Seroprevalence of bovine anaplasmosis caused by *Anaplasma marginale* in Malaysia.

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Abstract: Anaplasmosis, also known as yellow fever, is an infectious parasitic disease of cattle caused by the protozoan *Anaplasma marginale*. *Anaplasma marginale* infects the erythrocytes and causes severe anaemia, weakness, loss of appetite, fever, depression, abortion, decreased milk production, constipation, jaundice and sometimes death. In Malaysia, data on *A. marginale* infection is still behind compared to other parasites such as nematodes. Anaplasmosis in livestock has received little attention in Malaysia with only occasional reports in cattle. In addition, the determinants of tick- and fly-borne transmission are not well understood. Looking into this possibility, this study was carried out to investigate and to compare the prevalence rate of bovine anaplasmosis in Malaysia. A seroprevalence study on bovine anaplasmosis was conducted at the Veterinary Research Institute, Malaysia. Sera of various cattle breeds were collected from farms and abattoirs to be tested for the presence of *A. marginale*. Competitive enzyme-linked immunosorbent assay (c-ELISA) *Anaplasma* antibody test kits by VMRD, Inc. were used for this study, where antibodies to *A. marginale* from sample sera inhibit the binding of horseradish peroxidase (HRP)-labelled monoclonal antibody to the *Anaplasma* antigen coated on the plastic wells. A total number of 267 serum samples were tested and 79.4% were positive for bovine anaplasmosis. Results showed that the infection percentage is 100% in Pahang while the state with lowest infection percentage is Sabah with 59.2%. A comparison is done between Peninsular Malaysia with Sabah and Sarawak. The infection percentage in Peninsular Malaysia is higher at 87% while Sabah and Sarawak has an infection percentage of 60%. However, there is no significant difference in the rate of infections. The high number of cases in Peninsular Malaysia may be caused by the lack of strict control measures due to dependence on modern tools and drugs, while cases in Sabah and Sarawak may be due to traditional practices carried out frequently that may cause iatrogenic transmission. More samples should be obtained in order to validate the results. In addition, annual studies must be done to monitor the status of *A. marginale* prevalence in local cattle that is medically and economically significant to Malaysia.
Key words: Anaplasma marginale, bovine, disease, anaplasmosis.

Introduction

An effort to achieve the balance between food production and its demands is a major challenge. According to United Nation's Population Division (2009), there will be 9.5 billion people to feed in 2050; 1.3 times the population in 2010. With livestock products supplying about 12.9% of calories and 27.9% of protein consumed worldwide (Food and Agriculture Organization, 2009), the expanded population is expected to consume almost twice as much animal protein as compared to today. The demand grew so fast that it became almost impossible to maintain productivity. This matter can only become worse when livestock animals are susceptible to disease and natural disasters. Therefore, if these are not carefully addressed, the positive effects of livestock on food supply stability will decrease.

Bovine anaplasmosis is an intraerythrocytic disease that affects cattle (Palmer *et al.*, 1998) that is distributed worldwide. It is found mainly in tropical and subtropical areas, but it is further dispersed into temperate regions (Brandt, 2009). It is a disease caused by *Anaplasma sp.* but the most rampant tick-borne pathogen is *Anaplasma marginale* (Kocan *et al.*, 2004). This disease brings morbidity and mortality of cattle (European Bioinformatics Institute, 2012) as well as causing losses to cattle production (Centro Panamericano de Zoonosis, 1976). Research has shown that almost all of South Africa's cattle population was plagued by this disease (de Waal, 2000). In one year, losses of over 800 million dollars were reported in Latin America (Lonibardo, 1976) and 300 million dollars were reported in United States of America (McCallon, 1973). Bovine anaplasmosis is characterized by haemolytic anaemia (Ribeiro, Passos, and Guimarães, 1997), jaundice and haemoglobinuria (Wilkinson, 2005). Ticks from the genera Boophilus, Rhipicephalus and Hyalomma are the biological vectors in the transmission (James, 1979). Transmission by other blood-sucking insects is also possible via *Stomoxys calcitrans*, *Haematobia irritans* and *Tabanus sp.* (Wagner *et al.*, 1991).

Anaplasmosis have been prevented by using quarantine methods that needs a negative complement fixation test (CFT) reaction in every imported cattle. However, its complex technicalities are time-consuming which contributes to the need for a faster and more sensitive technique (Nakamura *et al.*, 1988). It was also reported that estimates of the accuracy of CFT in detecting persistently-infected cattle vary, and studies point out that CFT is no longer reliable for bovine anaplamosis regulation and surveillance programs (Bradway *et al.*, 2001). Competitive

enzyme-linked immunosorbent assay (c-ELISA) was shown to be more sensitive in carrier-cattle-detection and is specific with well-characterized cross-reactivity between *A. marginale* and *A. centrale* only. According to Nakamura (1988), major antibody levels were detected by ELISA and CFT at almost the same instance with experimental calf infections. However, antibodies against *A. marginale* were measurable for longer periods using ELISA as opposed to CFT.

In Malaysia, bovine anaplasmosis received little attention compared to other cattle diseases. The lack of information related to this disease is worrying when reports were only filed occasionally and inconsistently. The infection rate and status of bovine anaplasmosis is very important because it causes not only abortion in cattle but decreases milk yield and death. This will affect the local farmer's income, the country's economy as well as the people's nutrition. In 2009, the total amount of cattle in Malaysia was 755153 and this value has since increased by 19% to 931836 in the following year (World Organization for Animal Health, 2012), which shows that the cattle industry is growing to meet the demands of Malaysia's increasing population. However, the lack of research and limited information along with the inadequacy technology and proper management practices led to the increasing number of infection particularly in Malaysia.

Because of that, this study was carried out to determine the infection rate of bovine anaplasmosis throughout Malaysia. A comparison of results was also done between Peninsular Malaysia and Sabah and Sarawak. This research was carried out with the hope of filling the gap between livestock and its management, including their health and factors that contribute to the infection. An annual study on the prevalence of this disease should be performed in all farms around the nation to fully-grasp the medical and economical importance that is influences by these parasites, and to help provide enough important information regarding bovine anaplasmosis that could be helpful to farmers and veterinarians in the country.

Materials and Methods

Serum sample collection and storage

Serum samples were obtained from Kedah, Pahang, Johor, Penang, Terengganu, Perlis, Kelantan, Selangor, Sabah and Sarawak, with the exception of Negeri Sembilan, Melaka and Kuala Lumpur. Adult and cattle sera of Tenusu, Brahman, Kedah-Kelantan, Mafriwal, Kobe, and cross breeds were used. All samples were sent to the Veterinary Institute (VRI) in Ipoh and were kept in a serum bank at -6°C in the Serological Unite prior to use. The c-ELISA kit, consisting of reagents and plates, were kept refrigerated at a temperature between 2°C to 7°C.

Preparation

All serum samples, reagents and plates were brought to room temperature. Copies of the Setup Record were made and sample identifications were entered. Positive Control in duplicates and Negative Control in triplicates are done on every plate regardless of sample numbers. 1X Antibody-Peroxidase Conjugate was prepared by diluting one part of the 100X Antibody-Peroxidase Conjugate Concentrate with 99 parts of Conjugate Diluting Buffer. 1X Wash Solution was prepared by diluting one part of 10X Wash Solution Concentrate with nine parts of distilled water. Serum samples were tested undiluted.

Test Procedure

70µl of serum samples and controls were transferred to the coated-adsorption plate according to the setup record. The loaded plate was tapped at the side several times to make sure the samples coat the bottom of the wells. The plate was incubated for 30 minutes at room temperature and 50µl of the adsorbed serum samples were then transferred to the corresponding wells of the Anaplasma Antigen-Coated Plate. The loaded assay plate was tapped at the side several times to make sure the samples coat the bottom of the wells. The plate was then incubated for 1 hour in room temperature. After incubation, the plate was washed two times with the wash solution. 50µl of diluted Antibody-Peroxidase Conjugate was added to each well and the loaded assay plate was tapped several times to allow the conjugate to coat bottom of wells. The plate was incubated at room temperature for 20 minutes. After incubation, the plate is washed four times and 50µl of Substrate Solution was added to each well. The loaded assay plate was tapped several times to make sure the substrate coats the bottom of wells and was incubated away from sunlight for 20 minutes at room temperature. After incubation, 50µl of Stop Solution was added to each well and the well contents were mixed gently by tapping the side of

the loaded assay plate. The plate was then immediately read on a plate reader with the optical density reading wavelength of 620nm.

Test Validation

The mean optical density of the negative control must range from 0.04 to 2.10 and the inhibition percentage of the positive control must be ≥30%. The inhibition percentage (%I) is calculated as follow:

$$\%I = 100 - [(\text{Sample optical density} \times 100) \div (\text{Mean negative control optical density})]$$

In other words, serum samples with less than 30% inhibition are tested negative and those ≥30% are tested positive for bovine anaplasmosis.

Statistical analysis

The Student t-test was used to determine the statistical of significance between states. Differences between states were considered significant at $p<0.05$. All statistical analysis was calculated using GraphPad program.

Results and Discussion

A total number of 267 samples tested for bovine anaplasmosis infection were obtained from 10 states in Malaysia. 212 samples (74.9%) were tested to be positive for bovine anaplasmosis (Table 1).

Table 1: Percentage of bovine anaplasmosis infection in each state.

State	Number of sample	Positive sample	(%)
Kedah	19	17	89.5
Pahang	20	20	100
Johor	43	34	79.1
Penang	35	27	77.1
Terengganu	20	19	95
Perlis	21	18	85.7
Kelantan	14	13	92.9
Selangor	20	19	95
Sabah	49	29	59.2
Sarawak	26	16	61.5
Total	**267**	**212**	**79.4**

Results showed that the infection percentage is 100% in Pahang while the state with lowest infection percentage is Sabah with 59.2%. The total infection rate is 79.4% nationwide, with 212 samples that tested positive for the presence of *A. marginale* out of a total of 267 samples.

Table 2: Comparison of infection percentage between Peninsular Malaysia with Sabah and Sarawak

Region	Number of sample	Positive sample	(%)
Peninsular Malaysia	192	167	87
Sabah & Sarawak	75	45	60

A comparison was done between Peninsular Malaysia with Sabah and Sarawak. The infection percentage in Peninsular Malaysia is higher at 87% while Sabah and Sarawak has an infection percentage of 60%. However, there is no significant difference in the rate of infections at $p<0.05$. In this study, the detection of *Anaplasma marginale* is done via competitive enzyme-linked immunosorbent assay (c-ELISA); a diagnostic tool proven to be very sensitive and specific for the detection of *Anaplasma*-infected animals (Knowles *et al*., 1996; Ndung'u *et al*., 1995; Strik *et al.*, 2007; Visser *et al*., 1992). It proved to be more sensitive in carrier-cattle-detection and is specific with well-characterized cross-reactivity between *A. marginale* and *A. centrale* only. Also, antibodies against *A. marginale* were measurable for longer periods (Nakamura, 1988). According to World Organization for Animal Health (OIE), there were clinical diseases that have been reported between 2005 and 2009. From 2010 onwards, only confirmed infections were

reported without any clinical disease. Unfortunately, due to the unsystematically-reported epidemiological distribution, a proper comparison could not be done to monitor the exact status of this disease.

However, the high number of cases in Peninsular Malaysia may be caused by various reasons. The lack of strict control measures due to dependence on modern tools and drugs is one of them. There are reports of farmers that may have wrongly diluted stock solutions and failure to practice proper application. In fact, acaricides abuse may also cause ia reduction in efficacy, causing a high degree of tick resistance (Swai, 2002). Negligence by farmers is also a factor. Reports have shown that they sometimes may not notice the ticks feeding on cattle. Another reason for the cause of this disease is traditional practices such as bleeding, tagging, and vaccination. When carried out frequently, this may also cause iatrogenic transmission. Other factors such as location, herd management and vector incidences also affect the disease prevalence rate. Studies showed a higher incidence of bovine anaplasmosis during rainy season and lower incidences during dry season. Husbandry system and cattle age also affects the prevalence rate of this disease (Melendez and Forlano, 1997). Research has proven that prevalence of bovine anaplasmosis shows a positive correlation with tick incidence. In other words, high amounts of tick detected will usually result in higher amount of cases of *A. marginale* infections.

Since many farmers tend to be careless as many believed that technology will keep everything in order, extra caution has to be taken at early stages of cattle import. According to the Department of Veterinary Services (2008), quarantine of imported animals is necessary to protect the animal population by preventing the introduction and spreading of exotic diseases in this country. Although all imported animals have to be certified as healthy and free from diseases by the Veterinary Authority in their country of origin, quarantine is still crucial to ensure that animals are indeed healthy and not in fact, incubating any disease.

As a developing nation, it is no wonder that traditional practices are still valued highly although technology has improved so much. In fact, a group of professionals advocate for traditional methods to be used by small producers in Malaysia, in order to produce safe food while maintaining sustainability. They encourage the practice of indigenous farming, obtaining traditional knowledge from the community based on herbal and other non-chemical methods (Consumers Association of Penang, 2011). While sustainability is something that everyone should learn to practice, this issue should be handled with much care because no mistakes can be afforded as it will gravely affect the nation's economy and food resources.

Conclusions

Proper and valid documentation on bovine anaplasmosis outbreaks in Malaysia are scarce. Therefore, more comprehensive studies should be carried out to determine the prevalence rate and its effect on Malaysia's economy, mainly on beef and milk production.

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