Assessment of *in vitro* growth characteristics of *Mycoplasma ovipneumoniae*

This paper (No. 12052020-00164-EN) has been peer-reviewed, accepted, edited, and corrected by authors. It has not yet been formatted for printing. It will be published in issue **39** (3) of the *Scientific and Technical Review*, with a cover date of December 2020.

Z. Maksimović [(1)*], C. de la Fe [(2)] & M. Rifatbegović [(1)]

(1) Department of Microbiology and Infectious Diseases, Veterinary Faculty, University of Sarajevo, Zmaja od Bosne 90, 71000 Sarajevo, Bosnia and Herzegovina

(2) Department of Animal Health, Faculty of Veterinary Sciences, Universidad de Murcia, Campus de Espinardo s/n., 30100 Murcia, Spain

*Corresponding author: zinka.maksimovic@vfs.unsa.ba

Summary

Mycoplasma ovipneumoniae (*Movp*) is an emerging pathogen that causes respiratory disease in small ruminants worldwide. It is considered to be difficult and time consuming to grow, which complicates diagnostic and control measures including isolation (an essential step required prior to the characterisation of strains), antimicrobial susceptibility testing and the development of vaccines. The objectives of this study were to analyse *in vitro* growth patterns of *Movp* strains, and the effects of different media used to support their growth. The study was conducted on 20 ovine and caprine *Movp* strains, isolated using Thiaucourt's medium. The rapid growth phase varied among the strains from 24 h to 72 h, although 60% of strains (12 of 20) reached a peak at 48 h. All strains were viable at 72 h after incubation, and declining viability was observed at 96 h (13 of 20 remained viable; 65%), 120 h (9 of 20; 45%) and 144 h (4 of 20; 20%). Growth was not detected at 168 h. All strains were able to grow in modified tryptone soy broth, while PH mycoplasma medium-

Hayflick modified medium supported the growth of only two strains. Improved techniques of *Movp* cultivation require consideration of the growth variability among strains, the time of subculturing (during the first three days of incubation) and selection of appropriate media.

Keywords

Culture medium – Growth variability – *Mycoplasma ovipneumoniae*.

Introduction

Mycoplasma ovipneumoniae (*Movp*) is an emerging pathogen that causes respiratory disease in domestic and wild small ruminants worldwide, and consequently results in large financial losses (1, 2). Several studies have demonstrated a high degree of phenotypic and genotypic heterogeneity among *Movp* strains (3, 4, 5, 6). The protein, antigenic and genotypic differences between goat and sheep strains have been recently exposed, revealing a link between strains and host ruminant species (5, 7). Generally, mycoplasmas exhibit a high degree of variability among strains, but disease outbreaks have been attributed to single strains (6). However, where *Movp* is involved, multiple strains can be found within herds or within a single animal, and these infections may result in more severe disease (1, 3, 6). Evidence for strain-specific immunity to pneumonia complicates efforts to develop vaccines (7), while variation in antimicrobial susceptibility among strains (1) increases the difficulties in treatment of *Movp* infections. In addition, *Movp* is considered to be difficult and time consuming to grow, so that the actual status of the infection often remains unclear and additional detection tests are required, such as polymerase chain reaction (PCR) (8). Serological testing can be unreliable because of the antigenic heterogeneity of strains and variation in immune response (1). The variable results of *Movp* cultivation, limited growth in mycoplasma media when compared with other mycoplasmas, and the need for a medium free of ruminant-derived proteins has led to the development of new growth media. Among various media formulations tested for optimal growth of *Movp*, tryptone soy broth (TSB-1) medium produced significantly greater yields in a short period in comparison with other media (9).

All the above characteristics of *Movp* have complicated diagnostic and control measures including isolation (an essential step required prior to the characterisation of strains), virulence factor studies, epidemiology, as well as antimicrobial susceptibility testing and the development of vaccines. Considering that isolation can fail and give false negative results for *Movp*, previous studies have suggested improving culture methods for *Movp* and a comparison of media formulations (8). Information on the *in vitro* growth of *Movp* is limited and, to the authors' knowledge, there is no report on the growth characteristics of caprine *Movp* strains. The objective of this study was to analyse *in vitro* growth patterns of ovine and caprine *Movp* strains by assessing their growth curves and evaluating the differences among the strains. The effects of different media in supporting the growth of *Movp* were also investigated, in an attempt to improve culture techniques.

Materials and methods

Twenty field strains of *Movp* collected from sheep (n = 13) and goats (n = 7), with or without respiratory signs, were used in this study (Table I). The strains were isolated using Thiaucourt's medium (TH) (10), cloned, identified as *Movp* by PCR (11) and stored at –85°C until used.

Table I

***Mycoplasma ovipneumoniae* strains used in this study**

***Mycoplasma ovipneumoniae* strains (herds/flocks)**	**Animal**	**Clinical findings**	**Sample type**
274, 275 (F)	Goat	Asymptomatic	Lung
339 (E)	Goat	Severe respiratory disease	Lung
8, 15, 23, 29 (D)	Goat	Severe respiratory disease	Nasal swab
283 (C)	Sheep	Unknown	Lung
324 (G)	Sheep	Unknown	Lung
150, 152, 151 (A); 156, 160, 161, 162, 163, 164, 165, 169 (B)	Sheep	Respiratory disease	Nasal swab

F, E, D: goat herds
C, G, A, B: sheep flocks

To assess the growth curves of the strains, TH medium (2 ml) was inoculated with 0.2 ml of each frozen/thawed culture and incubated for 48 h in 5% CO_2 at 37°C. The cultures were then passaged in the same manner five times prior to testing. Finally, 200 µl samples were transferred into 2 ml aliquots of the medium and incubated for seven days. Quadruplicate samples were taken aseptically at 0, 24, 48, 72, 96, 120, 144 and 168 h to determine the number of colony forming units (CFU)/ml for each strain using a simple quantification method for viable mycoplasmas (12). The logarithm of CFU/ml was calculated for each strain for different periods of incubation.

Two different culture media were tested for their ability to support the growth of *Movp*: TSB-1, which was specifically developed to support the growth of *Movp* (9), with a slight modification (porcine serum was replaced with horse serum), and PH mycoplasma medium–Hayflick modified medium, which has been used for some other small ruminant mycoplasmas (13). The cultures were prepared, inoculated into the test media and incubated in the same manner as described above. The growth media were sampled at 0, 24, 48, 72, 96 and 120 h and plated. Viability was determined by considering the presence or absence of colonies on the solid medium.

Results

The rapid growth phase varied among the strains from 24 h to 72 h, although most strains reached a peak at 48 h. The duration of the rapid growth phase for the majority of strains was 48 h (12 of 20; 60%), followed by 72 h (6 of 20; 30%) and 24 h (2 of 20; 10%) (Table II, Fig. 1). All strains were viable at 72 h after incubation, and declining viability was observed at 96 h (13 of 20 remained viable; 65%), 120 h (9 of 20; 45%) and 144 h (4 of 20; 20%). Growth was not detected at 168 h. A more rapid decrease in CFU/ml after 72 h of incubation was observed for the goat strains when compared with the sheep strains (Fig. 1).

Table II

The mean, variance and range values of colony forming units/millilitre for *Mycoplasma ovipneumoniae* strains cultured in Thiaucourt's medium

	Log of CFU/ml		
Incubation period (hours)	**Mean**	**Variance**	**Range**
0	4.156	9.772	0.000–7.475
24	6.674	3.963	0.000–9.056
48	7.654	1.031	5.461–9.107
72	7.438	0.454	5.310–8.505
96	4.441	11.032	0.000–8.505
120	2.784	10.243	0.000–7.903
144	1.200	6.231	0.000–7.903
168	0.000	0.000	0.000–000

CFU/ml: colony forming units/millilitre

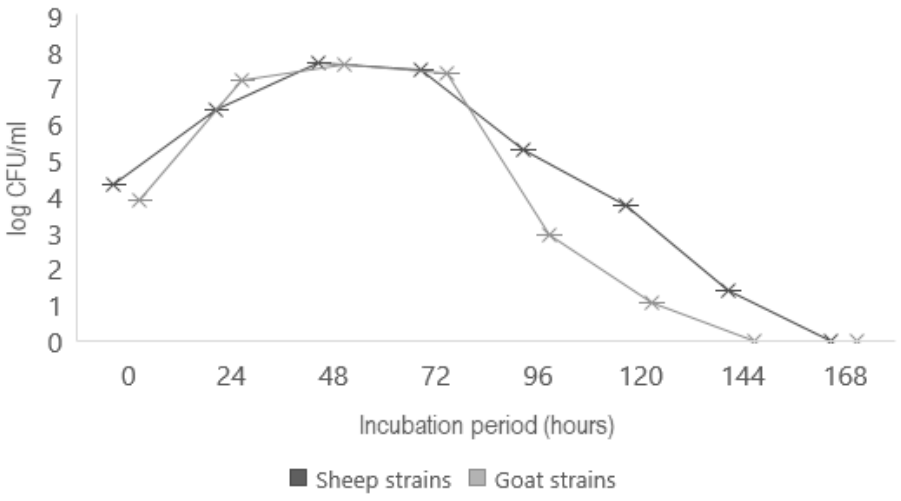

Fig. 1

Comparison of growth rates between sheep and goat *Mycoplasma ovipneumoniae* strains cultured in Thiaucourt's medium

CFU/ml: colony forming units/millilitre

All strains were able to grow in modified TSB-1 medium, but PH–Hayflick modified medium supported the growth of only two strains (10%).

Discussion

The results exposed differences in the growth characteristics among *Movp* strains. The growth variability among *Movp* strains is supported by previously demonstrated phenotypic and genotypic heterogeneity (3, 4, 5, 6). Significant differences in growth characteristics between caprine and ovine strains were not observed. However, the rapid decrease in CFU/ml after 72 h for the goat strains suggests that the first three days of incubation are optimal for the cultivation of most caprine *Movp* strains. The duration of the rapid growth phase differed among the strains; however, most strains reached a peak at 48 h incubation. Curiously, two strains reached maximum viability at 24 h. These findings are crucial for adequate subculturing and preparation of *Movp* strains for different tests. Moreover, they could be related to the generally low recovery of *Movp* isolates. The importance of incubation time was also revealed in a previous study in which incubation of field samples for 48 h seemed to improve the isolation of *Movp* (n = 9/13; 69%) when compared with 96 h (n = 5/23; 22%) (14).

In this study, the viability of the strains varied when observed at between three and six days of culture. A rapid loss of viability due to the accumulation of toxic products, the depletion of required substrates or a high tendency of mycoplasmas to undergo autolysis can result in failure to obtain growth in subculture (15). Different CFU/ml values at time 0 could affect the results because of the more rapid growth of some strains than others leading to faster substrate utilisation and the accumulation of toxic products. Nevertheless, the results indicate that the initial inoculum did not have a significant effect, because the strains with different initial numbers reached the peak of growth at the same time. The observed differences among strains should be carefully evaluated during the isolation of *Movp*. Considering the growth of all strains observed within 72 h, the results

indicate that subculturing during the first three days of incubation is essential for efficient cultivation of most *Movp* strains. However, the results should be closely examined because primary isolation and repeated passage on TH medium may have resulted in changes to the strains and their adaptation to this medium. Despite this limitation, significant differences in strain growth between the TSB-1 and PH–Hayflick media were observed, emphasising the importance of culture medium choice. The wide and varied metabolic activity of *Movp* has been reported, manifest by the metabolic heterogeneity of strains in terms of substrate utilisation patterns (1). However, it was also noted that all strains were able to utilise glucose and pyruvate (1, 16), substrates incorporated in TH medium (10), which highlights the advantages of this medium in comparison to other media without these components (9, 13). In the present study, a slightly modified TSB-1 medium was found to be another suitable substrate, supporting the growth of *Movp* strains; TSB-1 medium was formulated (9) for the production of a significant growth yield of *Movp*. The advantages of using this medium were observed during the isolation procedures. Incorporation of several contamination inhibitors in TSB-1 notably increased culture success (14). These findings suggest that TH and modified TSB-1 should be considered as the media of choice for *Movp* growth.

Conclusions

Improved techniques of *Movp* cultivation require consideration of the growth variability among strains, the time of subculturing (during the first three days of incubation), and selection of appropriate media.

References

1. Nicholas R., Ayling R. & McAuliffe L. (2008). – Respiratory diseases of small ruminants. *In* Mycoplasma diseases of ruminants (R. Nicholas, R. Ayling & L. McAuliffe, eds). CABI, Oxfordshire, United Kingdom, 169–198. https://doi.org/10.1079/9780851990125.0169.

2. Prats-van der Ham M., de la Fe C., Amores J., Paterna A., Tatay-Dualde J. & Gómez-Martín Á. (2015). – Contagious caprine pleuropneumonia (CCPP) and other emergent mycoplasmal diseases affecting small ruminants in arid lands. *J. Arid Environ.*, **119**, 9–15. https://doi.org/10.1016/j.jaridenv.2015.03.006.

3. Harvey M.E., Morrical D.G. & Rosenbusch R.F. (2007). – Sheep flock infections with *Mycoplasma ovipneumoniae* involve multiple strains. *Small Rum. Res.*, **73** (1–3), 287–290. https://doi.org/10.1016/j.smallrumres.2007.01.013.

4. Ionas G., Norman N.G., Clarke J.K. & Marshall R.B. (1991). – A study of the heterogeneity of isolates of *Mycoplasma ovipneumoniae* from sheep in New Zealand. *Vet. Microbiol.*, **29** (3–4), 339–347. https://doi.org/10.1016/0378-1135(91)90141-2.

5. Maksimović Z., de la Fe C., Amores J., Gómez-Martín Á. & Rifatbegović M. (2017). – Comparison of phenotypic and genotypic profiles among caprine and ovine *Mycoplasma ovipneumoniae* strains. *Vet. Rec.*, **180** (7), article 180. https://doi.org/10.1136/vr.103699.

6. Parham K., Churchward C.P., McAuliffe L., Nicholas R.A.J. & Ayling R.D. (2006). – A high level of strain variation within the *Mycoplasma ovipneumoniae* population of the UK has implications for disease diagnosis and management. *Vet. Microbiol.*, **118** (1–2), 83–90. https://doi.org/10.1016/j.vetmic.2006.07.005.

7. 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. https://doi.org/10.1002/jwmg.21172.

8. Weiser G.C., Drew M.L., Cassirer E.F. & Ward A.C.S. (2012). – Detection of *Mycoplasma ovipneumoniae* and *M. arginini* in bighorn sheep using enrichment culture coupled with genus- and species-specific polymerase chain reaction. *J. Wildl. Dis.*, **48** (2), 449–453. https://doi.org/10.7589/0090-3558-48.2.449.

9. Patel H., Mackintosh D., Ayling R.D., Nicholas R.A.J. & Fielder M.D. (2008). – A novel medium devoid of ruminant peptone for high yield growth of *Mycoplasma ovipneumoniae*. *Vet. Microbiol.*, **127** (3–4), 309–314. https://doi.org/10.1016/j.vetmic.2007.08.030.

10. Thiaucourt F., Bölske G., Leneguersh B., Smith D. & Wesonga H. (1996). – Diagnosis and control of contagious caprine pleuropneumonia. *In* Animal mycoplasmoses and control (J. Nicolet, ed.). *Rev. Sci. Tech. Off. Int. Epiz.*, **15** (4), 1415–1429. https://doi.org/10.20506/rst.15.4.989.

11. McAuliffe L., Hatchell F.M., Ayling R.D., King A.I.M. & Nicholas R.A.J. (2003). – Detection of *Mycoplasma ovipneumoniae* in *Pasteurella*-vaccinated sheep flocks with respiratory disease in England. *Vet. Rec.*, **153** (22), 687–688. https://doi.org/10.1136/vr.153.22.687.

12. Albers A.C. & Fletcher R.D. (1982). – Simple method for quantitation of viable mycoplasmas. *Appl. Environ. Microbiol.*, **43** (4), 958–960. https://doi.org/10.1128/AEM.43.4.958-960.1982.

13. Gómez-Martín Á., de la Fe C., Amores J., Sánchez A., Contreras A., Paterna A., Buendía A.J. & Corrales J.C. (2012). – Anatomic location of *Mycoplasma mycoides* subsp. *capri* and *Mycoplasma agalactiae* in naturally infected goat male auricular carriers. *Vet. Microbiol.*, **157** (3–4), 355–362. https://doi.org/10.1016/j.vetmic.2012.01.004.

14. Jennings-Gaines J.E., Edwards W.H., Wood M.E., Fox K.A., Wolfe L.L., Miller M.W. & Killion H.J. (2016). – An improved method for culturing *Mycoplasma ovipneumoniae* from field samples. *In* Proc. 20th Biennial Symposium of the Northern Wild Sheep and Goat Council (NWSGC) (R. Harris, ed.), 9–12 May 2016, Moscow, Idaho & Pullman, Washington. NWSGC, Bozeman, Montana, United States of America, 83–88. Available at: http://media.nwsgc.org/proceedings/NWSGC-2016/Jennings_NWSGC20_83-88.pdf (accessed on 25 February 2020).

15. Razin S. & Tully J.G. (1983). – Methods in mycoplasmology. **Vol. 1**: Mycoplasma characterization. Academic Press, New York, United States of America, 526 pp. Available at: www.sciencedirect.com/book/9780125838016/methods-in-mycoplasmology#book-info (accessed on 25 February 2020).

16. Lin Y.-C., Miles R.J., Nicholas R.A.J., 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. https://doi.org/10.1016/j.rvsc.2007.06.004.