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1 **A novel medium devoid of ruminant peptone for high yield growth of *Mycoplasma***  
2 ***ovipneumoniae*.**

3

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15 Running title: Growth medium for fastidious mycoplasmas devoid of ruminant peptide

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## Abstract

*Mycoplasma ovipneumoniae* is considered an emerging veterinary pathogen causing pneumonia in sheep and goats worldwide. Currently it has not been possible to define a growth medium that yields the maximum growth of *M. ovipneumoniae* within a short incubation period. Growth yields of *M. ovipneumoniae* in Eaton's medium are variable and not as consistently high as those seen with other *Mycoplasma* spp. This study investigated the ability of different *M. ovipneumoniae* field strains to grow in various media formulations, where PPLO broth was replaced by a vegetable protein source, and comparisons were made in terms of strain viability in Eaton's medium. Studies were also conducted to determine the optimal carbohydrate source for use in the *M. ovipneumoniae* medium. Generally, it was found that different strains showed good growth in all media tested, with growth yields at 24 hrs in TSB-1 medium higher than those observed with Eaton's medium. Growth yields reached  $10^8$  - $10^9$  cfu ml<sup>-1</sup> within 24hrs for particular field strains, with all strains achieving this growth level within 48 -72hrs.

Keywords: *Mycoplasma ovipneumoniae*; culture; diagnosis; emerging veterinary pathogen, vaccine.

## 1        **1. Introduction**

2

3        *M. ovipneumoniae* is an emerging veterinary pathogen isolated from the respiratory tract of  
4        sheep and goats and frequently associated with atypical pneumonia (enzootic pneumonia),  
5        chronic non-progressive pneumonia (CNP) and coughing syndrome (Alley *et al*, 1979; Jones  
6        *et al*, 1979; Sullivan *et al*, 1973). *M. ovipneumoniae* has been reported in 80-98% of  
7        pneumonic lungs but is also present in up to 30% of normal lungs (Davies, 1985). It has been  
8        suggested that even though *M. ovipneumoniae* is recognised as a cause of low grade  
9        pneumonia in sheep it may predispose to invasion by more serious lower respiratory  
10        pathogens, such as *Pasteurella* and para-influenza 3 virus (Jones *et al*, 1982).

11        Infection with *M. ovipneumoniae* has been reported worldwide, including USA,  
12        Australia, New Zealand and the UK and is often recognised as an endemic problem (Martin,  
13        1996). Improved culturing and diagnostic capability would contribute valuable information  
14        for evaluation of the economic impact of this organism.

15        Mycoplasmas are fastidious with a limited genome size, hence require a large array of  
16        precursors (Rodwell, 1983) and high concentrations of serum and energy sources.  
17        Mycoplasma media can contain peptone, beef heart infusion, DNA and/or aqueous yeast  
18        extract, animal serum and sodium chloride (Edwards, 1947). Even in such complex media,  
19        Mycoplasma growth rates and yields are typically low and broth cultures of many mollicutes  
20        do not reach visible turbidity (Miles, 1992a and Miles, 1992b).

21        In the UK *M. ovipneumoniae* is routinely grown in Eaton's medium containing  
22        ruminant protein (PPLO broth base). Growth yields of some strains are limited when  
23        compared with other *Mycoplasma* spp. in similar media. This study considers adaptations to  
24        Eaton's medium to improve growth yields of *M. ovipneumoniae* within shorter time periods.  
25        Importantly, substituting the ruminant protein source (PPLO broth base) with vegetable

1 peptone sources eliminates the potential iatrogenic transmission of transmissible spongiform  
2 encephalopathy agents (TSE) or prions via vaccination (Cashman., 2001, Ramasamy., 2004,  
3 Priola and Vorberg., 2004), facilitating the development of a safe 'vaccine medium' capable  
4 of producing increased growth yields.

5

## 6 **2. Materials and Methods**

### 7 **2.1 Organisms: source, growth and maintenance.**

8 The 11 strains of *M. ovipneumoniae* were obtained from the Veterinary Laboratories  
9 Agency, Surrey (UK), as frozen broth cultures. All cultures were grown in Eaton's broth  
10 medium and stored at -70°C until required. The strains used in this study were all field isolates  
11 from the UK (n=10) and Turkey (n=1) from the lungs of sheep presenting clinical signs of  
12 atypical pneumonia.

13

### 14 **2.2 Growth Media:**

15 Eaton's medium comprising: PPLO Broth Base (BD Diagnostics, Oxford, UK) 21 g L<sup>-1</sup>  
16 <sup>1</sup>; Glucose (Sigma, Dorset, UK), 10 g L<sup>-1</sup>; fresh yeast extract, 100ml L<sup>-1</sup>, heat inactivated  
17 horse serum (Sigma, Dorset, UK), 200ml L<sup>-1</sup>, DNA (Sigma, Dorset, UK), 0.02 g L<sup>-1</sup>,  
18 Penicillin (Sigma, Dorset, UK), 0.5ml L<sup>-1</sup> (200 000 IU ml<sup>-1</sup>) and Phenol red (Sigma, Dorset,  
19 UK), 0.2ml L<sup>-1</sup>, was used for the growth and maintenance of all strains. The test media  
20 formulations were modifications of Eaton's medium, with DNA, phenol red and penicillin  
21 omitted from the protocol. The media formulation of the novel media are referred to as VP-1,  
22 B.P, SL72, TSB, Tryp, NSP, TSB-1 and TSB-B respectively. The variations in media  
23 formulation relative to Eaton's media are detailed in Table 1. Where solid media was  
24 required, broths were supplemented with 1% molten sterile bacteriological agar No.1 (Oxoid,  
25 UK) and dispensed into sterile Petri dishes.

### 1 **2.3 Growth of organisms in novel media**

2 Eaton's medium (5 ml), (pH 7.6) was inoculated with 0.1ml of frozen/thawed culture,  
3 and incubated aerobically at 37°C for 24 hrs the cultures were passaged 10 times prior to  
4 testing. Following this initial incubation 0.05 ml aliquots of the cultures were aseptically  
5 transferred into 5ml aliquots of the given test media (shown in Table 1), and incubated  
6 aerobically at 37°C for a 72 hr period. Triplicate samples (10µl aliquots) were taken  
7 aseptically at 0, 24, 48 and 72 hrs to determine viable counts (cfu ml<sup>-1</sup>) (Miles and Misra,  
8 1938).

### 10 **2.4 Determination of Optimum Carbohydrate source in TSB media**

11 TSB medium described in Table 1 was adapted by replacing glucose with 5 different  
12 sugars, fructose, sucrose, mannose, maltose and lactose at 10g L<sup>-1</sup>. The media were tested on  
13 all strains with growth measured as described in section 2.3. The growth media was sampled  
14 at 0, 24, 48 and 72 hrs to determine viable counts (cfu ml<sup>-1</sup>).

### 16 **2.5 Determination of media adaptation by test organisms**

17 Organisms were cultured as described in section 2.3 with the following adaptations,  
18 organisms were cultured in either Eaton's, TSB or TSB-1 broth for 48 hrs and then  
19 subcultured into equal volumes of the appropriate test broths. The subsequent broths were  
20 incubated as described previously with samples being taken at 0, 24, 48 and 72hrs. The  
21 samples were then plated onto Eaton's, TSB and TSB-1 agar to determine viable counts.

### 23 **2.6 Statistical analysis.**

24 The variances in the data were statistically analysed using one way ANOVA and Tukey's  
25 high stringency multiple comparison test in the SPSS statistical package (Ver 14.00). The raw

1 data was presented in box whisker plots (Figure1, panel A-C). The plots comprise the  
2 following data aspects: data range (represented by the Y error bars), median, upper and lower  
3 quartile ranges. The mean of the data is also depicted.

4

### 5 **3. Results**

#### 6 **3.1 Growth in Novel Media**

7 Preliminary observations indicated that of the vegetable peptone sources used in the  
8 study Tryptone Soya Broth (TSB) showed a clear increase in yield on the basis of viable  
9 counts at 24, 48 and 72hrs (Figure 1, panel A-C). As a consequence of these observations  
10 TSB media was used as the base media for the determination of the optimum carbohydrate  
11 source investigation.

12

#### 13 **3.2 Determination of Optimum Carbohydrate source in TSB media**

14 A total of 6 different carbohydrates were added to TSB medium at  $10\text{g L}^{-1}$ . TSB  
15 medium supplemented with a range of carbohydrates were compared to TSB medium with  
16 glucose at  $10\text{g L}^{-1}$ . Table 2 shows the mean cfu  $\text{ml}^{-1}$  of all 11 strains grown in TSB  
17 supplemented with the 6 different carbohydrates over the 72hr period. The data indicates that  
18 TSB media supplemented with glucose or lactose at  $10\text{g L}^{-1}$  gave higher growth yields when  
19 compared to the other carbohydrates examined, when incorporated in a medium (Table 2).  
20 These results were used to formulate TSB-1 and TSB-B media as detailed in Table 1.

21

#### 22 **3.3 Characterisation of Growth Media**

23 After 24hrs incubation, there was a notable increase in the mean cfu  $\text{ml}^{-1}$  with all 11 strains  
24 tested in the various media formulations, ranging from  $3 \times 10^8 - 2 \times 10^9$  cfu  $\text{ml}^{-1}$  illustrating  
25 that all strains had shown significant growth in the 24hr period.

1           The data indicated that the novel media TSB-1 supported the growth of all the strains  
2 to a level in excess of  $1 \times 10^9$  cfu ml<sup>-1</sup> (Figure 1, panel A). This represents a significant  
3 increase in cell number in a 24hr time period compared with the other media variations tested.  
4 All of the media formulations tested, with the exception of NSP media showed increased  
5 growth yields over this time period (24hrs). Analysis using Tukey's multiple comparison tests  
6 indicated that all media variations showed significantly higher growth yields than that  
7 achieved in NSP medium ( $P > 0.005$ ). Additionally, the data also demonstrated that TSB-1  
8 medium produced significantly greater growth yields than all other media formulations  
9 including Eaton's medium ( $p > 0.005$ ).

10           With reference to Figure 1, panel B it is clear that the mean level of growth at 48hrs  
11 showed greater equivalence in terms of cfu ml<sup>-1</sup> achieved with the notable exception of NSP  
12 medium. The yield observed in NSP was significantly lower than that observed in all other  
13 media formulations. Multiple comparison (Tukey's) tests showed that Eaton's and TSB media  
14 showed no significant difference in growth yield. Interestingly, at 48hrs both TSB medium  
15 and Eaton's medium showed similar growth yields, whilst TSB-1 medium showed an increase  
16 in mean growth yield above that observed with both TSB and Eaton's media. Despite the  
17 apparent increased mean viable count at 48hrs TSB-1 did not show a statistically significant  
18 difference when compared to TSB or Eaton's media.

19           Despite earlier observations showing increased growth yields with either glucose or  
20 lactose, subsequent studies combining both carbohydrates included in a medium did not  
21 appear to support growth to the levels observed with either Eaton's, TSB or TSB-1 media.  
22 Following 24hrs incubation TSB-B medium produced lower growth yields than all other  
23 media variations except NSP. It was also observed that TSB-B medium growth yields were  
24 shown to be similar to those attained with VP-1 medium.

1           After 72hrs incubation the colony count data (Figure1, panel C), indicated that growth  
2 yields in all media (with the exception of NSP) showed similar mean values. Once again NSP  
3 showed significantly lower growth yield than all other media formulations tested ( $p>0.005$ ).  
4 The greatest growth yields could be observed in Eaton's media, TSB and TSB-1, with these  
5 media showing greater mean viable counts than all other media under examination. In  
6 addition, TSB-1 showed the greatest mean viable count. Neither of these observations were  
7 statistically significant at this time point.

8           Additionally, the number of culture passages undertaken ( $n=10$ ) appeared to have no  
9 real effect on the resultant cell number regardless of the media recipe/combinations tested.  
10

### 11 **3.4 Determination of media adaptation by test organisms.**

12           From the data presented in Table 3 the initial culture media does not appear to effect  
13 the growth of *M. ovipneumoniae* in the novel media (TSB and TSB-1). Indeed comparable  
14 numbers of organisms could be cultured regardless of whether the organisms were  
15 subcultured from similar or dissimilar media. In addition, no significant variation in cell  
16 number was observed when the subsequent broths were cultured onto solid media of any  
17 formulation (Table 3).

## 19 **4. Discussion**

20           The results presented here demonstrate that all peptone sources utilised in the media  
21 formulations supported the growth of field strains of *M. ovipneumoniae*, producing high  
22 growth yield in a 24 hr period. TSB produced the greatest growth yield as a peptone source  
23 and when combined with lactose and porcine serum, in the form of TSB-1 medium, produced  
24 significantly greater yields than those seen with any other media combination in 24 hrs.

25

1 It is evident that the other base peptones and different carbohydrate sources tested in the  
2 various media formulations supported the growth of *M. ovipneumoniae* with Tryptone Soya  
3 Broth acting as the optimal non ruminant peptone source and either glucose or lactose as the  
4 best carbohydrate source. The data demonstrated that glucose and lactose could not be used  
5 effectively together. Consequently a new media formulation is proposed for high yield culture  
6 of vaccine strains.

7 The results showed that with the exception of NSP all the other peptones supported the  
8 growth of the 11 strains tested to at least the levels achieved with Eaton's medium. Despite  
9 the success of TSB and TSB-1 media it was noted that TSB-B medium did not give growth  
10 yields comparable to those seen with the other TSB based media variations. The number of  
11 passages appeared to have no discernible effect on the resultant cell number in the media  
12 combinations tested.

13  
14 TSB-1 medium can also support the production of a significant growth yield in a short  
15 time period (24hrs); this is an improvement on previous media formulations such as Eaton's  
16 medium in the same time period. However, despite this early significant increase in yield,  
17 latter time periods (48hrs and 72hrs) show TSB-1 to have an increased mean viable count  
18 when compared to Eaton's medium, but shows a loss of statistical significance.

19 The data presented in this study indicated that TSB-1 medium represented an effective  
20 substitute for Eaton's medium when growing *M. ovipneumoniae*. It can therefore be suggested  
21 that TSB-1 medium might represent an improved medium for the detection, isolation and  
22 maintenance of *M. ovipneumoniae* cultures. Not only does TSB-1 medium demonstrate the  
23 potential for an improved growth yield of *M. ovipneumoniae* but, importantly, the removal of  
24 ruminant protein from the medium formulation may also represent an important stage in the  
25 development of a safe and efficacious vaccine production medium.

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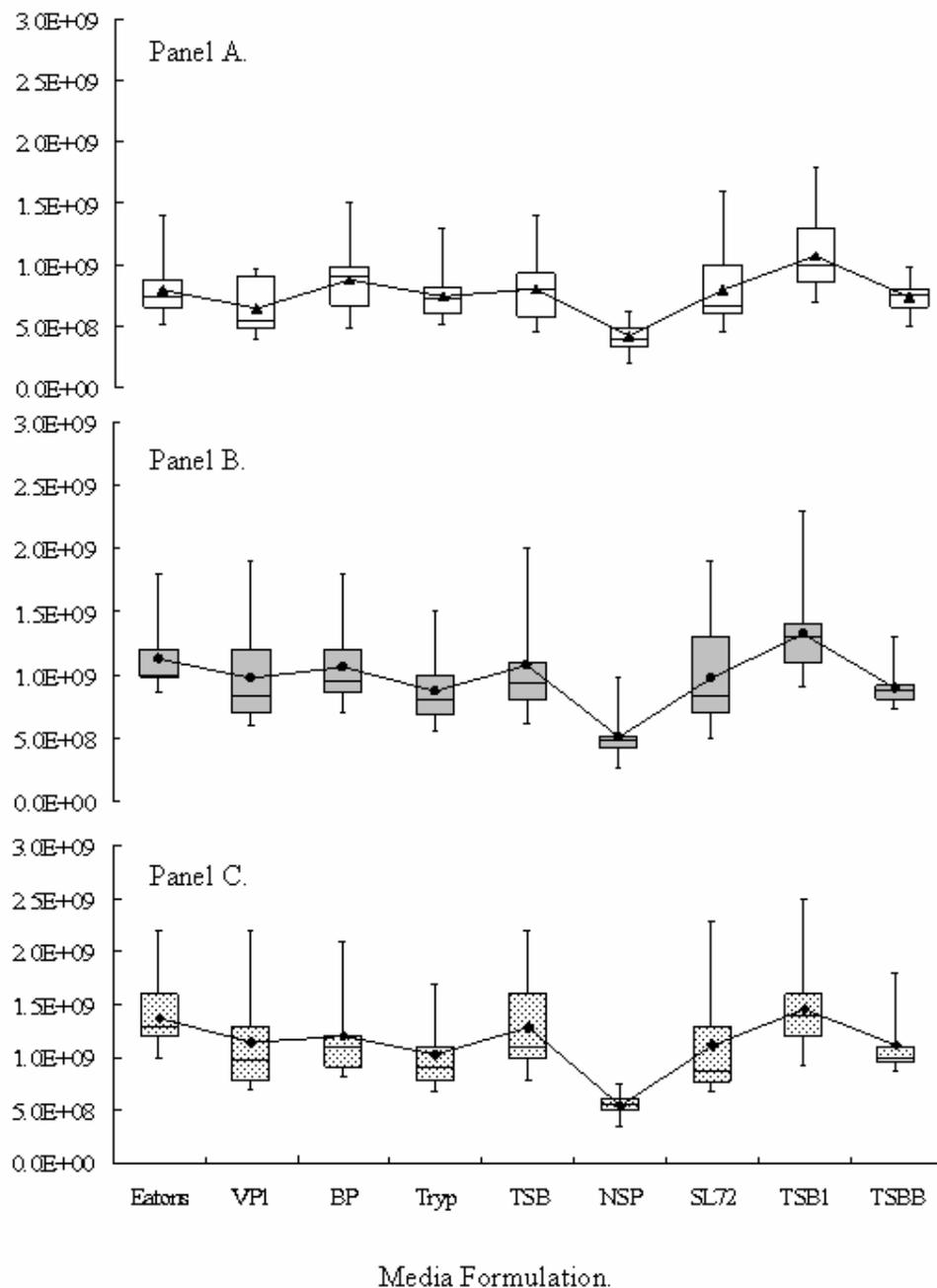
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**Figure 1. Analysis of the viable count data achieved following 24hr (Panel A), 48hr (Panel B) and 72 hr (Panel C) incubation of *M. ovipneumoniae* strains grown in different media formulations containing variations in peptone and/or carbohydrate inclusions (detailed in table 2). (Boxes represent 25% and 75% quartiles, bars represent median and error bars represent data range. Mean cfu ml<sup>-1</sup> values shown by ▲ in Panel A, ● in Panel B and ◆ in Panel C).**

**Table 1 – Individual Constituents of the proposed media as compared to standard Eaton’s medium. In each novel medium the PPLO broth from Eaton’s medium was replaced by the peptone source indicated. In the case of TSB-1 and TSB-B all constituents listed replaced the original components of Eaton’s medium.**

<b>Media</b>	<b>Constituents</b>			
<b>Eaton’s</b>	PPLO Broth, 21gL <sup>-1</sup>	Glucose, 10gL <sup>-1</sup>	fresh yeast extract, 100ml L <sup>-1</sup>	Horse serum 200ml L <sup>-1</sup>
<b>VP-1</b>	Vegetable Peptone-1, 20gL <sup>-1</sup>			
<b>B.P</b>	Bacteriological Peptone, 30gL <sup>-1</sup>			
<b>SL72</b>	Special Peptone L-72, 30gL <sup>-1</sup>			
<b>TSB</b>	Tryptone Soya Broth, 30gL <sup>-1</sup>			
<b>Tryp.</b>	Tryptose, 25gL <sup>-1</sup>			
<b>NSP</b>	Neutralised Soya Peptone, 21gL <sup>-1</sup>			
<b>TSB-1</b>	Tryptone Soya Broth, 30gL <sup>-1</sup> ;	Lactose, 10gL <sup>-1</sup> ;		Porcine serum, 200ml L <sup>-1</sup>
<b>TSB-B</b>	Tryptone Soya Broth, 30gL <sup>-1</sup>	Glucose, 5gL <sup>-1</sup> and Lactose, 5gL <sup>-1</sup>		Porcine serum, 200ml L <sup>-1</sup>

**Table 2. Represents the mean cfu ml<sup>-1</sup> of all 11 *M. ovipneumoniae* strains over a 72hr period in TSB medium with 6 different sugars.**

		TIME		
		24Hrs	48Hrs	72Hrs
SUGARS	Glucose	9.0 x 10 <sup>8</sup>	1.1 x 10 <sup>9</sup>	1.3 x 10 <sup>9</sup>
	Fructose	7.6 x 10 <sup>8</sup>	8.4 x 10 <sup>8</sup>	9.7 x 10 <sup>8</sup>
	Sucrose	6.0 x 10 <sup>8</sup>	6.9 x 10 <sup>8</sup>	8.1 x 10 <sup>8</sup>
	Mannose	7.0x 10 <sup>8</sup>	7.7 x 10 <sup>8</sup>	8.8 x 10 <sup>8</sup>
	Maltose	7.8 x 10 <sup>8</sup>	8.6 x 10 <sup>8</sup>	1.0 x 10 <sup>9</sup>
	Lactose	1.1 x 10 <sup>9</sup>	1.3 x 10 <sup>9</sup>	1.5 x 10 <sup>9</sup>

**Table 3. Represents the mean cfu/ml values for all 11 strains of *M. ovipneumoniae* grown in different media combinations. Cultures were passaged (n=10) and subsequently grown in the broth formulation indicated in the left hand panel of the table and then enumerated on either (a) Eatons Agar, (b) TSB agar or (c) TSB-1 agar.**

Broth Formulation		Time (Hrs)			
		0	24	48	72
<b>Eaton's</b>	<b>a</b>	$3.93 \times 10^7$	$7.84 \times 10^8$	$1.02 \times 10^9$	$1.23 \times 10^9$
	<b>b</b>	$3.96 \times 10^7$	$7.75 \times 10^8$	$1.02 \times 10^9$	$1.23 \times 10^9$
	<b>c</b>	$3.92 \times 10^7$	$7.75 \times 10^8$	$1.03 \times 10^9$	$1.23 \times 10^9$
<b>TSB</b>	<b>a</b>	$4.13 \times 10^7$	$8.34 \times 10^8$	$1.05 \times 10^9$	$1.22 \times 10^9$
	<b>b</b>	$3.98 \times 10^7$	$8.35 \times 10^8$	$1.03 \times 10^9$	$1.24 \times 10^9$
	<b>c</b>	$4.05 \times 10^7$	$8.21 \times 10^8$	$1.04 \times 10^9$	$1.21 \times 10^9$
<b>TSB-1</b>	<b>a</b>	$4.14 \times 10^7$	$9.89 \times 10^8$	$1.21 \times 10^9$	$1.39 \times 10^9$
	<b>b</b>	$4.06 \times 10^7$	$9.86 \times 10^8$	$1.23 \times 10^9$	$1.39 \times 10^9$
	<b>c</b>	$4.09 \times 10^7$	$9.91 \times 10^8$	$1.22 \times 10^9$	$1.38 \times 10^9$