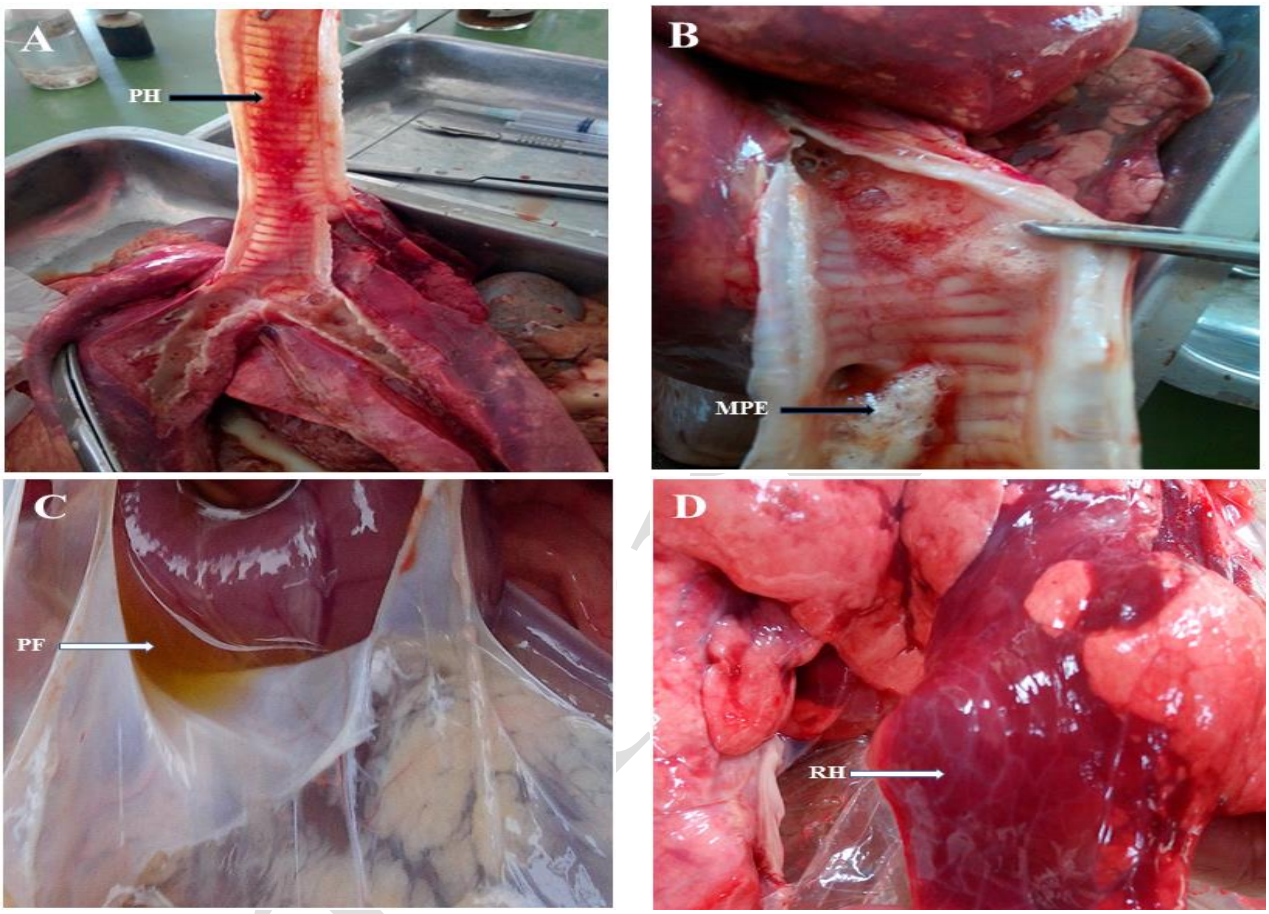


Fig 3: Gross pathological lesions revealed in necropsied goats infected with CCPP: (A), Showing Petechial haemorrhages (PH) on tracheal mucosae; (B), Showing mucopurulent exudates (MPE) with haemorrhages in trachea; (C), Showing presence of straw-colored pleural fluid (PF), in the thoracic cavity (D), Showing red hepatization (RH) of lung.

Table 5: Leukocytic profile of goats challenged with endemic Mccp isolate

DPI	Goat	TLC-10^9/L	NEU-10^9/L	LYM -10^9/L	MC-10^9/L
Day 0	NC	9.10 ± 0.10^{c}	3.00 ± 0.40^{c}	3.10 ± 0.50^{de}	1.00 ± 0.20^{c}
	Challenged	10.8 ± 0.30^{c}	2.30 ± 0.30^{c}	5.01 ± 1.10^{de}	0.50 ± 0.30^{c}
Day 6	NC	9.33 ± 0.25^{c}	2.43 ± 0.32^{c}	3.00 ± 0.43^{de}	1.00 ± 0.21^{c}
	Challenged	10.54 ± 1.13^{c}	2.28 ± 0.21^{c}	5.12 ± 0.98^{d}	0.59 ± 0.48^{c}
Day 12	NC	9.20 ± 0.30^{c}	2.66 ± 0.23^{c}	3.13 ± 0.20^{de}	1.03 ± 0.11^{c}
	Challenged	19.3 ± 0.69^{b}	4.66 ± 0.70^{b}	10.40 ± 1.41^{c}	2.06 ± 0.39^{b}
Day 18	NC	9.86 ± 0.20^{c}	2.93 ± 0.15^{c}	3.26 ± 0.15^{de}	1.00 ± 0.17^{c}
	Challenged	25.31 ± 3.20^{a}	7.23 ± 1.65^{a}	16.60 ± 3.20^{b}	2.51 ± 0.53^{ab}
Day 24	NC	10.00 ± 0.26^{c}	2.80 ± 0.26^{c}	3.33 ± 0.11^{de}	1.06 ± 0.15^{c}
	Challenged	27.82 ± 4.98^{a}	7.98 ± 1.37^{a}	19.36 ± 2.33^{b}	2.76 ± 0.61^{a}
Day 28	NC	10.18 ± 0.20^{c}	2.96 ± 0.20^{c}	3.33 ± 0.20^{de}	1.00 ± 0.17^{c}
	Challenged	28.06 ± 4.68^{a}	7.84 ± 1.55^{a}	20.0 ± 3.00^{a}	3.1 ± 0.50^{a}

^{a}, ^{b}, ^{c}, ^{d}, ^{e} means with various superscript within column are significantly different (P<0.05). DPI, day post infection; NC, Negative Control experimental goat; TLC = Total leukocytic count; NEU, Neutrophils; LYM, Lymphocytes; MC, Monocytes

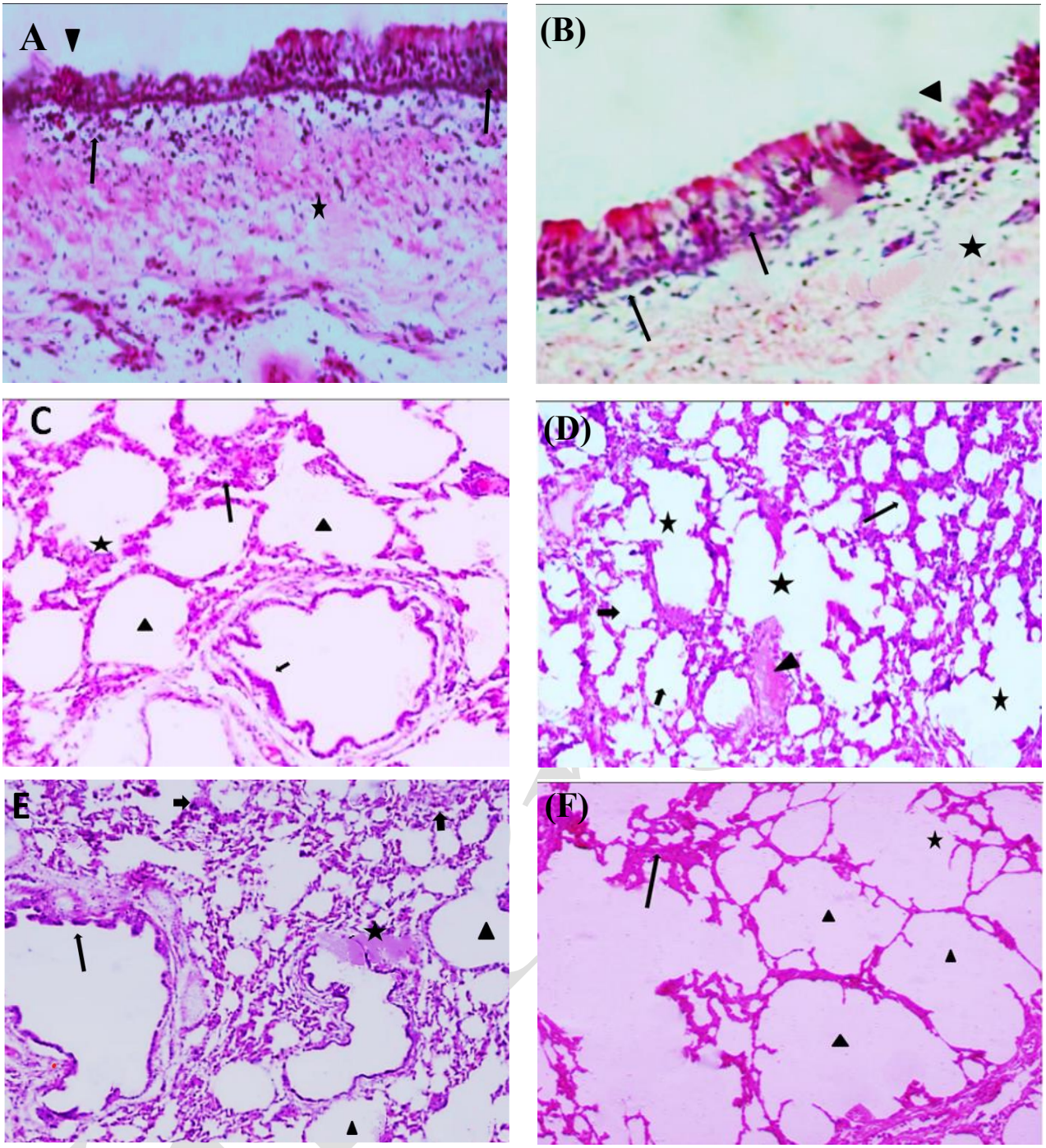


Fig. 4: Histopathological findings in tissue samples collected from goats infected with CCPP: (A&B), Trachea exhibiting sloughing of epithelial lining (arrowhead), oedematous swelling (star), leukocytic infiltration (arrows); (C), Lung showing emphysema (arrow heads), rupture of alveoli (star), thickening of alveoli septa (short arrow), sloughing of epithelial lining of bronchiole (long arrow), exudate (star); (D), Lung showing leukocytic infiltration (Long arrow), emphysema (arrowhead) thickening of alveolar septa (star), sloughing of epithelial lining of bronchiole (short arrow); (E&F), Lung showing severe emphysema (arrow heads), rupture of alveoli (star), thickening of alveoli septa (arrow) (H&E stain, 400X).

Table 6: Gross pulmonary lesions in Mccp-infected goats.

Group	Goat ID	Consolidation	Pleural adhesion	Coagulative necrosis	Tracheal hemorrhages/exudate	Pleural fluid
A	G01	+	-	-	-	+
	G02	+	-	-	+	-
	G03	+	+	+	+	+
	G04	-	+	+	-	-
	G05	+	-	-	+	-
	G06	+	+	+	+	+
	G07	+	+	-	+	+
B	G08	-	-	-	-	-
	G09	-	-	-	-	-
	G10	-	-	-	-	-

Table 7: Histopathological lesions in Mccp-infected goats.

Group	Goat ID	Alveolar exudation	Disruption of alveoli	Disruption of bronchiolar epithelium	Leukocytic infiltration	Thickening of Alveolar wall	Tracheal theepithelium and cilia disruption
A	G01	-	+	-	+	+	-
	G02	-	+	-	+	-	-
	G03	-	+	+	+	+	+
	G04	-	-	+	-	+	+
	G05	+	+	-	+	-	-
	G06	-	-	+	+	+	+
	G07	+	+	-	+	-	+
B	G08	-	-	-	-	-	-
	G09	-	-	-	-	-	-
	G10	-	-	-	-	-	-

Immunohistochemical localization of Mccp antigens: Immunohistochemical analyses revealed a lack of immuno-reactivity in the tracheal tissue sections (Fig. 5A); however, strong immuno-positivity (3+ intensity) was observed in the bronchiolar epithelium, notably 80–90% of ciliated columnar epithelial cells showed immuno-reactivity (Fig. 5B–D). No immune-staining was detected in the alveolar septa or pleural mesothelium, and antigen density was highest at the necrosis-inflammation interface (Fig. 5D). Moreover, immune staining was observed to be negative in areas of complete alveolar rupture.

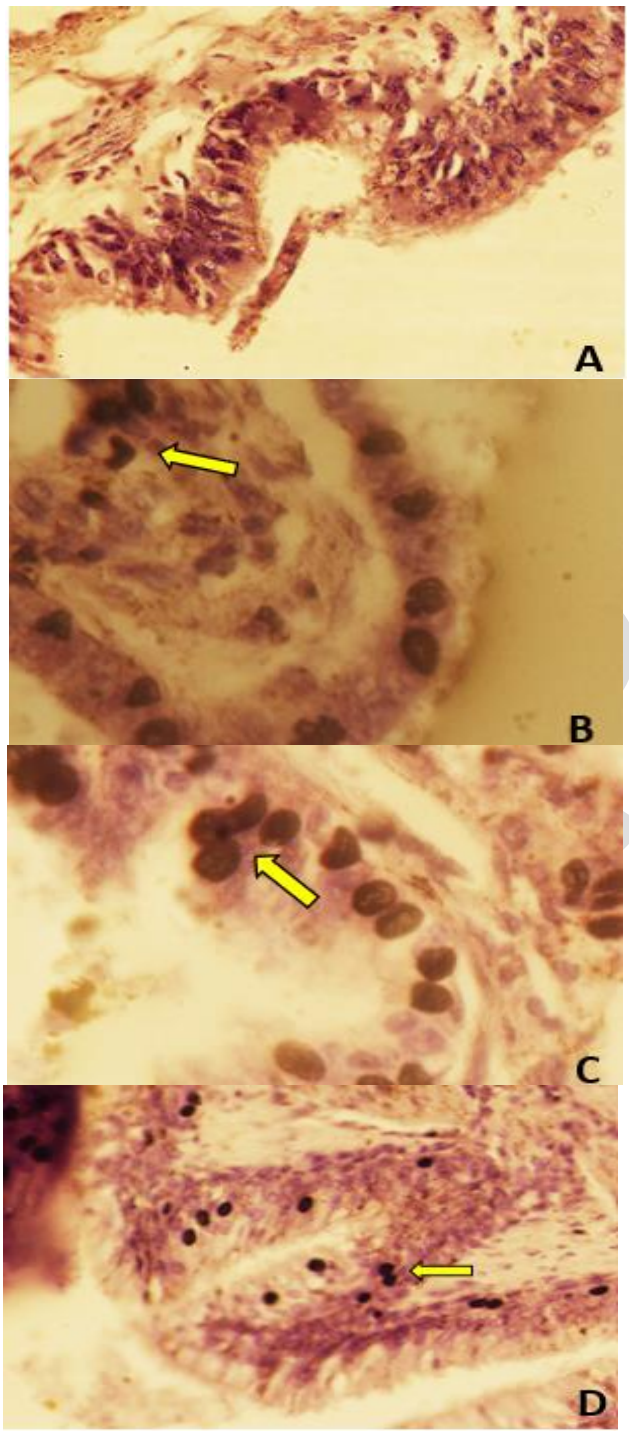


Fig. 5: Immunohistochemical staining of tissue samples from CCPP-infected goats: (A) The trachea of the affected lung shows no immune reactivity; (B-D) Positive immunostaining indicates the localization of Mccp in the lung tissue (arrows), particularly in the bronchiolar epithelium (40X & 100X).

DISCUSSION

The isolation and characterization of Mccp in this study provided critical insights about the pathogenesis of CCPP, aligning with and expanding upon previous findings while addressing key gaps in the current understanding of economically significant disease (CCPP). The current study findings (microbiological, hematological, and pathological) supported the previous concepts about Mccp infection in small ruminants, as well as exposed the novel characteristics of Mccp in the natural host and offered novel ideas for the therapeutic interventions.

Molecular Detection and Epidemiological Significance: The successful isolation and confirmation of Mccp strains employing PPLO media (broth and agar) and PCR respectively are consistent with previous reports, and therefore, supporting the reliability of these approaches for preliminary detection of Mccp (El Mahi and El Nasri, 1982; Ying *et al*., 2011; Nicholas and Churchward, 2012). The detected overall prevalence of Mccp (7.25%), with regional-variations (Northern zone-9.6% vs. Southern zone-5.6%) is contrary to the higher prevalence reported in East Africa (Kipronoh *et al*., 2016). Kenya (Africa) is reportedly having a seroprevalence of 63.9% in the Turkana-West region and 48.6% in Kajiado Central (Kipronoh *et al*., 2016). This emphasizes the wide-spread endemic-nature of CCPP in pastoral-systems, common in East-Africa. Likewise, in Ethiopia (Africa), CCPP is considered prevalent with regular occurrences since its detection back in 1983 (Teshome and Sori, 2021). This inconsistency may be attributed to differences in; (i) sampling methods, (ii) clinical vs. subclinical cases, (iii) diagnostic sensitivity, and (iv) environmental factors influencing the spread of disease (CCPP). Remarkably, lower rates of prevalence in the Middle East (Gulf countries) (Abdollah *et al*., 2023; Ali *et al*., 2024), are supporting the outcomes of the current study. Mccp strains identified in Oman are closely related to those found in Kenya, suggesting a shared phylogenetic group and potential cross-regional spread of the pathogen.

Interestingly, the lack of significant intra-zonal and inter-zonal differences [χ^2=3.82, p=0.281] suggests that Mccp strain (locally prevalent) is endemic across the regions studied in this research and is constantly circulating in goats, despite regional differences (Akhtar *et al*., 2022).

Hematological Alterations: Linking Pathology to Systemic Effects: The erythrocytopenia (↓38% TEC, ↓42% PCV) and leukocytosis (increased by 2.1 folds) observed in challenged goats (infected with Mccp) mirror findings in goats naturally infected with CCPP as reported elsewhere (Rodriguez *et al*., 1999; Iqbal *et al*., 2019), and supporting the hypothesis of Mccp linked hemolytic anemia and systemic infection in small ruminants. The detected alterations in RBCs count (lowered TEC and PCV) coupled with high TLC, reflects a strong immune-response to Mccp-infection and may contribute to the pathogenesis and severity of CCPP in the natural hosts by enhancing tissue damage through exaggerated immunological responses in Mccp infection.

The elevated RBCs indices (MCH and MCHC) diverged from some reports, where such increases were

either not observed or were attributed to different underlying mechanisms (Jain and Jaiswal, 2016). The variability in MCH and MCHC (indices) may be due to variations in disease stage (advance CCPP) or host response (Hardy *et al*., 2001). Specifically, the RBCs indices may vary as a compensatory mechanism (of host) in response to oxidative damage or compensatory erythropoiesis, where the body attempts to compensate for erythrocyte destruction (Richards *et al*., 2000; Chevalier *et al*., 2024). Additionally, oxidative damage to erythrocytes as supported by increased malondialdehyde, might be responsible for the observed changes in erythrocyte indices as reported previously (Perrone *et al*., 2012). Future studies should focus on measuring oxidative stress-markers (in the natural host) for clarity regarding the mechanisms that drives these hematological changes in Mccp infections (Wang *et al*., 2024).

Pathological Findings: Confirming and Expanding the Disease Model: The gross-pathological lesions including (i) tracheal hemorrhages, (ii) fibrinous pleuritis, and (iii) cranio-ventral consolidation are classical features of CCPP (caused by Mccp), were in consistency with previous reports (Sadique *et al*., 2012), indicating severe respiratory distress and tissue damages are classically associated with CCPP in goats. The extent of fibrin deposition (2-5mm) and the presence of multiple necrotic foci (0.5-3cm) in lungs observed in the current study exceed the measurements found elsewhere (Sadique *et al*., 2012), suggesting strain-specific virulence (Mccp vs. *M. mycoides capri*) or potential differences in host-susceptibility or differences in the individual animal immune response to the pathogen.

Histopathological-lesions linked with Mccp (3.5-fold alveolar septal thickening & bronchiolar epithelial sloughing) are aligned with prior studies (Yang *et al*., 2009; Sadique *et al*., 2012). However, the presence of emphysema (30–50% parenchymal involvement) in lung tissues depicted by this study is noteworthy and implies significant mechanical-compromise due to airways occlusion, a feature that was less emphasized in previous reports on CCPP pathogenesis. The evidence of emphysema suggests that; (i) Mccp may not only cause direct tissue destruction but, (ii) also lead to airflow limitations, and (iii) ultimately exacerbating respiratory dysfunction. Further investigation is warranted to understand these variations in the natural host caused by Mccp.

Immunohistochemical Localization: Resolving Key Questions: The detection of Mccp (antigens) by anti-Mccp antisera in the bronchiolar epithelium (80-90% positivity), coupled with its absence in the trachea and alveoli of the same host, provides clarity on the Mccp tissue tropism in natural host (goats). This observation reportedly resolves a long-debated issue linked with CCPP-pathogenesis. Earlier studies speculated about tracheal colonization by Mccp, however findings of the current study suggests that Mccp primarily targets the lower airways (especially terminal as well as respiratory bronchioles), which helps explain the severity of bronchiolar epithelium-multifocal necrosis observed in the infected goats. This tropism aligns with the known behavior of other respiratory pathogens including *Chlamydophila pneumoniae* (Mogilevski *et al*., 2002). The lack of immune-staining in alveolar-septa further supports the hypothesis that alveolar damage in CCPP is secondary to inflammation or hypoxia, as reported in *Mycoplasma pneumoniae* infections elsewhere (Lasbury *et al*., 2006). The focal antigen density observed at the necrosis-inflammation interfaces (necrotic-foci) mirrors findings in *M. mycoides* as reported previously (Tans *et al*., 2021), hinting at localized immune-evasion strategies adapted in the host (goat) by Mccp, as described elsewhere (Bahia *et al*., 2017).

Implications for Diagnosis and Control: The bronchiolar tropism (respiratory/terminal bronchiolar epithelium) of Mccp underscores the critical need for deep lung sampling like bronchoalveolar lavage (BAL), over conventional tracheal and nasal swabs for accurate diagnosis. BAL has proven to be highly effective in diagnosing lower-respiratory tract infections (bronchiolitis and pneumonia), providing a more comprehensive sample and higher diagnostic accuracy compared to nasal and tracheal swabs as suggested previously (Zhu *et al*., 2022).

In addition to PCR-based methods, the hematological changes observed in CCPP suggest that CBC could serve as early-indicators of Mccp infection. Integrating CBC with PCR could offer a dual-layered diagnostic approach, combining early detection of systemic changes with specific pathogen identification, enhancing the overall diagnostic process for CCPP (Loonen *et al*., 2024). Strain heterogeneity (Mccp strains) can be explored through comparative genomics of isolates from high- versus low-prevalence zones (Northren vs. Southern zone) to identify virulence markers (Data not published). Host-pathogen dynamics can be better understood using single-cell RNA sequencing of the bronchiolar epithelium to reveal the mechanisms of pathogen adhesion and invasion. Given the parallels with *M. haemofelis*, testing iron chelators or antioxidants as therapeutic options could help mitigate hemolysis. Finally, vaccine design efforts should focus on mucosal vaccines targeting bronchiolar immunity, as systemic vaccines have shown limited efficacy.

Conclusions: The findings of the present study have greatly advanced the CCPP paradigm by resolving long-standing controversies, such as Mccp tropism using endemic strain, and shedding light on understudied aspects, including emphysema and oxidative stress. This refined framework sets the stage for further research and improved CCPP control using endemic Mccp strains. Future work should emphasize the translational application of these findings to reduce the burden of CCPP in endemic regions.

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Author's contribution: MS and FAK conceived and designed the study. MS, SA, and MF executed the

experiment and analyzed the sera and tissue samples. MS, HK, and FAK analyzed the data. MS, RK, and FAK prepared the original draft of the Manuscript. RK, MAK, IA, and FAK review and edit the Manuscript. All authors critically revised the manuscript for important intellectual content and approved the final version.

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