Journal of Microbiological Methods 165 (2019) 105700

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Comparison of three methods of enumeration for *Mycoplasma ovipneumoniae*

Melissa G. Ackerman[a,*], David A. Schneider[b], Katherine N.K. Baker[a], Thomas E. Besser[a]

[a] Washington State University, PO Box 647060, Pullman, WA 99164-7060, USA
[b] USDA-Agricultural Research Service, Animal Disease Research Unit, 3003 ADBF, Pullman, WA 99164-6630, USA

1. Introduction

Mycoplasma ovipneumoniae is an important pathogen that contributes to pneumonia, a costly disease of domestic sheep (*Ovis aries*) worldwide.(Nicholas et al., 2008) *M. ovipneumoniae* causes variable morbidity with generally low mortality primarily in lambs, and clinical signs include lethargy, chronic coughing, rectal prolapse, and poor weight gain.(Nicholas et al., 2008) *M. ovipneumoniae* also initiates polymicrobial pneumonia in bighorn sheep (*Ovis canadensis*);(Besser et al., 2008) its key role in early pathogenesis of bighorn sheep pneumonia has only recently been clarified.(Besser et al., 2013) Morbidity is typically high with highly variable mortality (5–100%),(Cassirer et al., 2018) but subsequent recurrent lamb pneumonia epizootics can limit population growth in affected herds.(Cassirer et al., 2013; Cassirer and Sinclair, 2007; Butler et al., 2018) *M. ovipneumoniae* may also be an important wildlife pathogen, as recently it has been reported in other wild ungulates.(Highland et al., 2018; Rovani et al., 2019; Wolff et al., 2019; Handeland et al., 2014)

M. ovipneumoniae studies have focused on identifying virulence factors, mechanisms of damage to host cells, and effects related to host immune response.(Niang et al., 1998a; Niang et al., 1998b; Jiang et al., 2017; Li et al., 2016; Niang et al., 1997; Shahzad et al., 2010; Xue et al., 2015) Studies characterizing strain types and their relationship to virulence, pathogenicity, and transmission have also been conducted. (Besser et al., 2013; Wolff et al., 2019; Cassirer et al., 2017; Besser et al., 2017; Besser et al., 2012) Accurate enumeration of *M. ovipneumoniae* is important in such studies and any others where a standardized dose or inoculum is necessary, and where minimal dose required to induce disease is a consideration.(Marois et al., 2010) Because pathogen infective dose is widely variable, even by strain, quantifying *M. ovipneumoniae* is important for investigations exploring questions about virulence, infectivity potential, and diseases status.(Schmid-Hempel and Frank, 2007)

Obtaining accurate counts of mycoplasmas is generally difficult due to several factors. Methods relying on bacterial growth are time-consuming due to the slow growth of mycoplasmas. Colony forming units (CFU) is a common method of enumeration, but underestimates the number of viable cells, which is compounded by their small size (0.3–0.8 um diameter) and takes weeks to complete.(Calus et al., 2010; Razin and Hayflick, 2010) In the authors' experience, colonies of *M. ovipneumoniae* are small and indistinctive, making accurate counts difficult, likely due to the small size and centerless appearance.(Nicholas et al., 2008) Color changing unit (CCU) assays rely on cellular metabolism and acid production to estimate bacterial numbers using limiting dilutions, and are commonly employed for enumeration of some mycoplasma species, and may be considered the gold standard.(Calus et al., 2010; Poveda and Nicholas, 1998) The CCU technique is time-consuming, relying on growth to reach final titers and underestimates cell numbers, since one color changing unit may comprise more than one cell. A modified CCU method that determines the 50% endpoint, expressed as CCU_{50}, suggested greater accuracy than CCU by determining that one CCU_{50} contained 1–3 genome equivalents as compared to a single CFU, which contained 4–7 genome equivalents for two species of mycoplasmas.(Stemke and Robertson, 1982) Flow cytometry (FC) has recently been used to quantify mycoplasmas in broth media. (Assuncao et al., 2006) This method uses a nucleic acid fluorescent stain which allows for rapid quantification (under 1 h), and results correlate well with CCU and CFU.(Assuncao et al., 2006) Adenosine triphosphate (ATP) luminometry is a quantitative method using an enzymatic (luciferase) reaction to produce a light signal quantified by a luminometer. This signal is proportional to the amount of ATP, and thus estimates metabolizing cells in the reaction. This method has been used to quantify at least two mycoplasmas and correlates well with CCU.(Calus et al., 2010; Stemke and Robertson, 1990) In the authors' experience, accurate counts were difficult. Preliminary measurements displayed inconsistencies between replicates, and similar readings with negative control (media) samples and *M. ovipneumoniae* inoculated samples. Finally, quantitative polymerase chain reaction (qPCR) tests have recently been developed to quantify several mycoplasmas, including *M. ovipneumoniae*, and may produce results in < 1 day, given a suitable deoxyribonucleic acid (DNA) extraction method and thermocycler settings(Jiang et al., 2017; Yang et al., 2014; Ziegler et al., 2014).

Abbreviations: CFU, colony forming units; CCU, color changing unit; FC, flow cytometry; qPCR, quantitative polymerase chain reaction; DNA, deoxyribonucleic acid; SSC, side scatter; gDNA, genomic DNA; C_T, cycle threshold

* Corresponding author.
E-mail addresses: melissa.ackerman@wsu.edu (M.G. Ackerman), david.schneider1@usda.gov (D.A. Schneider), kayak@wsu.edu (K.N.K. Baker), tbesser@wsu.edu (T.E. Besser).

https://doi.org/10.1016/j.mimet.2019.105700
Received 20 May 2019; Received in revised form 21 August 2019; Accepted 21 August 2019
Available online 22 August 2019
0167-7012/ Published by Elsevier B.V.

The objective of this study was to compare *M. ovipneumoniae* growth as determined by qPCR, flow cytometry, and CCU_{50} in order to evaluate the relative accuracy, advantages, and limitations of each method compared to the gold standard, CCU_{50}.

2. Materials and methods

2.1. Mycoplasma ovipneumoniae propagation in broth culture

M. ovipneumoniae reference strain (American Type Culture Collection NCTC 10151 [Y98] ATCC 29419) was propagated according to manufacturer's directions and stored in buffered glycerol at −80 °C. *M. ovipneumoniae* was propagated for each experiment by scraping a pinhead size amount from a frozen aliquot and adding it to 2 ml SP4 broth media (Hardy Diagnostics, Santa Maria, CA) in a 15 ml polystyrene tube (VWR International, Radnor, PA). This medium contains a pH indicator (phenol red), which turns yellow with decreasing pH. The culture was incubated (agitating platform 200 rpm 37 °C) until a color change was observed and used as the inoculum in the quantitative assays described below.

2.2. Comparison study design

M. ovipneumoniae cultures as described above were inoculated into 50 ml SP4 broth media in a 50 ml sterile polystyrene tube (Fisher Scientific, Lenexa, KS) in three independent experiments using differing inoculum ratios (1:10,000, 1:50,000, and 1:500,000 vol:vol). For each experiment, negative control tubes were prepared using the same inoculation ratios with mock-inoculated media. Master inoculated culture and negative control media tubes were mixed by inversion and incubated (agitating platform 200 rpm 37 °C) for the duration of the experiment. Tubes were sampled by removal of aliquots (200 µl) immediately after preparation and at 24 h intervals thereafter for analysis by three methods (CCU_{50}, qPCR, and FC).

2.3. Flow cytometry

Flow cytometry protocol was modified from Assuncao et al. (2006). Triplicates of culture and media control samples (200 µl) at each time point were stained with SYBR green-I nucleic acid dye (Life Technologies Corporation, Eugene, OR) at a final concentration of 1:1000 of the commercial stock solution, then incubated in the dark for 15 min at room temperature. A Becton Dickinson FACSort or FACSCalibur cytometer (BD Immunocytometry Systems, San Jose, CA) was used for sample analysis on the "Lo" setting (12 µl/min ± 3), and CELLQuest software (BD Biosciences, San Jose, CA) was used to acquire data for 10 s, with FSC detector voltage set at "E02". Side scatter (SSC) and green fluorescence parameter (FL1 detector) were used to characterize the cells during acquisition, and FCS Express version 4 software (De-Novo Software, Glendale, CA) was used to analyze the data. Gating strategy was based on presence of fluorescent-labelled events in SSC-FL1 dot plot seen in *M. ovipneumoniae* culture samples compared to media controls (Fig. 1). The total number of SYBR green-labelled events within the gate was determined using the FCS Express software statistics application. Results are expressed as the base 10 logarithm of number of events per 1 ml. For all zero values, 1 was added to allow for a data point to be plotted on the log scale for growth curves (Fig. 2A).

2.4. Quantitative PCR for Mycoplasma ovipneumoniae

Quantitative PCR tests were performed on a StepOne RealTime PCR Detection system (Applied Biosystems, ThermoFisher Scientific, Foster City, CA), and analyses were performed with StepOne Software v2.3 (Applied Biosystems, ThermoFisher Scientific, Foster City, CA). For each time point, qPCR was carried out for quantification of *M. ovipneumoniae* culture or media control samples in triplicate. Extraction of DNA from each 200 µl aliquot was carried out using a commercial kit (QIAamp DNA Mini Kit, Qiagen, Redwood City, CA) according to manufacturer's instructions for bacterial cultures, except that centrifugation speed and time for pelleting bacteria was modified to 16,100 x *g* for 30 min to better reflect that typically used with mycoplasmas. (Almeida et al., 1992; Clyde Jr, 1964) Extracts were eluted with 200 µl elution buffer and stored at −20 °C.

Standard curves were constructed for each qPCR assay *M. ovipneumoniae* reference strain Y98 genomic DNA (gDNA), quantified by fluorometer (Qubit, Invitrogen, ThermoFisher Scientific, Foster City, CA) using a fluorescent double-stranded DNA dye kit (AccuBlue High Sensitivity dsDNA Quantitation Kit, Biotium, Inc., Fremont, CA) according to the manufacturer's directions, and stored at −20 °C. The concentration of gDNA stock was determined based on the *M. ovipneumoniae* genome size (1,020,601 base pairs).(Yang et al., 2011) For each assay, a stock aliquot was thawed and eight 10-fold serial dilutions were prepared for qPCR analysis. The qPCR was conducted as described by Manlove et al. (2019). The genome copy numbers in each reaction and their corresponding cycle threshold (C_T) values were used to plot the standard curve and to obtain genome copy numbers for samples at each time point. Each qPCR assay included a positive amplification control (*M. ovipneumoniae* reference strain Y98 gDNA) and a no-template control (PCR-grade water). A negative extraction control (PCR-grade water) was included for each time point. Results were reported as the base 10 logarithm of the template copy number per 1 ml. For any $C_T = 40$ (undetected), the value was considered zero and 1 was added for plotting on the log scale for growth curves (Fig. 2B).

2.5. CCU_{50}

The CCU_{50} was determined using the Spearmann-Karber formula and the microtitration method modified from Litamoi et al. (1996). In sterile 96-well polystyrene microtiter plates (Greiner Bio-One North America Inc., Monroe, NC), or in 1.5 ml sterile microcentrifuge tubes (USA Scientific, Inc., Ocala, FL), 180 µl of SP4 broth media was placed into columns 2–12 in each of 5 rows. Aliquots (200 µl) from the culture or media control were placed into the first well of each of the 5 rows. Serial 10-fold dilutions were made for all 5 rows up to 10^{-11}, mixing by pipetting and using sterile pipette tips for each dilution. Assays were incubated for 3 weeks (5% CO_2, 37 °C) and monitored for color change. The CCU_{50} was calculated as described below (Litamoi et al., 1996):

$$\text{Log}_{10}\,\text{CCU}_{50} = (\mathbf{X_0} - (\mathbf{d}/\mathbf{2})) + \mathbf{d}(\boldsymbol{\Sigma}\,\mathbf{r_i}/\mathbf{n_i})$$

where: $\mathbf{X_0} = \log_{10}$ of the reciprocal of the lowest dilution at which all 5 wells have demonstrated color change (i.e. positives); $\mathbf{d} = \log_{10}$ of the serial dilution factor (here d = 1); $\mathbf{n_i}$ = number of wells per dilution; $\mathbf{r_i}$ = number of positive wells at each dilution; $\boldsymbol{\Sigma}\ \mathbf{r_i}/\mathbf{n_i}$ = sum of the proportion of positive wells starting at the lowest dilution that demonstrates 5/5 positives and including all higher dilutions with positives.(Litamoi et al., 1996) Results are reported as base 10 logarithm of CCU_{50} values expressed in units per 1 ml. For all zero values where there was no growth, 1 was added to allow for a data point to be plotted on the log scale for growth curves (Fig. 2C).

2.6. Statistical analysis

The relationships of bacterial growth as measured by flow cytometry, qPCR, and CCU_{50} were evaluated by linear regression analysis (JMP statistical software version 14, SAS Institute, Inc., Cary, NC). The period of growth analyzed was defined as time points preceding the rapid senescence detected by the CCU_{50} method (Fig. 2C, time points < 120h). For flow cytometry, two-sample *t*-tests (JMP statistical software version 14, SAS Institute, Inc., Cary, NC) were used to determine the time point range in which events/ml measured from inoculated tubes were significantly greater than events/ml from negative media control tubes (Fig. 2A, detection range 72–216 h, $p < .05$).

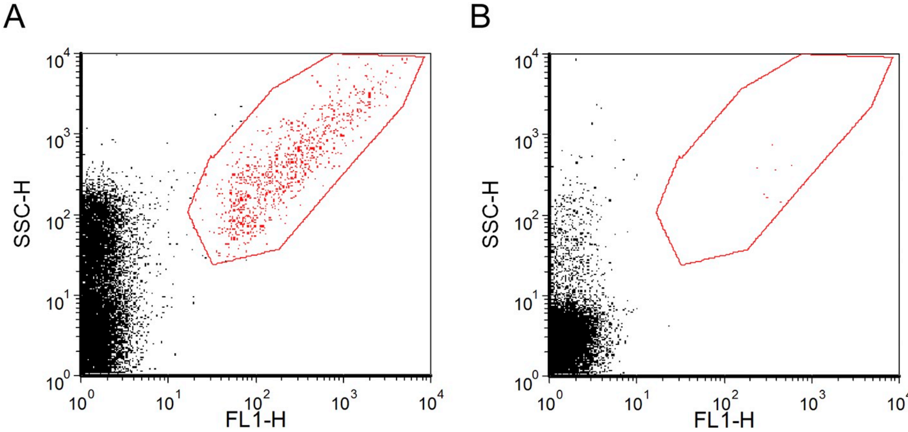

Fig. 1. Dot plots representing gating strategy of SYBR-labelled events. A) SSC-FL1 dot plot from an *M. ovipnuemoniae* culture sample demonstrating gate with SYBR-labelled events included. B) SSC-FL1 dot plot from a time-matched broth media sample with the same gate.

Background-corrected values were then calculated as the difference of average event counts from bacterial culture and media-only controls, with final conversion to logarithm base 10 number of events per 1 ml as the flow cytometry estimate of *M. ovipneumoniae* counts/ml.

3. Results

Log phases of *M. ovipneumoniae* growth were recognized with each quantitative method (Fig. 2A-C). The apparent stationary phases recognized with qPCR and FC after time 120 h were demonstrated by the CCU_{50} method to be senescence with rapid loss of cell viability (Fig. 2A-C). The FC method measured *M. ovipneumoniae* over the smallest quantitative range and was apparently unable to determine concentrations exceeding about 10^7 cells/ml, while CCU_{50} and qPCR methods determined concentrations up to approximately 10^9 cells/ml (Fig. 3).

The FC quantitation of bacterial culture tubes did not differ from values determined in negative media-only control tubes through the first 48 h of incubation, whereas the CCU_{50} and qPCR methods detected *M. ovipneumoniae* in inoculated tubes beginning at time 0. The qPCR method produced C_T values < 40 (range 33.4–36.9) for eight media negative control aliquots and four negative extraction controls aliquots in the first experiment, and for one media negative control aliquot from the second experiment (35.99). When qPCR was repeated on these tubes, similar values were obtained from 8/13 of the aliquots from the first experiment; 5/13 aliquots produced C_T values = 40.

Amplification efficiency for qPCR was > 95% for all experiments with slopes ranging from −3.36 to −3.42; the coefficient of determination (R^2) was > 0.996. A linear range was demonstrated with the standard dilution series over eight orders of magnitude for each assay. The 10^1 standards were consistently detected with C_T values ranging from 33.58 to 37.02, consistent with the detection of as few as 10 copies of *M. ovipneumoniae* template per reaction.

Both flow cytometry and qPCR values were strongly correlated with CCU_{50} during log phase growth, although the coefficient of determination was higher for qPCR than for flow cytometry (0.95 and 0.81, respectively, Table 1, and Fig. 3).

4. Discussion

Quantitation of the log growth phase of *M. ovipneumoniae* in broth culture by both FC and qPCR were closely correlated and linear in comparison with CCU_{50}, the gold standard quantitative method. However, the methods varied with respect to specificity, time, bacteria

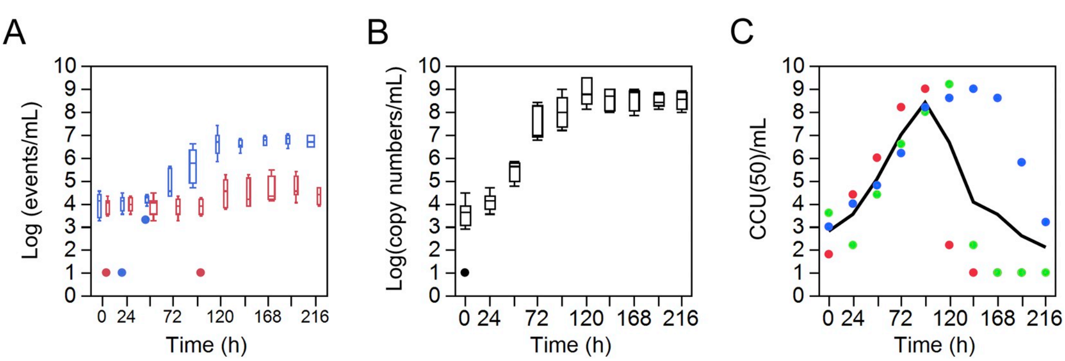

Fig. 2. *M. ovipneumoniae* growth over time, 3 methods of enumeration. Fig. 2A: Flow cytometry results. Flow cytometry values for *M. ovipneumoniae* culture (blue) and media (red) reported as the base 10 logarithm number of events per 1 ml. All replicates are plotted for each experiment. For any media or culture zero value, 1 was added and is represented as “1” in this plot. Median values are represented by the horizontal lines inside each box. The lower and upper ends of each box represent the 1st and 3rd quartiles, respectively. The lower and upper whiskers represent the 1st quartile – 1.5 (interquartile range), and the 3rd quartile +1.5 (interquartile range), respectively; if data points lie within this range, the whiskers are determined by upper or lower data point values. Outliers are identified as data points outside the box. Fig. 2B: qPCR results. *M. ovipneumoniae* qPCR values measured at different time points for 3 experiments reported as base 10 logarithm number of copies per 1 ml. Box and whisker values are as described for Fig. 2A. Where Ct = 40, the concentration was considered as zero and these data points are represented as “1” in this plot. Fig. 2C: CCU50 results. *M. ovipneumoniae* CCU_{50} values measured at different time points for 3 experiments reported as CCU_{50} per 1 ml, as described above. For any zero value where there was no growth, 1 was added and is represented as “1” in this plot. The mean for each time point is represented by the solid line, and each independent experiment with varying inoculum ratios is represented by a different color; 1:10,000 (red), 1:50,000 (blue), and 1:500,000 vol:vol (green).

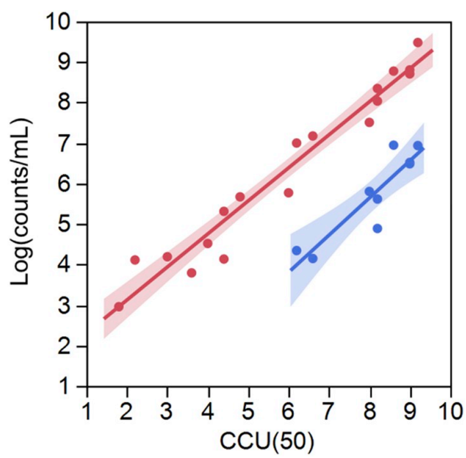

Fig. 3. Linear regression analysis comparing qPCR and flow cytometry to the gold standard, CCU_{50}. Base 10 logarithm of counts per 1 ml for flow cytometry (represented in blue) and qPCR (represented in red) are plotted against base 10 logarithm of CCU_{50} per 1 ml. Linear regression lines are shown with the shaded areas representing the 95% confidence intervals for the fit. Flow cytometry data points were excluded where culture and media means were not significantly different (i.e. times 0, 24, 48 as described in Fig. 2A). Flow cytometry data at all given time points represent (culture replicate mean events) – (media replicate mean events) per 1 ml. All data points were excluded from the comparison if the CCU_{50} value demonstrated *M. ovipneumoniae* senescence or death. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Linear regression analysis correlation coefficients including coefficient of determination (R^2), slope, intercept, and *P* value for the lines.

Quantitative method	R^2	Slope	Intercept	P value
Flow cytometry	0.81	0.92	−1.71	0.0009
qPCR	0.95	0.82	1.488	< 0.0001

viability, and range of detection.

Others have reported successful enumeration of mycoplasmas, including CFU for *M. ovipneumoniae*, with CFU and ATP luminometry methods.(Calus et al., 2010; Stemke and Robertson, 1990; Lin et al., 2008) We experienced difficulty obtaining counts for CFU, considering the small, indistinct colonies. In our hands, the ATP luminometry assay displayed inconsistencies with replicates and high background, as discussed earlier. For these reasons, quantitation by both CFU and ATP luminometry were not examined in this comparison, however we do not discount the value of these methods. Of the methods evaluated here, CCU_{50} had the widest range of detection, and was the only one informative about *M. ovipneumoniae* viability. While the media used in this study favors *M. ovipneumoniae* growth, it is not species-specific, and contamination with other viable bacteria capable of glucose fermentation would be expected to have non-specific results. Additionally, CCU_{50} is time-consuming, taking 2–3 weeks for *M. ovipneumoniae* growth. Quantitative PCR is the only method studied here which is specific for detection of *M. ovipneumoniae*. *M. ovipneumoniae* senescence was not detected by qPCR, which detected post-senescence non-viable cells as an apparent 'stationary phase'. The range of detection of qPCR was large and comparable to that of CCU_{50}. Assay time for qPCR depends upon the DNA extraction and thermocycler conditions utilized, but was generally < 2 days, much shorter than CCU_{50}. In two of the experiments reported here, one or more negative control samples produced weak qPCR signals (C_T 33.58–37.02), indicating potential contamination or probe breakdown. All samples were run in triplicate, and no media negative control produced a signal for all three replicates. The master media-only negative control tubes and all CCU_{50} negative controls remained red for all three experiments, consistent with no *M. ovipneumoniae* growth. Therefore if the qPCR signal was due to contamination, it only occurred after the qPCR aliquot was taken from the master tube, and did not occur in any aliquots used in CCU_{50}. While this qPCR protocol demonstrated detection of as few as 10 *M. ovipneumoniae* genome copies, these weak false positive signals indicate that caution must be observed in interpreting quantitation of low concentrations of *M. ovipneumoniae*.

Flow cytometry was consistently unable to detect *M. ovipneumoniae* growth until 72 h of incubation, equivalent to about 10^6 CCU_{50}, demonstrating that this method is poorly sensitive compared to CCU_{50} or qPCR. Across the concentration range at which FC was able to detect *M. ovipneumoniae*, it consistently underestimated the concentration by about two logs compared to either of the other methods. Nevertheless, its reasonably strong linear correlation with quantitation results produced by qPCR and CCU_{50} indicate it could provide useful rapid estimation of *M. ovipneumoniae* numbers. This flow cytometry protocol was not specific for *M. ovipneumoniae* and would not be appropriately used in mixed or contaminated cultures. Flow cytometry also failed to detect *M. ovipneumoniae* senescence, although protocols have been described that discriminate between live and dead mycoplasmas.(Assuncao et al., 2005) However, this method provides the fastest enumeration, giving results within about an hour.

The wide dynamic ranges of the CCU_{50} and qPCR tests suggest that either method would be suitable for studies necessitating quantification of a standardized inoculum or exploration of minimum infective dose of *M. ovipneumoniae*, but any information regarding viability is limited to the CCU_{50} method. If rapid results are required, flow cytometry may be used with caution for pure cultures, and with correction for the consistent underestimation this method provides.

Declarations of Competing Interest

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. No other declarations of interest exist.

Funding

This work was supported by the USDA Agricultural Research Service [CRIS Project funds 2090-32000-036-00D], by donations made to Washington State University from Veterinary Teaching Hospital Agricultural Animal Hospital clients and the American Sheep Industry to advance research in animal health, by the Wild Sheep Foundation, U.S.A., grant WSU003366, by the Idaho Department of Fish and Wildlife, U.S.A., grant AG0120-SB-886973, and by the USDA Forest Service, U.S.A., grant 17-CS-11132422-258. The donors had no role in study design, collection, analysis, or interpretation of data, in writing the report, or in the decision to publish.

Acknowledgements

The authors would like to acknowledge Margaret Highland for assistance with protocol development, and Cristina Cunha for research samples.

References

Almeida, R.A., Wannemuehler, M.J., Rosenbusch, R.F., 1992. Interaction of mycoplasma dispar with bovine alveolar macrophages. Infect. Immun. 60 (7), 2914–2919.
Assuncao, P., Diaz, R., Comas, J., de Galarreta, C.M., Gonzalez-Llamazares, O.R., Poveda, J.B., 2005. Evaluation of mycoplasma hyopneumoniae growth by flow cytometry. J. Appl. Microbiol. 98 (5), 1047–1054.
Assuncao, P., Rosales, R.S., Rifatbegovic, M., et al., 2006. Quantification of mycoplasmas in broth medium with sybr green-I and flow cytometry. Front. Biosci. 11, 492–497.
Besser, T.E., Cassirer, E.F., Potter, K.A., et al., 2008. Association of Mycoplasma

ovipneumoniae infection with population-limiting respiratory disease in free-ranging Rocky Mountain bighorn sheep (Ovis canadensis canadensis). J. Clin. Microbiol. 46 (2), 423–430.
Besser, T.E., Highland, M.A., Baker, K., et al., 2012. Causes of pneumonia epizootics among bighorn sheep, Western United States, 2008-2010. Emerg. Infect. Dis. 18 (3), 406–414.
Besser, T.E., Frances Cassirer, E., Highland, M.A., et al., 2013. Bighorn sheep pneumonia: sorting out the cause of a polymicrobial disease. Prev Vet Med. 108 (2–3), 85–93.
Besser, T.E., Cassirer, E.F., Potter, K.A., Foreyt, W.J., 2017. Exposure of bighorn sheep to domestic goats colonized with mycoplasma ovipneumoniae induces sub-lethal pneumonia. PLoS One 12 (6), e0178707.
Butler, C.J., Edwards, W.H., Paterson, J.T., et al., 2018. Respiratory pathogens and their association with population performance in Montana and Wyoming bighorn sheep populations. PLoS One 13 (11), e0207780.
Calus, D., Maes, D., Vranckx, K., Villareal, I., Pasmans, F., Haesebrouck, F., 2010. Validation of ATP luminometry for rapid and accurate titration of mycoplasma hyopneumoniae in Friis medium and a comparison with the color changing units assay. J. Microbiol. Methods 83 (3), 335–340.
Cassirer, E.F., Sinclair, A.R.E., 2007. Dynamics of pneumonia in a bighorn sheep metapopulation. J. Wildl. Manag. 71 (4), 1080–1088.
Cassirer, E.F., Plowright, R.K., Manlove, K.R., et al., 2013. Spatio-temporal dynamics of pneumonia in bighorn sheep. J. Anim. Ecol. 82 (3), 518–528.
Cassirer, E.F., Manlove, K.R., Plowright, R.K., Besser, T.E., 2017. Evidence for strain-specific immunity to pneumonia in bighorn sheep. J. Wildl. Manag. 81 (1), 133–143.
Cassirer, E., Manlove, K., Almberg, E.S., et al., 2018. Pneumonia in Bighorn sheep: risk and resilience. J. Wildl. Manag. 82 (1), 32–45.
Clyde Jr., W.A., 1964. Mycoplasma species identification based upon growth inhibition by specific antisera. J. Immunol. 92, 958–965.
Handeland, K., Tengs, T., Kokotovic, B., et al., 2014. Mycoplasma ovipneumoniae–a primary cause of severe pneumonia epizootics in the Norwegian muskox (Ovibos moschatus) population. PLoS One 9 (9), e106116.
Highland, M.A., Herndon, D.R., Bender, S.C., Hansen, L., Gerlach, R.F., Beckmen, K.B., 2018. Mycoplasma ovipneumoniae in wildlife species beyond subfamily Caprinae. Emerg. Infect. Dis. 24 (12), 2384–2386.
Jiang, Z., Song, F., Li, Y., et al., 2017. Capsular polysaccharide of mycoplasma ovipneumoniae induces sheep airway epithelial cell apoptosis via ROS-dependent JNK/P38 MAPK pathways. Oxidative Med. Cell. Longev. 2017, 6175841.
Li, Y., Jiang, Z., Xue, D., et al., 2016. Mycoplasma ovipneumoniae induces sheep airway epithelial cell apoptosis through an ERK signalling-mediated mitochondria pathway. BMC Microbiol. 16 (1), 222.
Lin, Y.C., Miles, R.J., Nicholas, R.A., Kelly, D.P., Wood, A.P., 2008. Isolation and immunological detection of mycoplasma ovipneumoniae in sheep with atypical pneumonia, and lack of a role for mycoplasma arginini. Res. Vet. Sci. 84 (3), 367–373.
Litamoi, J., Palya, V.J., Sylla, D., Rweyemamu, M.M., 1996. Quality Control Testing of Contagious Bovine Pleuropneumonia Live Attenuated Vaccine Standard Operating Procedures. Food and Agriculture Organization of the United Nations Animal Production and Health Paper, pp. 128.
Manlove, K., Branan, M., Baker, K., et al., 2019. Risk factors and productivity losses associated with *Mycoplasma ovipneumoniae* infection in United States domestic sheep operations. Prevent. Veter. Med. 168, 30–38.
Marois, C., Dory, D., Fablet, C., Madec, F., Kobisch, M., 2010. Development of a quantitative real-time TaqMan PCR assay for determination of the minimal dose of mycoplasma hyopneumoniae strain 116 required to induce pneumonia in SPF pigs. J. Appl. Microbiol. 108 (5), 1523–1533.
Niang, M., Rosenbusch, R.F., Lopez-Virella, J., Kaeberle, M.L., 1997. Expression of functions by normal sheep alveolar macrophages and their alteration by interaction with mycoplasma ovipneumoniae. Vet. Microbiol. 58 (1), 31–43.
Niang, M., Rosenbusch, R.F., DeBey, M.C., Niyo, Y., Andrews, J.J., Kaeberle, M.L., 1998a. Field isolates of mycoplasma ovipneumoniae exhibit distinct cytopathic effects in ovine tracheal organ cultures. Zentralbl Veterinarmed A. 45 (1), 29–40.
Niang, M., Rosenbusch, R.F., Andrews, J.J., Kaeberle, M.L., 1998b. Demonstration of a capsule on mycoplasma ovipneumoniae. Am. J. Vet. Res. 59 (5), 557–562.
Nicholas, R., Ayling, R., McAuliffe, L., 2008. Mycoplasma Diseases of Ruminants. CABI, Norfolk, UK.
Poveda, J.B., Nicholas, R., 1998. Serological identification of mycoplasmas by growth and metabolic inhibition tests. In: Miles, R., Nicholas, R. (Eds.), Mycoplasma Protocols. vol. 104. Humana Press, Totowa, NJ, pp. 105–112.
Razin, S., Hayflick, L., 2010. Highlights of mycoplasma research–an historical perspective. Biologicals 38 (2), 183–190.
Rovani, E.R., Beckmen, K.B., Highland, M.A., 2019. Mycoplasma ovipneumoniae associated with Polymicrobial pneumonia in a free-ranging yearling barren ground Caribou (Rangifer tarandus granti) from Alaska, USA. J. Wildl. Dis. 55 (3), 733–736.
Schmid-Hempel, P., Frank, S.A., 2007. Pathogenesis, virulence, and infective dose. PLoS Pathog. 3 (10), 1372–1373.
Shahzad, W., Ajuwape, A.T., Rosenbusch, R.F., 2010. Global suppression of mitogen-activated ovine peripheral blood mononuclear cells by surface protein activity from mycoplasma ovipneumoniae. Vet. Immunol. Immunopathol. 136 (1–2), 116–121.
Stemke, G.W., Robertson, J.A., 1982. Comparison of two methods for enumeration of mycoplasmas. J. Clin. Microbiol. 16 (5), 959–961.
Stemke, G.W., Robertson, J.A., 1990. The growth response of mycoplasma hyopneumoniae and mycoplasma flocculare based upon ATP-dependent luminometry. Vet. Microbiol. 24 (2), 135–142.
Wolff, P.L., Blanchong, J.A., Nelson, D.D., et al., 2019. Detection of mycoplasma ovipneumoniae in Pneumonic Mountain goat (Oreamnos americanus) kids. J. Wildl. Dis. 55 (1), 206–212.
Xue, D., Ma, Y., Li, M., et al., 2015. Mycoplasma ovipneumoniae induces inflammatory response in sheep airway epithelial cells via a MyD88-dependent TLR signaling pathway. Vet. Immunol. Immunopathol. 163 (1–2), 57–66.
Yang, F., Tang, C., Wang, Y., Zhang, H., Yue, H., 2011. Genome sequence of mycoplasma ovipneumoniae strain SC01. J. Bacteriol. 193 (18), 5018.
Yang, F., Dao, X., Rodriguez-Palacios, A., et al., 2014. A real-time PCR for detection and quantification of mycoplasma ovipneumoniae. J. Vet. Med. Sci. 76 (12), 1631–1634.
Ziegler, J.C., Lahmers, K.K., Barrington, G.M., et al., 2014. Safety and immunogenicity of a mycoplasma ovipneumoniae bacterin for domestic sheep (Ovis aries). PLoS One 9 (4), e95698.