Pertanika J. Trop. Agric. Sci. 39 (2): 257 - 265 (2016)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

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

Zarina, M.[1], Zamri-Saad, M.[1]*, Latiffah, H.[2], Shahrom, M. S.[3] and Norlida, O.[4]

[1]Research Centre for Ruminant Diseases, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Malaysia
[2]Department of Veterinary Laboratory Diagnosis, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Malaysia
[3]Department of Veterinary Preclinical Sciences, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Malaysia
[4]Regional Veterinary Laboratory, 16150 Kota Bharu, Kelantan, Malaysia

ABSTRACT

Contagious Bovine Pleuropneumonia (CBPP) is a highly infectious disease of cattle caused by *Mycoplasma mycoides* subsp. *mycoides* Small Colony (*Mmm*SC). 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 *Mmm*Sc 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 *Mmm*SC by PCR and immunoperoxidase. Thus, the northeastern part of Peninsular Malaysia remains positive serology for CBPP without isolation.

ARTICLE INFO

Article history:
Received: 10 July 2015
Accepted: 26 January 2016

E-mail addresses:
nana6344@gmail.com (Zarina, M.),
mzamri@upm.edu.my (Zamri-Saad, M.),
latiffah@upm.edu.my (Latiffah, H.),
shahrom@upm.edu.my (Shahrom, M. S.),
norlida@dvs.gov.my (Norlida, O.)
* Corresponding author

ISSN: 1511-3701 © Universiti Putra Malaysia Press

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 (*Mmm*SC) (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 uncleared (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) (OIE, 2014b). The findings can be used to make reliable estimates on the distribution of the CBPP, which enables the implementation of a national CBPP control programme. Isolation usually uses culture media of heart-infusion broth or Bacto pleuropneumonia-like organisms (PPLO), while identification can be done by biochemical and immunological tests, and the polymerase chain reaction (PCR) (Bashiruddin et al., 1994; FAO, 2002; Swai et al*.,* 2013; OIE, 2014b). This study uses the serological test to estimate the prevalence of CBPP in the northeastern part of Peninsular Malaysia before positive animals were sampled for isolation and identification of *Mmm*SC.

MATERIALS AND METHODS

Sampling

A total of 3,242 serum samples from 428 cattle farms, both private and government farms, were collected from Kelantan and Terengganu located at the northeastern part of Peninsular Malaysia for the CBPP National Surveillance Programme. Kelantan and Terengganu recorded the most number of cattle and most number of animal movements in Peninsular Malaysia, while none of the farms had programme against bovine pleuropneumonia. A total of 2,325 serum samples were taken from 218 herds in Kelantan and 917 samples were extracted from 210 herds in Terengganu. The samples were subjected to CBPP 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 *Mmm*SC 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% CO_2 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 *Mmm*SC 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_2O_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

State	No. of herd	No. of positive herd	Prevalence %
Kelantan	218	53	24.31[a]
Terengganu	210	22	10.48[b]
Total	428	75	17.52

[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

State	No. of animals	No. of positive samples	Prevalence %
Kelantan	2325	220	9.46[a]
Terengganu	917	46	5.02[b]
Total	3242	266	8.20

[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 *Mmm*SC 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 turbit 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 turbit 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 3
Yearly prevalence of CBPP in Kelantan

Year	No. of samples	No. of positive samples	Prevalence %
2011	132	0	0
2012	460	12	2.61
2013	1110	166	14.95
2014	623	42	6.47

Table 4
Yearly prevalence of CBPP in Terengganu

Year	No. of samples	No. of positive samples	Prevalence %
2011	113	5	4.42
2012	477	2	0.42
2013	292	36	12.33
2014	35	3	8.57

Table 5
Results of attempted isolation of MmmSC in broth as shown by increased turbidity of the PPLO broth

Type of samples	No. of sample	No. of samples showing turbidity
Nasal swabs	204	10 [4.9%]
Lung tissue	163	19 [11.6%]
Lymph node tissue	163	12 [7.4%]
Total	530	41 [7.7%]

of *Mmm*SC 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 *Mmm*SC 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

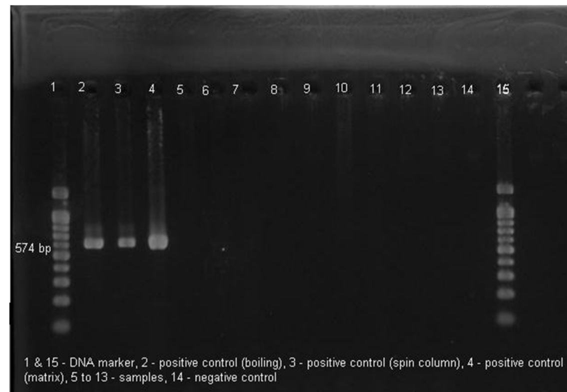

Figure 1. Results of PCR on the suspected samples where none of the samples (Lanes 5-14) were positive while the controls (Lanes 2-4) revealed the 574 bp bands

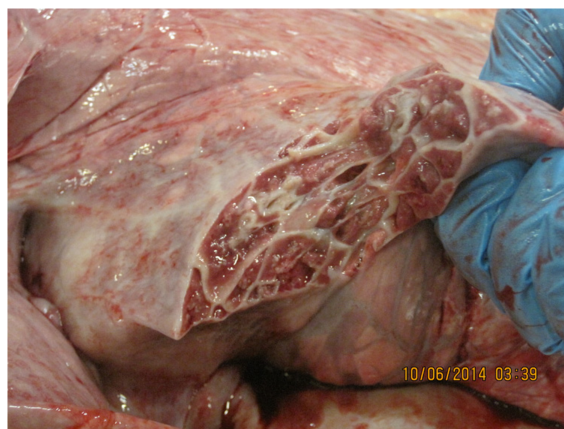

Figure 2. Gross lesions of the affected lung of cattle of suspected contagious bovine pleuropneumonia showing visibly distended interlobular septa

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 *Mmm*SC in this study was subjected to PCR since it could detect the *Mmm*SC 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 *Mmm*SC 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 *Mmm*SC (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 *Mmm*SC, which was likely due to the absence of this organism in this country.

ACKNOWLEDGEMENTS

The authors acknowledge the Department of Veterinary Services, Malaysia, and Kota Bharu Regional Veterinary Laboratory, for allowing the access to their records. Dr. Azri Adzhar, Mr. Faisal Ismail and Mr. Che Mud are acknowledged for their valuable contributions.

REFERENCES

Ameera, M. A., Shallali, A. A., Rania, S. G., Faisal, M. H., Jala, A. E. O., & NourElDin, T. M. (2010). Identification of *Mycoplasma mycoides subsp mycoides* SC (Small Colonies) Types by PCR in the Sudan. *Sudan Journal of Veterinary Research, 25,* 9-14.

Bashiruddin, J. B., Taylor, T. K., & Gould, A. R. (1994): A PCR-based test for the specific identification of

Mycoplasma mycoides subspecies *mycoides* SC. *Journal of Veterinary Diagnostic Investigation, 6*(4)*,* 428-434.

Chazel, M., Tardy, F., Le Grand, D., Calavas, D., & Poumarat, F. (2010). Mycoplasmoses of ruminants in France: recent data from the national surveillance network. *BMC Veterinary Research, 6*(1)*,* 1.

Dominique, L. G., Saras, E., Blond, D., Solsona, M., & Poumarat, F. (2004). Assessment of PCR for routine identification of species of the *Mycoplasma mycoides* cluster in ruminants. *Veterinary Research, 35*(6)*,* 635–649

Food and Agriculture Organization of the United Nations (FAO) (2002). *Animal Health Manual No. 13* (Rev. 1) 2002.

Hotzel, H., Sachse, K., & Pfutzner, H. (1996). A PCR scheme for differentiation of organisms belonging to the *Mycoplasma mycoides* cluster. *Veterinary Microbiology, 49*(1)*,* 31-43.

McAuliffe, L., Ellis, R. J., Ayling, R. D., & Nicholas, R. A. J. (2003). Differentiation of *Mycoplasma* species by 16S rDNAPCR and DGGE fingerprinting. *Journal of Clinical Microbiology, 41*(10)*,* 4844–4847.

Msami, A. (2009). *Pathology of contagious bovine pleuropneumonia: meat inspection perspective. Animal Disease Research Institute, Dar es Salaam*. Paper presented at Meat Inspectors on CBPP recognition in Lake Zone held in Mwanza on 21 to 23 July 2009.

Mondal, D., Pramanik, A. K., & Basak, D. K. (2004). Clinico-haematology and pathology of caprine mycoplasmal pneumonia in rain fed tropics of West Bengal. *Small Ruminant Research, 51*(3)*,* 285–295.

Office International des Epizooties (2014a). *Terrestrial Animal Health Code* (Chapter 11.8.1). Office International des Epizooties, Paris, France.

Office International des Epizooties (2014b). Contagious bovine pleuropneumonia. In *Manuals of standards for diagnostic tests and vaccines*. Office International des Epizooties, Paris, France.

Okaiyeto, S. O., Danbirni, S., Allam, L., Akam, E., Pewan, S. B., & Kudi, A. C. (2011). On-farm diagnosis of contagious bovine pleuropneumonia in nomadic herds using latex agglutination test (LAT). *Journal of Veterinary Medicine and Animal Health, 3*(5)*,* 62 – 66.

Provost, A., Perreau, P., Breard, A., Goff, L. E., Martel, J. L., & Cottew, G. S. (1987). Péripneumonie contagieuse bovine. *Scientific and Technical Review of the Office International des Epizooties, 6,* 565-624.

Ramanoon, S. Z., Robertson, I. D., Edwards, J., Hassan, L., & Isa, K. M. (2013). Outbreaks of foot-and-mouth disease in Peninsular Malaysia from 2001 to 2007. *Tropical Animal Health and Production, 45*(2)*,* 373-377.

Sadique, U., Chaudhry, Z. I., Younas, M., Anjum, A. A., Hassan, Z. U., Idrees, M., ... & Sabtain, S.M. (2012). Molecular characterization of contagious caprine pleuropneumonia (CCPP) in small ruminants of Khyber Pakhtunkhwa, Pakistan. *Journal of Animal and Plant Science, 22*(Suppl), 33-37.

Schubert, E., Sachse, K., Jores, J., & Heller, M. (2011). Serological testing of cattle experimentally infected with *Mycoplasma mycoides* subsp. *mycoides* Small Colony using four different tests reveals a variety of seroconversion patterns. *BMC Veterinary Research, 7*(1)*,* 1.

Stark, K. D., Vicari, A., Kihm, U. & Nicolet, J. (1995) Surveillance of Contagious Bovine Pleuropneumonia in Switzerland. *Scientific and Technical Review of the Office International des Epizooties, 14*(3)*,* 621-629.

Swai, E., Mwezimpya, I., Ulicky, E., Mbise, A., & Moshy, W. (2013). An abattoir survey of contagious bovine pleuropneumonia lesions in slaughtered cattle in selected districts in Northern Tanzania. *Asian Pacific Journal of Tropical Biomedicine, 3*(4), 303-306.

Taylor, T. K., Bashiriddin, J. B., & Gould, A. R. (1992). Application of a diagnostic: DNA probe for the differentiation of the two types of *Mycoplasma mycoides* subsp. *mycoides*. *Research in Veterinary Science, 53*(2)*,* 154-159.

Woubit, S., Lorenzon, S., Peyraud, A., Manso-Silva, L., & Thiaucourt, F. (2004). A specific PCR for the identification of *Mycoplasma capricolum* subsp. *capripneumoniae*, the causative agent of contagious caprine pleuropneumonia (CCPP). *Veterinary Microbiology, 104*(1)*,* 125–132.