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Bovine tracheal organoids for studying *Mycoplasma bovis* respiratory infections

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ABSTRACT

In vitro three-dimensional organoid models simulate key aspects of the structure and function of *in vivo* organs and have been used to study physiology, host-pathogen interactions, pathogenesis and pharmacodynamics. Although most organoid studies have been developed using human or mouse tissues, recent advancements have enabled the establishment of intestinal and respiratory tract organoids from domestic animal samples. *Mycoplasma bovis* causes chronic respiratory tract infections in cattle with significant health and economic consequences. The pathogenesis and virulence factors of *M. bovis* have been studied in several *in vitro* infection models, but the use of organoids has not been examined previously. In this study, we assessed the feasibility of using a matrix-embedded bovine tracheal organoid system to study respiratory infections with *M. bovis*. Bovine tracheal organoids were inoculated with *M. bovis* strain MbovMil and incubated for 72 hours to investigate the ability of *M. bovis* to proliferate, attach and invade the organoids. *M. bovis* was able to infect the organoids, resulting in a mean 260-fold increase in the titre of viable *M. bovis* by 72 hours post-inoculation. Examination of the infected organoids using transmission electron microscopy revealed the presence of mycoplasmas within the organoid cells and membrane bound clusters of *M. bovis* inside the intercellular junctions. Our findings indicate that bovine tracheal organoids can be used as a model system for studying respiratory tract infections caused by *M. bovis*.

1. Introduction

Organoids are self-organised, differentiated clusters of cells growing *in vitro* that mimic the structure and function of an *in vivo* organ (Corrò et al., 2020). They can be grown from either embryonic, induced pluripotent or adult stem cells (Corrò et al., 2020). Organoids have diverse applications, including research in developmental biology, pathogenesis, host-pathogen interactions, pharmacodynamics, toxicology and phylogenetics (Pain, 2021; Tran et al., 2022). Although there have been more studies performed with human and rodent organoids (Bartfeld and Clevers, 2015), intestinal, pancreatic, mammary, reproductive tract and airway organoids (Quah et al., 2023; Kawasaki et al., 2022) have also been generated from production animals to study the physiology of these organs, the pathogenesis of different infectious diseases and innate immune responses during infections (Quah et al., 2023; Beaumont et al., 2021).

Mycoplasma bovis is an important cause of bronchopneumonia in calves and feedlot cattle (Caswell and Archambault, 2007). It can also

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invade joints and cause arthritis in calves (Gagea et al., 2006), presumably as a result of hematogenous dissemination from the respiratory tract (van der Merwe et al., 2010). Although invasion of *M. bovis* into airway epithelial cells, erythrocytes and other peripheral blood mononuclear cells has been previously observed (van der Merwe et al., 2010; Nunoya et al., 2020), the mechanisms involved in the survival of *M. bovis* on mucosal surfaces, invasion of the cells at these surfaces, and penetration through the mucosal barrier have not been elucidated. Studies of the pathogenesis of infections with *M. bovis* and the virulence factors of the organism are needed to advance our understanding about the mechanisms underlying its persistence on mucosal surfaces and its capacity to penetrate these surfaces to cause systemic disease. This will enable the development and implementation of more effective control methods. Only a few *in vivo* studies using experimentally infected calves have investigated respiratory tract infections caused by *M. bovis* (Kanci et al., 2017). To date, studying interactions between *M. bovis* and its host in experimentally infected calves has been challenging mainly due to welfare concerns and the high costs associated with housing and maintaining live animals (Calcutt et al., 2018). As a result, *in vitro* infection models have been developed to mitigate the problems associated with the use of these *in vivo* infection models (Gaudino et al., 2023).

Primary bovine cells, such as embryonic bovine lung cells, embryonic bovine tracheal cells and synovial cells, have been used in host-pathogen interaction studies of *M. bovis* (Nishi et al., 2021; Thomas et al., 2003). In addition, cell lines, such as Madin-Darby bovine kidney cells (MDBK), and organ explants, such as tracheal organ cultures and precision cut lung slices (PCLS), have also been used (Gaudino et al., 2023; Thomas et al., 2003; Thomas et al., 1987; Premachandre et al., 2024). There are no previous reports of using organoids developed from the bovine respiratory tract to study infections with *M. bovis*. However, organoids derived from the respiratory tract tissues of other species have been used to study a variety of pathogens, including influenza viruses, SARS-CoV-2, *Mycobacterium tuberculosis* and *Mycobacterium abscessus* (Chen and Na, 2022; Iakobachvili et al., 2022). Recently, Quah et al. (2023) established a matrix-embedded bovine tracheal organoid system to study infection with bovine herpesvirus 1 (BHV-1), a pathogen contributing to bovine respiratory disease (BRD). The tracheal organoids were infected with BHV-1 and used to investigate the epithelial cellular response to the pathogen.

The aims of the study described here were to examine whether matrix-embedded bovine tracheal organoids could be infected with *M. bovis* and whether they were a suitable *in vitro* model for studying respiratory diseases caused by *M. bovis*.

2. Materials and methods

2.1. Isolation of bovine tracheal epithelial cells

Two bovine tracheas from beef cattle were obtained from an abattoir. The tracheas were collected from discarded offal, and, from their size, we estimated that the animals were about 12 months of age. The tracheas were processed to obtain suspensions of primary cells from each of the tracheas, as previously described by Quah et al. (2023). The concentration of viable cells in the primary bovine epithelial cell suspensions was determined using a haemocytometer by Trypan Blue (0.4 % w/v) exclusion (Sigma-Aldrich, USA). The cell pellets were resuspended and diluted with medium based on the pellet size. A 10 μL aliquot of the cell suspension was mixed with an equal volume of 0.4 % Trypan Blue and the stained cell mixture was then loaded into the haemocytometer chamber. Viable cell counts were performed by counting four squares within the haemocytometer grid. The total number of cells per mL was calculated using the following formula:

Cell concentration/mL = average cell count per square x dilution factor x 10^4.

Each haemocytometer square has a volume of 0.1 mm^3, the multiplication factor of 10^4 converts the count from cells per 0.1 mm^3 to cells per mL.

Then the primary bovine epithelial cells were stored in liquid nitrogen at a density of 1 × 10^7 cells/mL for both animals and were labelled Animal 1 and Animal 2.

2.2. Preparation of bovine tracheal organoids

Bovine tracheal organoids, prepared as described by Quah et al. (2023), were used in this study. Briefly, bovine tracheal epithelial cells were resuspended in 1 mL of PneumaCult ExPlus medium (STEMCELL Technologies, Canada) and a small aliquot of cell suspension was used to determine the cell concentration using a haemocytometer as described above. Then the cells were diluted with ExPlus medium to achieve a concentration of 2.5 x 10^5 viable cells/mL and transferred to T-25 cell culture flasks pre-coated with PureCol-S (Advanced BioMatrix, USA) type 1 collagen and incubated at 37 °C in 5 % CO_2. The medium was replaced with fresh warm medium on Day 3 and Day 5 and the cells were harvested when they reached 60 – 80 % confluency, on Day 7. The cell pellet was then resuspended in 10 mL of ice cold RPMI and an aliquot of the cell suspension was used to determine the cell concentration using the Trypan Blue exclusion method, as described above. Aliquots of cells required to set up organoid cultures were centrifuged for 5 min at 300 x g at 4 °C and resuspended in ice cold Cultrex reduced growth factor basement membrane extract, type 2 (BME-2) (R&D Systems, USA), at a concentration of 10,000 cells/50 μL. The cell suspensions in BME-2 (50 μL) were inoculated onto the surface of each well of a 24-well plate that had been warmed for 15 min in the incubator. Pneumacult Airway Organoid Seeding Medium (STEMCELL Technologies, Canada) was then added to each well and the plate was incubated at 37 °C in 5 % CO_2 for 7 days. The medium was switched from the seeding medium to PneumaCult Airway Organoid Differentiation Medium (STEMCELL Technologies, Canada) after 7 days. Organoids were cultured in differentiation medium for 16 – 21 days prior to infection.

2.3. Bacterial strains and culture conditions

The Australian wildtype strain of *M. bovis*, Millmerran_QLD_AUS_2009 (MbovMil), formerly known as strain 3683 (Kanci et al., 2017), was cultured at 37 °C in *M. bovis* (MB) medium [6 g Bacto Heart infusion broth (BD, USA)/L, 10 g Bacto proteose peptone broth (BD, USA)/L, 5 g sodium chloride/L, 37 mL of 15 % yeast extract (Gibco, USA)/L, 100 mL inactivated swine serum (Selborne, Australia)/L, 4 mL of 1.6 % phenol red solution/L, 859 mL distilled water/L, 300 mg penicillin G/L] for 24 h until the stationary phase of growth. Titres of viable mycoplasmas were determined by dilution of samples in MB medium in 96 well plates, observation for a colour change in the medium reflecting growth of the mycoplasmas, and calculation of the most probable number (MPN) at the endpoint, as described previously (Meynell and Meynell, 1970). The titre was expressed as colour changing units per millilitre (CCU/mL). The *M. bovis* culture stocks were stored as broth aliquots at −70 °C. The titres of viable mycoplasmas in the culture stocks were determined prior to storage at −70 °C.

2.4. Infection of organoids

The stock culture of *M. bovis* was inoculated into MB medium at a ratio of 1:10 (v/v) and incubated at 37 °C for 24 h. The bacterial cells were pelleted by centrifugation at 10,000 x g for 5 min at room temperature, washed with phosphate buffered saline (PBS), centrifuged at 13,000 x g for 5 min at room temperature, and resuspended and diluted in PneumaCult Airway Organoid Differentiation Medium. The inoculum was then titrated to confirm the concentration of viable *M. bovis* using the MPN method for determination of the concentration of viable organisms (Meynell and Meynell, 1970). The PneumaCult Airway

Organoid Differentiation Medium was then removed without disturbing the BME-2 dome containing the tracheal organoids and the organoids were then inoculated with 10^3 CCU of *M. bovis* in 500 µL of PneumaCult Airway Organoid Differentiation Medium and incubated with the inoculum for 2 h at 37 °C in 5 % CO_2. The volume of the medium in each of the wells was topped up with 500 µL of warm differentiation medium and the plates were then incubated at 37 °C in 5 % CO_2 for 72 h. *M. bovis* was also inoculated into PneumaCult Airway Organoid Differentiation Medium without any organoids (medium only control) to assess the ability of *M. bovis* to proliferate in the medium. Uninfected organoids were included as negative controls. After 72 h incubation, the medium in each well was collected and retained. The infected bovine tracheal organoids were washed with cold Dulbecco's Modified Eagle Medium (DMEM) (Sigma) to dissolve the matrix and the organoids were collected from each well of the cell culture plate into conical-bottomed tubes (Corning, USA), which were then centrifuged at 300 x g for 5 min at 4 °C to separate the matrix from the organoids. Three cycles of washes with cold DMEM and centrifugation were performed to dissolve any residual matrix and separate it from the organoids. The organoids and the differentiation medium from the corresponding wells were mixed and titres of viable *M. bovis* in the homogenate were determined after one freeze-thaw cycle, using the MPN method as described previously (Meynell and Meynell, 1970), with the titre expressed as CCU/mL. The organoids derived from Animal 1 and Animal 2 were infected with different *M. bovis* inocula on different days. The steps involved in infection, incubation and sample collection were similar for both infection assays. Three technical replicates were included for organoids prepared from each animal and for the differentiation medium for all time points.

2.5. Transmission electron microscopy of bovine tracheal organoids

Transmission electron microscopy (TEM) was used to examine the organoids to determine where *M. bovis* were localised in the tracheal organoid cells. Bovine tracheal organoids were prepared on Lab-Tek slides as described above and induced to differentiate for 3 weeks in PneumaCult Airway Organoid Differentiation Medium. The organoids were either inoculated with 10^3 CCU of *M. bovis* and incubated for 72 h as described above, or mock inoculated with medium alone. After 72 h incubation, the BME-2 matrix was removed using cold DMEM and the organoids were collected into 15 mL tubes, as described above. The organoids were washed once with PBS+ + (phosphate buffered saline with Ca^{2+} and Mg^{2+}) and fixed with 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) (ProSciTech, Australia) overnight at 4 °C in 15 mL tubes. Infected and uninfected organoids were added to 5 % low melting temperature agarose solution and incubated for 30 min at 4 °C to cast the organoids within an agarose block. Each agarose block was cut into small pieces (approximately 3 mm x 2 mm x 1 mm) to obtain samples containing samples of the organoids in a small amount of agarose gel. These organoids were then processed for electron microscopy using the high contrast reduced osmium-thiocarbohydrazide-osmium (rOTO) staining method described by Deerinck et al. (2022), using microwave-assisted tissue processing as described by Giberson et al. (2003), with modifications. The organoids were washed twice with ice cold 0.175 M cacodylate in a tissue processing microwave (PELCO BioWave Pro+, Ted Pella Inc. USA) at 150 W for 40 s. The gel pieces were then fixed in potassium ferricyanide reduced osmium tetroxide for 1 h at 4 °C, and then heated 3 times for 2 min each at 100 W under a vaccum in the microwave, with the microwave off for 2 minutes between each treatment. The samples were then treated with freshly prepared 1 % thiocarbohydrazide (THC) and further osmicated with 2 % non-reduced osmium tetroxide in milliQ water. The *en-bloc* staining was carried out with 1 % uranyl acetate and the samples were then treated with Walton's lead aspartate. These treatments were performed using 2 cycles of 2 min of heating at 100 W in the tissue processing microwave and 2 min with the microwave off. Dehydration of the samples, resin infiltration and embedding were then performed as described previously (Deerinck et al., 2022; Giberson et al., 2003). The resin blocks were sectioned using a Leica EM UC7 ultramicrotome (Leica Microsystems, Germany). TEM images were obtained using a Talos L120C transmission electron microscope (Thermo Fisher Ltd, USA). The number of *M. bovis* cells inside the organoid cells and the number of *M. bovis* cells in the intercellular junctions were counted in images obtained at x 5300 magnification of three different 115 μm^2 fields, using FIJI v2.14.0.

2.6. Statistical analyses

The fold increment in the *M. bovis* titre after 72 hours of infection was calculated to determine whether *M. bovis* had proliferated within the organoid cultures. Statistical analyses were performed using GraphPad Prism version 10.2.3 for Windows (GraphPad Software, San Diego, CA). A two-way ANOVA and Tukey's multiple comparison tests were used to compare the $\log_{10}$ titres of *M. bovis* in organoids prepared from different animals. A P value ≤ 0.05 was considered significant.

3. Results

3.1. M. bovis can proliferate in bovine tracheal organoids

The titres of *M. bovis* increased by 182-fold in the organoids prepared from tracheal tissue from Animal 1 and by 372-fold in the organoids prepared from tracheal tissue from Animal 2 (Fig. 1). There was a significant difference between the initial (T0) titres of *M. bovis* in the organoid samples and the titres at 72 h (T72) after inoculation in organoids prepared from Animal 1 ($P < 0.001$) and Animal 2 ($P < 0.0001$). No significant difference was detected in the titres of *M. bovis* at T72 between the organoids prepared from the two different animals, and no viable *M. bovis* were detected in the medium only controls after incubation for 72 h (Fig. 1).

3.2. M. bovis can invade bovine tracheal organoid cells

In TEM, *M. bovis* appear as round to oval structures with varying cytoplasmic electron densities within a single, bounding cytoplasmic membrane (Fig. 2). Microvillus-like cytoplasmic processes were

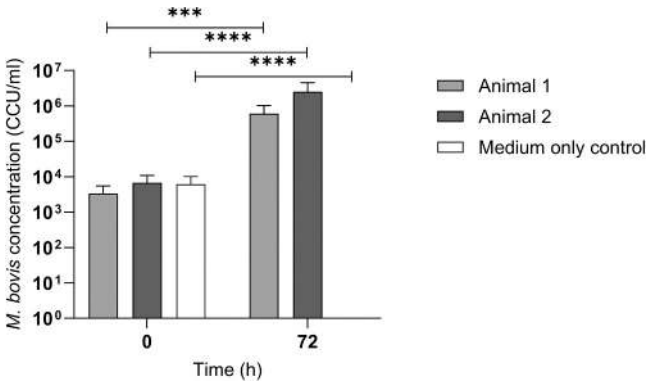

Fig. 1. Titres of viable *Mycoplasma bovis* MbovMil in bovine tracheal organoid infection assays. Tracheal cells from two animals were grown into differentiated organoids by culturing for 21 days in BME-2 matrix. The organoid cultures were infected with 10^3 CCU of *M. bovis*. Medium and organoids were collected at 2 hours after inoculation for use as time 0 samples (T0), and at 72 hours after inoculation, medium and organoids were collected for use as 72 hour samples (T72). Medium only controls were included to assess the ability of *M. bovis* to proliferate in culture medium alone in the absence of organoids. No viable *M. bovis* were detected at T72 in the medium only control. Data represent the means and standard deviations of three technical replicates. * ** significantly different at $P < 0.001$, * ** * significantly different at $P < 0.0001$; CCU, colour changing units.

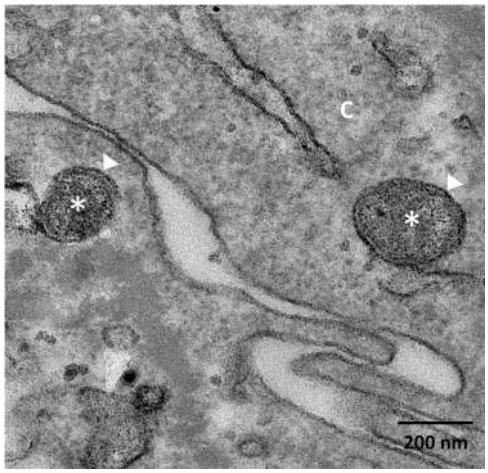

Fig. 2. Transmission electron micrograph of *M. bovis* in bovine tracheal organoid cells. C, cytoplasm of tracheal organoid cell; *, *Mycoplasma bovis*; arrowhead, cell membrane of *M. bovis.*

observed in the intercellular junctions between the organoid cells (Fig. 3 A-D, red arrow head), but the type of intercellular junction could not be determined. Clusters of *M. bovis* cells were observed at these intercellular junctions of the tracheal organoid cells, within a membrane bound structure (Fig. 3 A, B). The *M. bovis* cells in the intercellular clusters had an approximate diameter of 0.18–0.45 μm. Individual *M. bovis* cells, with an approximate diameter of 0.18 – 0.30 μm, were observed inside the cytoplasm of organoid cells (Fig. 3 A, B). The mean number of intracellular mycoplasmas was 41 ± 15 per field, and the mean number of mycoplasmas per field in the intercellular junctions was 45 ± 28. No observable changes were detected in the ultrastructure of the organelles of the bovine tracheal cells associated with *M. bovis* infection.

4. Discussion

In the studies described here, we demonstrated the feasibility of using bovine tracheal organoids to study infection with *M. bovis*. Although organoids developed from human tissues have been used to study the pathogenesis of and host responses to infections (Chen and Na, 2022), few studies have used organoids to study infectious diseases in other animals (Chen et al., 2023). Organoids have been developed from an increasing number of different animal species, including cattle, pigs, chickens, sheep, horses, dogs and cats, and from a range of organ systems, including the respiratory, digestive, urinary and reproductive systems, and from the mammary gland (Kawasaki et al., 2022). However, relatively few of these have been used to investigate the pathogenesis of infectious diseases (Chen et al., 2023), with the majority of studies using organoids focused on studying physiology and nutrition (Kawasaki et al., 2022; Chen et al., 2023). Kirchhoff et al. (2014) used air-liquid interface (ALI) cultures of bovine airway epithelial cells to investigate the bovine respiratory disease complex, but our study is the first to use matrix-embedded bovine tracheal organoids to study *M. bovis*, an important contributor to the development of the bovine respiratory disease complex.

We have shown that *M. bovis* can infect and proliferate in tracheal organoids, resulting in a greater than 250-fold increase in titre by 72 h

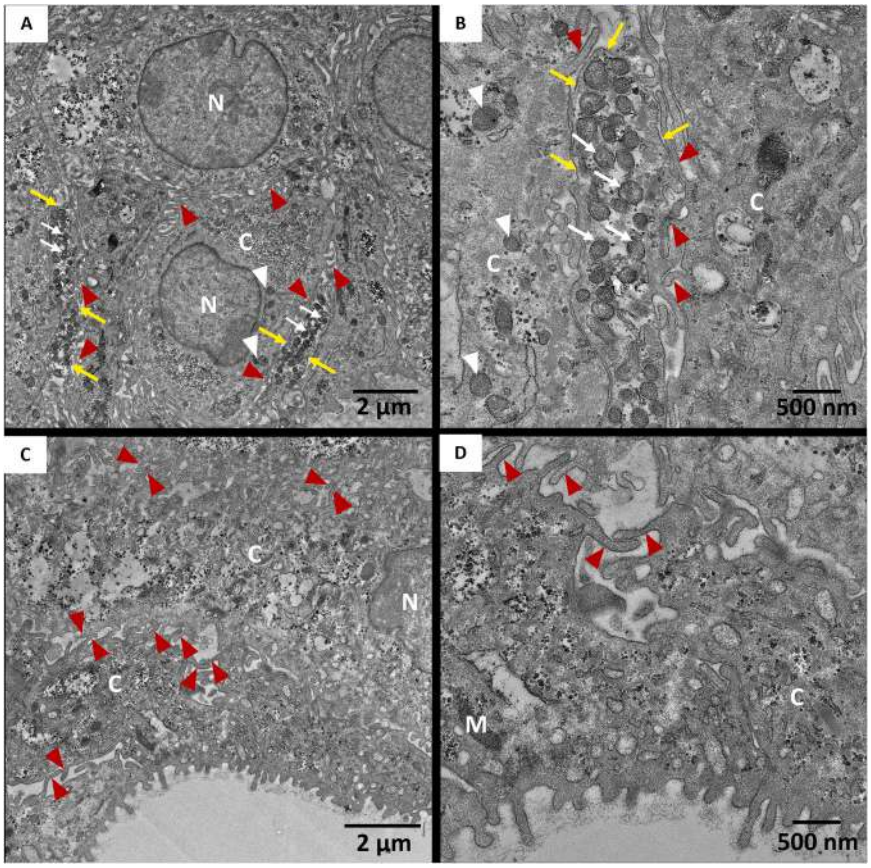

Fig. 3. Transmission electron micrographs of bovine tracheal organoids. (A) and (B), bovine tracheal organoid infected with *M. bovis*; (C) and (D), uninfected bovine tracheal organoid. B and D are magnified images of A and C, respectively. N, nucleus; M, mitochondria; C, cytoplasm; white arrowheads, intracellular *M. bovis*; white arrows, *M. bovis* clusters at intercellular junctions; red arrow heads, cytoplasmic processes of the organoid cells; yellow arrow, membrane surrounding the *M. bovis* clusters at the intercellular junctions.

after inoculation. In our previous co-culture studies in MDBK cells, there was a greater than 2,500-fold increase in the titre of *M. bovis* strain MbovMil at 72 h after inoculation (Premachandre et al., 2024). Quah et al. (2023) also observed a greater increase in the titre of BHV-1 in tracheal cell monolayer assays than in organoid infection assays. The differences in the increases in titre between the cell monolayer infection assays and the organoid infection assays may have resulted from differences in the compositions of the media used in the different assays. In addition, the presence of the BME-2 matrix in the organoid model may have influenced proliferation and recovery of *M. bovis* prior to titration. Mucus secretion by and ciliary movements of the organoid cells, which may have reduced the attachment of and colonisation by the pathogen could also have reduced the titres obtained in the organoid infection assays. In order to examine the effect of ciliary motility on the colonisation and multiplication of *M. bovis*, an infection assay could be conducted in organoids with reduced ciliary function. This reduction could be achieved using inhibitors such as glucose oxidase or bacterial products such as pyocyanin and 1-hydroxyphenazine (Jackowski et al., 1991).

Clusters of *M. bovis* were observed in between cells, with individual *M. bovis* cells detected in the cytoplasm of infected organoids by TEM. In a previous study, Thomas et al. (1987) detected the infiltration of *M. bovis* between epithelial cells and accumulation of the organisms in the lamina propria of the trachea. The clusters observed in the junctional regions may have been *M. bovis* migrating through the paracellular pathway towards the basal epithelial cell layers, similar to what has been observed for *M. pneumoniae* (Prince et al., 2018). Infection assays using polarized airway organoids, which have apical and basal surfaces, using confocal microscopy (Iakobachvili et al., 2022) to monitor migration through the paracellular pathway, will be required to confirm this. The intercellular clusters of *M. bovis* could also be a consequence of a predilection for binding actin fibres in this region. Prince et al. (2014) have previously observed colocalization of *M. pneumoniae* with actin at the intercellular junctions and a recent study has shown that actin is a receptor for *M. hyopneumoniae*, with its adherence and colonisation reduced in the absence of actin on host cell surfaces (Raymond et al., 2018). Thus, further investigation of the role that actin may play in the pathogenesis of infections with *M. bovis* may be warranted.

A key finding in our studies was the presence of a membrane surrounding the clusters of *M. bovis* between the organoid cells. Although mycoplasmas have not previously been observed in membrane bound clusters in the intercellular junctions, *M. bovis* has been observed intracellularly within a membrane-like structure (Nishi et al., 2021; Liu et al., 2021). *M. hyopneumoniae* has also been observed inside PK-15 cells within vesicle-like structures (Raymond et al., 2018), and disruption of the tight junctions in pig bronchial epithelial cells by *M. hyopneumoniae* has been detected using confocal microscopy (Wang et al., 2020). Therefore, *M. bovis* appears to be able to invade the organoid cells and enter cytoplasmic processes within the junctional complexes and multiply, resulting in these membrane-bound clusters of *M. bovis* cells in between the host cells. These intercellular mycoplasmas may be able to subsequently infect adjacent host cells. Further studies, including time course cell invasion experiments to monitor invasion, intracellular proliferation and migration of *M. bovis* between adjacent cells, are needed to understand the mechanisms underlying the formation of these clusters of membrane-bound *M. bovis* and their potential role in the pathogenesis of mycoplasmosis in cattle. The intercellular junctions observed in between the organoid cells did not have the typical structure of intercellular junctions observed between epithelial cells *in vivo*. The types of junctional complexes between cells in three dimensional organoids have not been studied widely, and the junctions within the organoids may not have been fully developed, as they are probably at early stages of development. Therefore, it was difficult to differentiate the type of junctions found between the organoid cells.

In our study, we observed *M. bovis* in the cytoplasm of the tracheal organoid cells, but there were no vesicle-like structures surrounding them, as was observed by Liu et al. (2021) and Nishi et al. (2021). Endocytosis had been identified as one of the mechanisms used by *M. bovis* to invade host cells (Nishi et al., 2021; Liu et al., 2021), but it is not the only mechanism that could be used for cell invasion (Nunoya et al., 2020; Nishi et al., 2021). It is clear that a mechanism other than endocytosis may have played a role in the invasion of the organoid cells in our study. Future investigations should focus on the mechanisms mycoplasmas use to invade the cytoplasm and the strategies they employ to survive within host cells. Since our study demonstrates that *M. bovis* can invade and survive inside bovine tracheal organoid cells, these organoids represent a valuable *in vitro* model for enhancing our understanding of host cell invasion by *M. bovis*.

Organoids are less commonly used in veterinary research compared to medical research, but they offer several advantages over other *in vitro* models (Chen et al., 2023). Organoids can be established from single cells, which can be expanded, dissociated, and re-cultured to obtain more organoids (Varani et al., 2021), enabling use in high throughput screening experiments (DeZeeuw et al., 2020), and genetic manipulation for disease modelling (Matano et al., 2015). These features make organoids a better *in vitro* infection model than *in vitro* organ culture models, which are also comprised of differentiated cells and also exhibit the functions of an *in vivo* organ. The lack of some components of tissues, including vasculature, immune cells and stroma, may be disadvantages of organoids, but recent advances in stem cell technologies have paved the way for mitigating some of these limitations by co-culturing organoids with stem cell derived immune cells within the 3D matrix and introducing flow enhanced vascularisation of the organoids (Holloway et al., 2019; Homan et al., 2019), creating more physiologically relevant model systems. Kirchhoff et al. (2014) found that disruption of tight junctions is required to facilitate the infection of the ALI system with BHV-1, while Quah et al. (2023) showed that such manipulation was not required for infection of matrix-embedded tracheal organoids with BHV-1, and that the cell differentiation and the distribution of differentiated cells in these two models were quite similar. Therefore, based on the findings of our study and those of Quah et al. (2023), matrix-embedded bovine tracheal organoids appear to be a more convenient and adaptable infection model for studying the pathogens involved in the bovine respiratory disease complex.

5. Conclusions

In conclusion, *M. bovis* was shown to be able to proliferate in tracheal organoids and invade bovine tracheal organoid cells, indicating that these organoids represent a valuable *in vitro* model for studying respiratory tract infections caused by *M. bovis*. Since this model has also been used to study infection with BHV-1 (Quah et al., 2023), it could be valuable for co-infection studies involving different viral and bacterial pathogens in the bovine respiratory disease complex, allowing for exploration of their interactions.

CRediT authorship contribution statement

Elizabeth Vincan: Writing – review & editing, Validation, Resources, Methodology, Conceptualization. **Pin Shie Quah:** Writing – review & editing, Validation, Resources, Methodology, Conceptualization. **Bang Manh Tran:** Writing – review & editing, Validation, Resources, Methodology, Conceptualization. **David C. Jackson:** Funding acquisition, Resources. **Patrick C. Reading:** Funding acquisition, Resources. **Chintha K. Premachandre:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Paola K. Vaz:** Writing – review & editing, Validation, Supervision, Resources, Conceptualization. **Nadeeka Kumari Wawegama:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition, Conceptualization. **Andrés Diaz-Méndez:** Methodology, Conceptualization. **Glenn F. Browning:** Writing – review & editing, Validation, Supervision, Resources, Conceptualization.

Georgia Deliyannis: Writing – review & editing, Validation, Resources, Methodology, Conceptualization. **Chinn Yi Wong:** Methodology, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. Nadeeka Wawegama reports financial support was provided by Australian Research Council. Georgia Deliyannis reports financial support was provided by Australian Research Council. Bang Manh Tran, Elizabeth Vincan reports financial support was provided by National Health and Medical Research Council. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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