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# Sero-Prevalence of Caseous Lymphadenitis Evaluated by Agar Gel Precipitation Test among Small Ruminant Flocks in East Coast Economic Regions in Peninsular Malaysia

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**Abstract:** Seroepidemiological survey of Caseous Lymphadenitis (CLA), a bacterial infection in sheep and goats was conducted in East Coast Economic Regions (ECER) in peninsular Malaysia. A total of 422 animals (n = 422) from 15 small ruminant farms (sheep and goats) in the four states were screened during the period from January to April 2011. The management practices that could be associated with CLA occurrence was also analyzed. The objective of this survey was to determine the overall status of CLA infections in small ruminants. AGPT was conducted on serum samples collected from animals. About 47 samples out of 422 yielded a positive reaction for AGPT test. The frequency of CLA infections was estimated at 11.1%. Management practices were analyzed through questionnaire. All farmers (15/15) had semi-extensive rearing system; 27.6% (13/47) of farmers had periodical veterinary services; 10.6% (5/47) took note of animals with clinical signs of CLA; 14.8% (7/47) were aware of the zoonotic potential of this disease. Lack of sanitary measures and epidemiological studies led the dissemination of this disease in Malaysia. Moreover, the economic importance of this disease seems to be overlooked by both individual and state level allowing existence of endemic nature of the disease in this country. Therefore, further epidemiological studies using reliable diagnostic tools and application of appropriate management practices need to be implemented for aiding control and eradication programs for this disease.

**Key words:** *Corynebacterium pseudotuberculosis*, CLA, seroprevalence, caseous lymphadenitis, AGPT, Malaysia

## INTRODUCTION

*C. pseudotuberculosis* is a causative agent of chronic infections in a number of different mammalian species, the most significant of which is Caseous Lymphadenitis (CLA) or chessy gland, a chronic granulomatous infectious disease of sheep and goats that is characterized by abcsessation of one or more lymph nodes. The disease is distributed globally with cases being reported in Europe, Australia, North and South America, Africa and the Middle East (Paton *et al*., 2005; Dorella *et al*., 2006).

CLA causes considerable economic losses for ovine and caprine breeders due to loss of body condition (wasting), subsequently reduced meat, wool, milk yields, segregation of affected animals, condemnation of downgraded of affected carcasses and skins in abattoirs (Stoops *et al*., 1984; Pacheco *et al*., 2007). The organism is a gram-positive rod bacterium, mycolic acid containing, facultative anerobe, intracellular pathogen and often associated with the development of abscesses in a range of mammalian hosts (Songer *et al*., 1988; Peel *et al*., 1997; Dorella *et al*., 2006). It is most commonly causes CLA in sheep and goats and less commonly, pneumonia, hepatitis, mastitis, arthritis, orchitis, subcutaneous abscess, abortion, still birth and prenatal mortalities and mastitis in cattle and buffaloes. The mode transmission of *C. pseudotuberculosis* is not known for certain; however, the pathogen will definitely be released due to a rupture of external or internal abscesses; the following contamination of skin scratches such as those caused by shearing and fighting injuries with purulent material has been suggested as the corereason of transmission. Nevertheless, an a erogenous spread has also been considered Kaba *et al*. (2001).

In Malaysia, the first case of caseous lymphadenitis was reported in 1970 when the organism isolated from a goat at the Veterinary Research Institute, Ipoh (VRI). About 1 year later, the organism was again isolated from

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ovine case. In the early sixties, however, the condition was detected in imported sheep during meat inspection at the Johor Bahru abattoir (http://agrolink.moa.my/jph). The average prevalence of CLA In Malaysian small ruminants (sheep and goats) was found to be 30% using two (AGPT and ELISA) combined diagnostics tests (Komala *et al*., 2008).

Despite growing interest in small ruminant integrated farming in Malaysia and the economic impact of CLA to small ruminant industry few epidemiological studies were carried out in Malaysia. Moreover, earlier studies were conducted only in one out eleven states in peninsular Malaysia. The serological status of the herd is an indication of the presence of the infectious agent and can be used to orient control programs so that they are compatible with the actual infection rate.

Moreover, the small ruminant population in Malaysia has been steadily increasing over the past 10 years with acquisition of animals from other regions or other countries where the disease might prevalent resulting in considerable transit of animals in East Coast Economic Regions (ECER) in Peninsular Malaysia. Therefore, the aim of this study was to assess the current seroprevalence of CLA among small ruminant flocks in East Coast Economic starts in Malasyia.

## MATERIALS AND METHODS

**Study area and sampling:** The East Coast Economic Region (ECER) covers the states of Kelantan, Terengganu and Pahang as well as the district of Mersingin Johor (Fig. 1).

ECER is also, one of the three development regions formed in Peninsular Malaysia. It has an estimated area of 66,000 km$^2$ (51%) land area of Peninsular Malaysia, a mostly tropical climate and a mean temperature is 32°C throughout the day and 22°C at night. Annual rainfall average in the country is around 85.8 inches with well-defined dry and monsoon seasons.


Fig. 1: Sampled area of East coast economic regions in peninsular Malaysia

Sampling was organized at two levels: Farms and animals. To calculate the number of herds that should be sampled, simple sampling with an estimated prevalence of 50%, a confidence interval of 95% and an error of 5% has been used (Noordhuizen *et al*., 1997).

Based on a combined list of goat and sheep farms from the Department of Veterinary Service (DVS) and Veterinary Research Institute, Ipoh, 15 small ruminant farms were selected randomly, representing the four states of ECER. Animals were randomly selected and a fixed sampling 10-15% animal from each property was used (Bennett *et al*., 1991) for properties with <10-15% animals all were sampled. The total animal sample was calculated as 422 sheep and goats. Blood samples were collected by jugular vein puncture and the serum was separated and stored at -20°C until used for analysis.

**Sample size determination and sampling method:** The calculation of sample size required was calculated based on Thrusfield *et al*. (2001) with 50% expected prevalence, 95% confidence level and 5% precision ($\alpha$ = 0.05). The calculation was done based on the assumptions that no previous study has been done in the area.

**Data animals:** A earlier tested questionnaire was filled out for each herd, demanding data on the farm, the herd, the farmer. Management and the farm animal population is shown in Table 1. All farms were found to be practiced semi-intensive management where the animals were allowed to graze during the day for 4-8 h and housed in a raised floor wooden shed at night.

**Bacteriological examination:** The organism was previously isolated from cases infected with CLA at TPU farm in UPM (Jesse *et al*., 2008).

Table 1: General information on the 15 small ruminant farms involved in the study of seroprevalence

| States | No. of farms | Type of animal | Farm management | No. of animal sampled |
|---|---|---|---|---|
| Johor | J1 | Sheep | Semi-Intensive | 24 |
| | J2 | Goat | Semi-Intensive | 36 |
| | J3 | Sheep | Semi-Intensive | 25 |
| Kelantan | K1 | Goat | Semi-Intensive | 59 |
| | K2 | Sheep | Semi-Intensive | 8 |
| | K3 | Goat | Semi-Intensive | 32 |
| | K4 | Goat | Semi-Intensive | 21 |
| | K5 | Goat | Semi-Intensive | 44 |
| Pahang | P1 | Sheep | Semi-Intensive | 32 |
| | P2 | Goat | Semi-Intensive | 5 |
| | P3 | Goat | Semi-Intensive | 13 |
| Terengganu | T1 | Goat | Semi-Intensive | 31 |
| | T2 | Goat | Semi-Intensive | 46 |
| | T3 | Goat | Semi-Intensive | 27 |
| | T4 | Goat | Semi-Intensive | 19 |

*C. pseudotuberculosis* was then sub-cultured under complete aseptic conditions and used for both direct smear and isolation of the organism by culturing onto 10% sheep blood agar and MacConkey's agar plates then incubated at 37°C for 48 h aerobically as well as in 5% $CO_2$ incubator according to the method described by Bailey and Scott. The resultant colonies were then subjected to biochemical tests (Table 2).

**Preparation of Staphylococcal beta-lysin (*Staphylococcus aureus*):** The Staphylococcal β-lysin was initially prepared by mixing 10 g Proteose peptone (Difco), 2.5 g sodium chloride (NaCl) (BDH), 0.5 g anhydrous potassium dihydrogen orthophosphate ($KH_2PO_4$)-(BDH), 0.5 g anhydrous dipotassium hydrogen orthophosphate ($K_2HPO_4$)-(BDH), 0.1 g hepatahydate magnesium sulphate ($MgSO_4.7H_2O$)-(BDH), 0.05 g dihydarte calcium chloride ($CaCl_2.2H_2O$)-(BDH), 2.15 mL syrupy ammonium lactate ($CHOH.COONH_4$)-(BDH) and 500 mL distilled water. All these mixtures was covered and boiled for 10 min with constant stirring. The medium was then filtered using filter paper and cooled at room temperature. The pH was then adjusted to 6.8.

Approximately, 200 mL amounts of solution were then dispend to 500 mL flask and autoclaved at 121°C for 15 min. The medium was incubated aerobically at 37°C for checking sterility and then stored at 4°C. Each bottle of the two medium was inoculated with approximately 5 mL of a 3-5 h culture of *Staphylococcus aureus* (β-haemolytic) grown in peptone water and tryptose soy broth, respectively. The inoculated bottles were then incubated at 37°C for 48 h in atmosphere 80% $CO_2$ and 20% $O_2$ (using anaerobic in Baird and Totlock wit out Cayaly). The solution was centrifuged at 8000 rpm for 20 min and the supernatant filtered through a cellulose acetate membrane with an Average Pore Diameter (APD) of 0.2 μm (Nalgene) and stored at 4°C.

**Titration of Staphylococcal beta-lysin (*S. aureus*):** The Beta-lysin (*S. aureus*) was titrated by making double dilution of 0.5 mL of the lysin in 0.5 mL normal saline except tube control saline where it contains 1 mL of normal saline. All tubes were then added about 0.5 mL of 3% washed bovine RBCs, gently mixed and placed in incubator at 37°C for 1 h after which all tubes were removed from incubator, gently mixed and placed in a fridge at 4°C for minimum 3 h or overnight. Observation was then taken by reading the end point of tested tubes as the highest dilution of lysine which gives complete haemolysis (Fig. 2).

Table 2: Biochemical charactristics of *C. pseudotuberculosis*

| Acid production /Hydrolysis | Results |
|---|---|
| Glucose | + |
| Fructose | + |
| Trehalose | - |
| Sucrose | - |
| Urea | + |
| Nitrate | - |
| Catalase | - |
| Oxidase | - |
| Blood broth | + |

**Preparation of *C. pseudotuberculosis* toxin (PLD):** About 500 mL of *C. pseudotuberculosis* toxin was extracted by mixing 100 g grinded bovine heart, 100 g grinded bovine liver, 5 g pepsin, 10 mL concentrated hydrochloric acid and 1000 mL distilled water. All ingredients were properly mixed and allowed to macerate by placing in water bath for 20 h at 56°C. The mixture was then placed in a steamer, approximately 90°C for 15 min and left it at room temperature overnight. The media were then filtered through fine filter paper, pH adjusted up to 7.6 with Sodium hydroxide and left for 2 h at 4°C. The media (500 mL) was equally distributed into two flasks where each flask contained about 250 mL media each. The flasks were then autoclaved at 110°C for 15 min and stored at 4°C until used.

**Toxin procedure:** The frozen media were thawed prior inoculation of *C. pseudotuberculosis* by placing on level of surface at room temperature for 1-2 h. Each flask of medium was inoculated with 48 h blood agar culture of *C. pseudotuberculosis* with concentration estimated to the standard dose of $1\times10^4$ CFU $mL^{-1}$ of the organism. The flask were incubated aerobically for 7 days at 37°C in slanting postion 15°-20° to be horizontal as described.

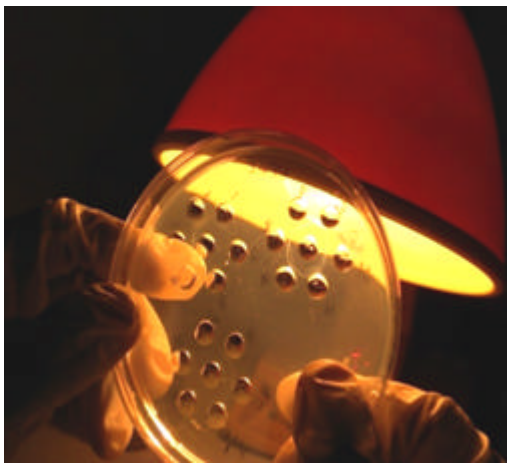

Fig. 2: AGPT showing the precipitation lines between the toxin (central well) and the sera

Both 2 cultures developed a pellicle and were centrifuged at 3000 rpm for 30 min. The supernatant pooled and passed through sterile cellulose membrane filter (0.2 μm) and stored 50 mL amounts in sterile bottles to check whether or not it develops a pellicles. The sediment was also checked for purity by sub culturing onto several blood agar plates.

**Titration of exotoxin produced by *C. pseudotuberculosis*:** The toxin was titrated by making double dilution of 0.5 mL of toxin in all tubes contained 0.5 mL normal saline except tube control saline where, it contains 1 mL of normal saline and lysine control tube. Bovine RBCs 3% was then added to all tubes including tubes (lysine control and normal saline control). All tubes were gently mixed and placed in incubator at 37°C for 4 h. Examination of haemolysis was carried out after the tubes were carefully removed from the incubator and added at the same time about 0.5 mL 2 MHD lysine to all tubes except saline control only. Following this, all tubes, after incubation of 1 h were gently mixed and left in a fridge at 4°C overnight. The end point is read as highest dilution which shows complete agglutination.

**Interpretation of the toxin results:** The reaction was accomplished when there is complete agglutination in those tubes contained the toxin. PLD toxinof *C. pseudotuberculosis* normally inhibits the activation of lysine (*Staphylococcus beta aureus*). If the extracted toxin is sufficiently effective, it would definitely inhibit any activation of lysine in examining tubes (Fig. 2). Additionally, the saline control tube should show absolutely no haemolysis (Fig. 2). In contrast, the lysine will show complete haemolysis of the bovine red blood in the interested tubes unless toxin was added in those tubes (Fig. 2).

**Agar Gel Precipitation Test (AGPT):** The gel diffusion test for measuring seropositivity against *C. pseudotuberculosis* was carried out. This test involves a precipitation reaction between toxin and antitoxin in a serum solid agar (Fig. 3). The objective of the gel diffusion test is to bring together through diffusion in optimal concentration of antigen and antibody or toxin and anti toxin to form visible line precipitation.

**Preparation of agar gel:** Ion Agar Plates (IAP) were prepared by mixing 5 g Agar No. 2 (OXOID) purified agar, 8 g sodium chloride (BDH), 2.5 mL phenol (BDH) and 500 mL distilled water. The mixture solution was allowed to remain at room temperature for half an h to permit imbibator of the agar. The solution was then gently

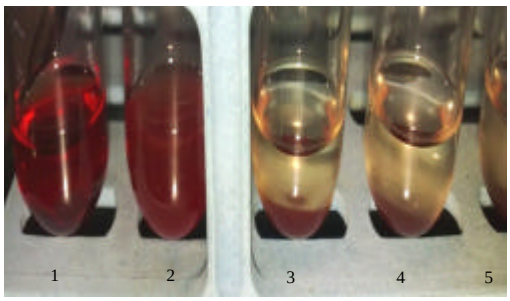


Fig. 3: Titration of *C. pseudotuberculosis* toxin: 1: Saline control, 2: Lysin control, 3-5: Titration of double diluted toxin, 1/1024, 1/512 and 1/256, respectively

heated to boiling point and autoclaved at 121°C for 15 min. The ion agar solution was cooled and poured into sterile 80 mm diameter plastic Petri dish. The plates were placed on level surface of dust free environment at room temperature until the agar set after which they were stored at 4°C for minimum 24 h before use.

**Cutting wells in the agar:** The stored agar plates were checked just prior to use to ensure that they have not dried out nor covered with moisture. The plates were then cut after it had hardened sufficiently using template for making wells where the peripheral wells were in standard hexagonal patterns surrounding a central well. Care was being taken to ensure that the cut edges did not break down during the process of removing agar plugs. The agar plugs were removed from the wells by sectioning using a glass cannula drawn to small opening (1-2 mm in diameter) connected to vacuum line. The plates were used the same day they were cut.

**Agar gel procedure:** Rozen sera were thawed prior to testing. The thawed samples were mixed by inverting tube several times before used. A earlier extracted toxin (80 mL) from *C. pseudotuberculosis* was placed in the central well while the test sera (80 μL) were placed in the outside wells. A single positive control serum was placed in one well on each plate. All testes plates were then left in a moist temperature for 3-5 days after which they were examined for precipitation lines using a bright light source against a black background.

**Interpretation of the results:** The reaction was completed when a visible line of precipitation was formed between sample and extracted toxin of *C. pseudotuberculosis* which had earlier placed in the central well. Positive samples will form distinct lines between the central well (toxin) and the outside well of the positive sample while

the negative sera will not definitely form a line (Fig. 3). The reaction was observed using a bright light source against a black background.

**Statistical analysis:** The statistical package SPSS Software 17 (PASW Version 17; Inc., Chicago, IL, USA) was used. Seroprevalence values were summarized and subjected to Kruskal-Wallis test. The following variables: individual identification of animals, technical assistance, husbandry system, requisition of sanitary certification when purchasing animals were tested to see how these affected the prevalence of lymphadenitis using the Software WinEpiscope® 2.0 (Noordhuizen *et al*., 1997; Thrusfield *et al*., 2001). An error level of 0.05 was use.

## RESULTS AND DISCUSSION

Questionnaires were filled out and serum samples collected from 422 animals from 11 farms in 4 states in peninsular Malaysia. All sera collected from these states were processed by utilization of Agar Gel Precipitation Test (AGPT). The results obtained from several of biochemical tests of the organism were tabulated in Table 2.

About fourty seven samples out of 422 yielded a positive reaction for the specific toxin of *C. pseudotuberculosis* (Table 3). The overall prevalence rate of CLA in these animals within the period of 2 months, namely January and February, 2011 was 11.1 as shown in Table 3. Seropositivity rates differed significantly ($p<0.005$) between sheep and goats (Fig. 4).

Among the states sampled, the seroprevalence of CLA in Johor state was found significantly differed ($p<0.05$) from other states where it showed the highest seroprevalence of CLA followed by Pahang and the lowest seroprevalence was observed in Trengganu (Fig. 5). The main management practices identified in small ruminant herds are shown in Table 4. Results of toxin extraction showed a complete agglutination in all tubes through inhibition of activation of Staphylococcal Beta-lysin (Fig. 3).

The current study is considered as the first sero-epedemiological study of caseous lymphadenitis in small ruminant flocks in East Coast Economic States (ECER) in Malaysia. The prevalence we found in examined animals using Agar Gel Precipitation test was relatively high with seropositivity of 11.1%. Similar studies carried out in another state within peninsular Malaysia recorded sero-positivity of 8.5% (Komala *et al*., 2008). The relatively high serological prevalence observed in the study, compared with those results of CLA reported by Komala *et al*. (2008) indicates that this disease is neglected in this country. In agreement with

Table 3: Frequency distribution of seropositive small ruminant based on AGPT for *C. pseudotuberculosis* in ECER'states, Malaysia 2011

| States | No. of farms | No. of samples | No. of positive samples | Percentage Positive (%) |
|---|---|---|---|---|
| Johor | J1 | 24 | 6 | 25.0 |
| | J2 | 36 | 10 | 27.0 |
| | J3 | 25 | 2 | 8.0 |
| Kelantan | K1 | 59 | 5 | 8.4 |
| | K2 | 8 | 0 | 0.0 |
| | K3 | 32 | 1 | 3.0 |
| | K4 | 21 | 7 | 33.0 |
| | K5 | 44 | 3 | 6.8 |
| Pahang | P1 | 32 | 5 | 16.0 |
| | P2 | 5 | 0 | 0.0 |
| | P3 | 13 | 0 | 0.0 |
| Terengganu | T1 | 31 | 2 | 0.0 |
| | T2 | 46 | 5 | 6.2 |
| | T3 | 27 | 1 | 11.0 |
| | T4 | 19 | 0 | 3.7 |
| Total | 15 | 422 | 47 | 11.1 |

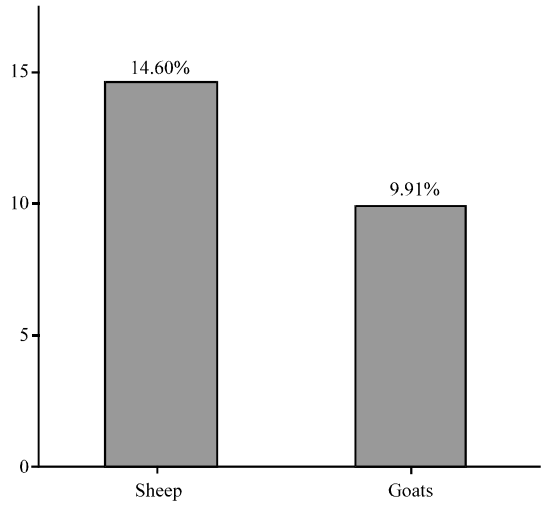


Fig. 4: Seroprevalence of CLA according to species, in ECER'S States, Malaysia

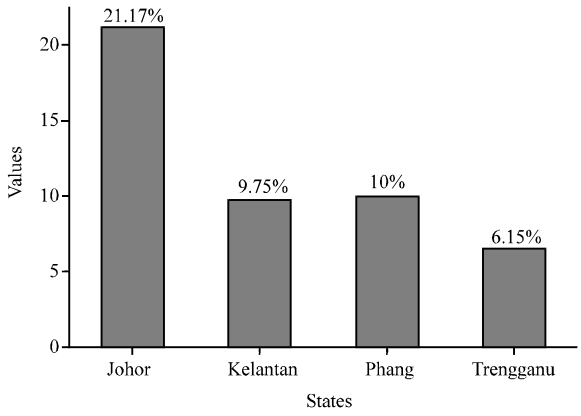


Fig. 5: Seroprevalence of CLA according to the states (ECER) Malaysia

Table 4: Management practices that could be associated with CLA identified among 47 small ruminant herds in ECER' states, Malaysia in 2011

| Management measures | n | % |
|---|---|---|
| Vaccination against CLA | 0 | 0.0 |
| Declared that they had CLA in their herd | 5 | 10.6 |
| Doesn't separate animals with clinical signs of caseous lymphadenitis | 37 | 79.0 |
| Doesn't identify animals individually | 31 | 66.0 |
| Ask for sanitary certificate when buying animals (sheep and goats) | 8 | 17.0 |
| Extensive/semi-extentensive rearing system | 27 | 30.0 |
| No veterinary assistance | 34 | 72.3 |
| Aware of zoonotic potential of CLA | 7 | 14.8 |

Chirino-Zarraga *et al*. (2006), lack of the appropriate sanitary measures which lead to dissemination of CLA and lack of veterinary care may be responsible for this.

Several other factors including the long incubation period (up to 180 days), the subclinical nature of CLA (Williamson, 2001; Valli and Gentry, 2007; Dorella *et al*., 2006) contributed the increasing risk of dissemination of this infectious agent.

There have been a very few epidemiological studies of CLA conducted in Malaysia. Perhaps the only study was that reported by Komala *et al*. (2008). They examined 579 animals from 2 districts in Perak, namely Kinta and Hilir Perak where they found a frequency of positivity of 8.5% among samples subjected for Agar Gel Precipitation Test (AGPT).

The variations in the disease frequency between the study and those reported by Komala *et al*. (2008) may be attributed to the differences in the management systems and climatic conditions in each study. Also, the endemic nature of the disease which leads to a variation in animal immunity and the degree of animal susceptibility.

The lack of serological studies to determine the prevalence of caseous lymphadenitis in other Malaysian states and lack of reliable figures for the specific financial losses caused by this public health concern disease (CLA) to farms and slaughterhouses reinforces the concept that the actual economic importance of this disease for small ruminant holders in this country is underestimated. In Australia where financial losses related to this disease have been extensively studied, costs for the meat industry due to CLA infections has been estimated around 12-15 million Australian dollars per annum (Paton *et al*., 2005). This is a part of subclinical loses which has itself causes economic impacts associated with animal production (Paton *et al*., 2005).

The estimated sero-prevalence of CLA in east coast states was acceptable but larger than that estimated by Komala *et al*. (2008) in another state. This reflects the lack of homogeneity in the distribution of the disease in each flock. The prevalence of infection in this study ranged from 0-33 among the 15 farms involved in the study. Two farms; K4 and J2 were found to have high prevalence 33 and 27%, respectively. Prevalence of CLA was significantly higher ($p<0.05$) in sheep than in goats. Similar results were obtained by Wo (1988) and Al-Gaabary and El-Sheikh (2002). The higher prevalence in sheep than goats may be attributed to the process of shearing which is the main risk factor for occurrence of CLA.

Among the states sampled, Johor state was found to have high frequency of herds with infected sheep this may be attributed to its geographic boundaries which also is the second largest sheep population in east coast economic states.

Determining risk factors associated with prevalence helps with the planning of control programs, researchers analyzed management practices used on these farms that could be associated with CLA occurrence. In this, however, researchers found that there is no any evidence showing vaccination against CLA. This would probably allow continuation spread of this pathogenic organism (*C. pseudotuberculosis*) among small ruminant population in these states involved in the study; as vaccination is one of the mos important measures for disease control.

Among the 47 small ruminant herds studies, 72.3% of farmers had no veterinary services. This high percentage indicates a presence of poor sanitary control program. Thus could be a consequence of future financial losses in Malaysian small ruminant industry.

In this study, we also found that almost 80% of owners did not separate their animals as very few of them only took notes of animals with clinical signs of CLA. This could be attributed to low level of training and education at farm owner level as well as lack of interest in sanitary control of the herd. Moreover, few farmers (14.78%) were aware the zoonotic potential of CLA to humans. This ignorance further increases the risk of human infection.

## CONCLUSION

The findings indicate that prevalence of caseous lymphadenitis was significantly higher in sheep than in goats as the study also supports the hypothesis that the disease is significantly related to deficient sanitary management detected in the farms involved in the study. Moreover, this study also elucidates that much of epidemiological mechanisms should be generated at national level to help prevent any possible future economic losses in animal industry in Malaysia, Consequently, researchers conclude that caseous lymphadenitis is amply disseminated in the small ruminant population in east coast economic states and is overlooked by both individual and state level leading existence of endemic nature of the disease.

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