Seroprevalence and Detection of Contagious Bovine Pleuropneumonia (CBPP) in Northeast States of Peninsular Malaysia

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ABSTRACT
Contagious Bovine Pleuropneumonia (CBPP) is a highly infectious disease of cattle caused by Mycoplasma mycoides subsp. mycoides Small Colony (MmmSC). It is a disease recognised by OIE that needs to be controlled or eradicated through surveillance system. This study establishes the sero-prevalence of CBPP, as well as attempts to isolate and identify the agent of CBPP from cattle in Kelantan and Terengganu, the northeastern states of Peninsular Malaysia, where cattle movement was high. A total of 3,242 sera from 428 farms were processed between 2011 and 2014 using the competitive ELISA (c-ELISA). The animal-level prevalence ranged between 5% (46/917) in Terengganu and 9% (220/2325) in Kelantan, whereas the herd prevalence ranged between 12% (22/210) in Terengganu and 24% (53/218) in Kelantan. The overall animal-level prevalence was 8% (266/3242), while the herd prevalence was 17% (75/428). Two hundred and four nasal swabs from 18 positive herds in Kelantan and 163 lung and mediastinal lymph node samples were processed for isolation of MmmSC before confirmation by PCR and immunoperoxidase. Forty-one samples showed turbidity in PPLO broth and 15 lung and mediastinal lymph node samples had ‘fried-egg colony’ growth on the PPLO agar, which were suggestive of Mycoplasma infection. However, all were negative for MmmSC by PCR and immunoperoxidase. Thus, the northeastern part of Peninsular Malaysia remains positive serology for CBPP without isolation.
Keywords: Contagious bovine pleuropneumonia (CBPP), MmmSc, prevalence, isolation, detection

INTRODUCTION

Contagious bovine pleuropneumonia (CBPP) is a highly infectious acute, sub acute and chronic disease of cattle caused by *Mycoplasma mycoides* subsp. *mycoides* Small Colony (MmmSC) (Okaiyeto et al., 2011; Schubert et al., 2011; OIE 2014a, 2014b). CBPP is one of the diseases recognised by OIE that needs to be controlled or eradicated through a national surveillance protocol (OIE, 2014a). Disease transmission requires close contact up to 200 metres, particularly during animal movements (Okaiyeto et al., 2011; Schubert et al., 2011; OIE 2014a, b). CBPP is currently important in many parts of Africa, but the status in Asia is unclear (OIE, 2014a).

The standard test for screening of CBPP is the Campbell and Turner complement fixation test (CFT) or competitive enzyme-linked immunosorbent assay (c-ELISA) kit to detect the specific antibodies to *Mycoplasma mycoides* subspecies *mycoides* Small Colony (MmmSC) according to the manufacturer’s (IDEXX, Switzerland) guidelines. Following cELISA serological testing, 204 deep nasal swabs from 18 cELISA-positive herds were collected. Furthermore, a total of 163 cELISA-positive cattle were slaughtered for consumption before lesions in the lung, while mediastinal lymph node samples were noted and collected for laboratory investigations. The nasal swabs were collected using the modified Dacron swab before they were swirled into the PPLO broth with supplement and...
antibiotics (OIE, 2014a). All the samples were kept refrigerated at 4°C for 24 h or at −20°C for longer storage. The suspected positive cultures showing classical ‘fried-egg’ appearance were used to identify *Mycoplasma mycoides* cluster using the polymerase chain reaction (PCR). The same lung and lymph node samples were fixed in 10% formalin for immunoperoxidase (IP) studies.

**MmmSC Isolation**

To isolate *MmmSC* from the organ samples, approximately 1 g of the tissue was minced in broth medium using a sterile scissor before the sample was diluted ten-folds. The sample was then inoculated into 3 ml of broth. The nasal swab was inoculated directly into the broth. Three serial dilutions were made before all the inoculated broths were incubated aerobically at 37°C. During incubation, the broths were checked daily for growth, indicated by a colour change from pink to yellow and appeared cloudy with silky, fragile filaments.

The broth culture was harvested at 3 to 7 days post-inoculation with a sterile syringe and filtered through a 25-mm filter containing 0.45-μm membranes to reduce contaminating bacteria. Approximately 0.2 ml of the filtrate was spread onto agar plates, while another 100 μl was re-introduced into the PPLO broth to passage further. The plates were incubated at 37°C in 5% CO₂ incubator. The plates were examined daily with inverted microscope (30X) for the presence of *Mycoplasma* colonies between 3 and 14 days of incubation. The plates were discarded if there was no growth. On agar medium, the *MmmSC* colonies appeared small (1 mm in diameter) and had the classical appearance of ‘fried eggs’ with a dense centre (OIE, 2014b).

**Detection of MmmSC by polymerase chain reaction**

The DNA was extracted by using InstaGene Matrix (Biorad, USA) according to the manufacturer’s instructions. Quick Taq® HS DyeMix (Toyobo Life Science, Japan) was used as master mix reagent. The *M. mycoides* species-specific primers MM450 (Sequence: 5’-GTA-TTT-TCC-TTT-CTA-ATT-TG-3’) and MM451 (Sequence: 5’-AAA-TCA-AAT-TAA-TAA-GTT-TG-3’) were used (OIE, 2014b).

The optimal amplification cycle for both primer sets was performed in a thermal cycler consisted of pre-denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min, and a final extension for 5 min at 72°C and hold at 4°C indefinitely. The 1.5% agarose (Pronadisa, Spain) was prepared and electrophoresis was carried out at 100 volts for 30 min. The amplified product of 574 bp was observed in positive samples by ultraviolet transillumination.

**Detection of MmmSC by immunoperoxidase staining**

The paraffin-embedded tissue samples were sectioned at 3 – 4 μm thick, collected on coated silanized slides, placed in an oven at 60°C for 15 min, and de-parafinised using
xylene and rehydrated by 100% alcohol. A commercially available kit (Nichirei, Japan) was used for the immunoperoxidase staining according to the manufacturer’s recommendation. Slides were quenched by soaking in 3% H$_2$O$_2$ solution and washed with 1x Phosphate Buffer Solution (PBS) twice. The rehydrated slides were immersed in a citrate buffer solution (Dako, USA) and digested using the autoclave method at 121°C for 10 min. The slides immersed in PBS were then applied 5% goat serum before the primary antibody was applied for 30 min. After washed with Phosphate Buffer Solution-Tween 20 (PBST), the tissues were incubated with a commercial secondary antibody N-Histofine Simple Stain MAX PO (MULTI) (Universal Immuno-peroxidase Polymer, Anti-Mouse and Anti-Rabbit by Nichirei, Japan) for 30 min at room temperature. Then, 3-amino-9-ethylcarbazole chromogen (AEC) (Nichirei, Japan) was applied for 5 min at room temperature and counter stained with Mayer’s Hematoxylin staining solution for 1 min. The immunoperoxidase staining was considered positive if there was a red colored deposit, particularly in macrophage-like cells in the lung and lymph nodes.

RESULTS

Sero-Prevalence

Tables 1 and 2 show the results of c-ELISA on sera collected from Kelantan and Terengganu between 2011 and 2014. Terengganu showed significantly (p<0.05) lower herd and animal prevalence. The herd prevalence was 10% in Terengganu and 24% in Kelantan (Table 1), while the animal prevalence was 5% in Terengganu and 9% in Kelantan (Table 2). The overall animal prevalence of CBPP in Kelantan and Terengganu between 2011 and 2014 was 8% (266 of 3242), while the overall herd prevalence was 17% (75 of 428).

Table 1
The prevalence of CBPP among herds in Kelantan and Terengganu between 2011 and 2014

<table>
<thead>
<tr>
<th>State</th>
<th>No. of herd</th>
<th>No. of positive herd</th>
<th>Prevalence %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kelantan</td>
<td>218</td>
<td>53</td>
<td>24.31$^a$</td>
</tr>
<tr>
<td>Terengganu</td>
<td>210</td>
<td>22</td>
<td>10.48$^b$</td>
</tr>
<tr>
<td>Total</td>
<td>428</td>
<td>75</td>
<td>17.52</td>
</tr>
</tbody>
</table>

$^{a,b}$Different superscripts indicate significant difference (p<0.05)

Table 2
The prevalence of CBPP in animals from Kelantan and Terengganu between 2011 and 2014

<table>
<thead>
<tr>
<th>State</th>
<th>No. of animals</th>
<th>No. of positive samples</th>
<th>Prevalence %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kelantan</td>
<td>2325</td>
<td>220</td>
<td>9.46$^a$</td>
</tr>
<tr>
<td>Terengganu</td>
<td>917</td>
<td>46</td>
<td>5.02$^b$</td>
</tr>
<tr>
<td>Total</td>
<td>3242</td>
<td>266</td>
<td>8.20</td>
</tr>
</tbody>
</table>

$^{a,b}$Different superscript indicates significant difference (p<0.05)
In Kelantan, there was an increasing trend in the animal sero-prevalence during the first 3 years (2011 to 2013) of surveillance from 0% in 2011 to 14.95% in 2013. The prevalence, however, decreased to 6.47% in 2014 (Table 3). Terengganu showed slight fluctuating pattern with a drop in prevalence in 2012 and 2014 (Table 4).

**Isolation and Identification of MmmSC**

Table 5 shows the results of attempted isolation of MmmSC in broth, as indicated by the turbidity in the broths. A total of 41 samples showed broth turbidity; 10 (4.9%) of the 204 samples of nasal swab, 19 (11.6%) of the 163 samples of lungs and 12 (7.4%) of the 163 samples of lymph nodes. The overall percentage of samples showing turbidity was 7.7%.

The 41 turbid broths were subsequently re-inoculated onto agar and these resulted in 15 samples showing growth of small colonies of 1mm in diameter with classical ‘fried-egg’ appearance of *Mycoplasma*. They were 9 (5.5%) of the lungs and 6 (3.7%) of the lymph node samples. Similarly, the 41 suspected positive samples of turbid broth, which were subjected to PCR, revealed no amplification (Figure 1).

Out of the 163 lung and lymph node samples obtained from the sero-positive animals, two (1.2%) lungs had lesions.

<table>
<thead>
<tr>
<th>Year</th>
<th>No. of samples</th>
<th>No. of positive samples</th>
<th>Prevalence %</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>132</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2012</td>
<td>460</td>
<td>12</td>
<td>2.61</td>
</tr>
<tr>
<td>2013</td>
<td>1110</td>
<td>166</td>
<td>14.95</td>
</tr>
<tr>
<td>2014</td>
<td>623</td>
<td>42</td>
<td>6.47</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Year</th>
<th>No. of samples</th>
<th>No. of positive samples</th>
<th>Prevalence %</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>113</td>
<td>5</td>
<td>4.42</td>
</tr>
<tr>
<td>2012</td>
<td>477</td>
<td>2</td>
<td>0.42</td>
</tr>
<tr>
<td>2013</td>
<td>292</td>
<td>36</td>
<td>12.33</td>
</tr>
<tr>
<td>2014</td>
<td>35</td>
<td>3</td>
<td>8.57</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Type of samples</th>
<th>No. of sample</th>
<th>No. of samples showing turbidity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal swabs</td>
<td>204</td>
<td>10 [4.9%]</td>
</tr>
<tr>
<td>Lung tissue</td>
<td>163</td>
<td>19 [11.6%]</td>
</tr>
<tr>
<td>Lymph node tissue</td>
<td>163</td>
<td>12 [7.4%]</td>
</tr>
<tr>
<td>Total</td>
<td>530</td>
<td>41 [7.7%]</td>
</tr>
</tbody>
</table>
of *MmmSC* infections, which included widening of the interlobular septa and hepatisation of the lung parenchyma with a mixture of oedematous fluid and fibrin (Figure 2). However, immunoperoxidase did not reveal positive staining.

**DISCUSSION**

This study revealed that sero-prevalence of CBPP in Malaysia increased each year and this was likely due to animal movements, which happened regularly in the states of Kelantan and Terengganu (Ramanoon et al., 2013). In the infected herds, some sero-positive animals had lung lesions, while others did not show lesions and were identified as *MmmSC* carriers. The ability to detect antibodies is optimal during the first month but showed decreasing ability in the following 6 months (Schubert et al., 2011; OIE, 2014a, b). Thus, c-ELISA is suitable in an epidemiological study as it is possible to detect new infected herd and estimate the distribution of CBPP for

![Figure 1](image1.png)

*Figure 1.* Results of PCR on the suspected samples where none of the samples (Lanes 5-14) were positive while the controls (Lanes 2-4) revealed the 574 bp bands

![Figure 2](image2.png)

*Figure 2.* Gross lesions of the affected lung of cattle of suspected contagious bovine pleuropneumonia showing visibly distended interlobular septa
implementation of control programme. Other than the serological surveillance, abattoir surveillance is also suitable to estimate the prevalence of CBPP (Msami, 2009). Nevertheless, a definitive diagnosis is made by isolation and identification of the organism from tissues of sick, freshly dead or euthanised animals (McAuliffe et al., 2003).

The conventional methods of diagnosis are based on culture and serological tests (McAuliffe et al., 2003). This is because there is no single conventional or serological test that can detect all infected animals. On the other hand, PCR can overcome some problems of the conventional tests, particularly issues pertaining to time-consuming, cross-reaction and insensitivity (Ameera et al., 2010). Therefore, suspected positive MmmSC in this study was subjected to PCR since it could detect the MmmSC even though the antibiotic treatment had been done (Taylor et al., 1992; Bashiruddin et al., 1994; Hotzel et al., 1996; Dominique et al., 2004; Woubit et al., 2004; OIE, 2014b). In this study, both PCR and immunoperoxidase staining could not detect MmmSC from any of the suspected positive samples. This might be due to the chronic stage of the disease during sampling (Sadique et al., 2012) and error in the preservation and transportation of samples that reduced the chances of isolating MmmSC (Dominique et al., 2004; Mondal et al., 2004). Furthermore, Chazel et al. (2010) revealed that if there was several Mycoplasma species present in one sample, the isolation step tends to prefer the most rapidly growing species such as M. bovirhinis and M. arginini and suppress the slower growing that are mostly pathogenic species including Mycoplasma mycoides cluster. However, Provost et al. (1987) concluded that the failure might due to the fact that the disease is not present in the country and the pathological findings might have been confused with other causes of pleuropneumonia such as Pasteurella species (Stark et al., 1995).

CONCLUSION
In conclusion, this study indicated that low cattle populations in the Northeastern States of Peninsular Malaysia showed sero-prevalence to CBPP. However, none revealed the presence of MmmSC, which was likely due to the absence of this organism in this country.

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