DEVELOPMENT OF INDIRECT IgG ELISA FOR DETECTION OF LEPTOSPIRAL INFECTION IN CATTLE USING MALAYSIAN LOCAL ISOLATE *Leptospira kmetyi* serovar *malaysia* strain Bejo-Iso9

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SUMMARY

Cattle is one of an important maintenance hosts involved in the transmission of leptospirosis. Serological method is always implemented to detect current or past leptospiral infection in cattle. The results can be obtained immediately compared to isolation method which need more longer time. An in-house IgG enzyme-linked immunosorbent assay (ELISA) using heat-killed whole cells of local isolate *Leptospira kmetyi* serovar *malaysia* strain Bejo-Iso9 (ELISA-Bejo-Iso9) was developed in this study. The perfomance of ELISA-Bejo-Iso9 was compared with in-house IgGELISA using reference strain 117123 (*hardjo bovis*) antigen (ELISA-117123) and CUSABIO® commercial ELISA. It was found that, the performance of ELISA-Bejo-Iso9 was promising to compete with commercial ELISA. The specificity and sensitivity was 98.75% and 53.75%, respectively. The sensitivity of ELISA-Bejo-Iso9 was lower than commercial ELISA. However, the specificity of ELISA-Bejo-Iso9 was higher than commercial ELISA. Due to economically and availability factors, this finding suggested that the ELISA-Bejo-Iso9 can be used as an alternatif method for serological diagnosis of leptospirosis in cattle.

Keywords:cattle, leptospirosis, whole cells, local isolate,in-house ELISA-Bejo-Iso9.

INTRODUCTION

Leptospirosis is a zoonotic disease cause by pathogenic *Leptospira sp.* and this disease is endemic in Malaysia (Benacer *et al.,* 2013). Occurence of leptospirosis cases are frequently obeserved in tropical and sub-tropical region (Perez *et al.,* 2011). Livestock such as cattle, goat, sheep, buffalo and pigs can get infected by *Leptospira sp.* and may become main source of leptospiral infection in human. Cattle had the highest prevalence of leptospiral infection among other livestock particularly in Malaysia (Samsi *et al*., 2013; El Jalii 2008; Bahaman *et al*., 1987). The microscopic agglutination test (MAT) is the serological 'gold standard' for diagnosis of leptospirosis (Niloofa *et al.,* 2015). However, MAT is time-consuming and hazardous to human because of the exposure of live bacteria (Sugunan *et al.,* 2004). The ELISA is often used as an additional test or an alternative test to the MAT. The success of ELISA is probably that the method provides highly useful information on class-specificity antibodies, which is of clinical importance (Terpstra *et al.,* 1985) and it detects antibodies reacting with a broadly reactive genus-specific antigen (Musso and Scola, 2013). Immunoglobulin M (IgM)-antibodies are compatible with current or recent infection that manifested with clinical symptoms, while immunoglobulin G (IgG)-antibodies are compatible with past infection or chronic phase of the disease (Bennett *et al.,* 2014). Since, all the cattle tested in this study were apparently healthy, detection of specific IgG-antibodies was selected to screen whether the cattle had exposure to leptospiral infection. Since, the prevalence of leptospiral serogroups varies geographically, therefore, an in-house IgG ELISA using local strain Bejo-Iso9 (ELISA-Bejo-

Iso9) was developed in this present study. The aims of this study were to develop ELISA using local isolate of *Leptospira kmetyi* serovar *malaysia* strain Bejo-Iso9 as antigen and to evaluate its performance compared to ELISA using *L. borgpetersenii* serovar *hardjobovis* strain 117123 antigen and CUSABIO® commercial ELISA.

MATERIAL AND METHODS

Serum samples

A total of 160 cattle sera were selected from 80 seropositive MAT and 80 seronegative MAT. The serum samples were obtained from cattle in Kelantan state of Malaysia.

In-house ELISA-Bejo-Iso9 (ELISA-Bejo-Iso9)

The antigens were prepared from *L. kmetyi* serovar *malaysia* strain Bejo-Iso9 for development of in-house ELISA-Bejo-Iso9 (ELISA-Bejo-Iso9). The protocol of preparing antigen and coating plate was according to the protocol described by Goris *et al*. (2012) and Tan *et. al*. (2014). Briefly, the cultures were grown in liquid EMJH medium and incubated for 7 days at 30⁰C. After incubation, leptospires were killed with 0.5% formalin, heated in a boiling water bath for 30 min and centrifuged for 30 min at 10,000 rpm. The supernatant was collected and used as antigen. The antigen (100 µl) was pipetted into each well of 96 wells microtiter plate and was then left to evaporate at room temperature until complete evaporation of the fluid has taken place. Before the plate being used, it was washed with PBS containing 0.05% Tween 20. Diluted serum samples with dilution of 1:50, 1:100 and 1:200 were added to the coated plates and incubated for 30 min at 37°C. The plate was washed again and subsequently 100 µl of peroxidase-conjugated goat

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anti-bovine IgG (KPL, USA) was added to the wells. The plate was incubated 30 min at 37°C and washed again. Next, 100 µl of ABTS peroxidase substrate (KPL, USA) was added and the plate was incubated for 30 min at 37°C. The density of the suspensions was read at 405 nm using Sunrise Microplate Absorbance Reader (TECAN, Switzerland). The samples were tested in triplicates and an average optical density was obtained for each sample. The same protocol was implemented for *L. borgpetersenii* serovar *hardjobovis* strain 117123 antigen for ELISA-117123.

Commercial ELISA

All the 160 serum samples were also tested using ELISA Kit (CUSABIO®, US) for bovine leptospira antibody (IgG). The assay procedure has been performed as in manufacturer's instructions. The density of the suspensions was read at 405 nm using Sunrise Microplate Absorbance Reader (TECAN, Switzerland).

Statistical analysis

Establishment of in-house IgG ELISA: Statistical analysis were performed with the statistical software MedCalc® version 2014. Receiver operating characteristic (ROC) curves were used to determine the optimal level of the in-house IgG ELISA for serum dilutions of 1:50, 1:100 and 1:200. The area under the curve (AUC) with 95% confidence intervals was used to compare the predictive capability of the three serum dilutions tested using in-house IgG ELISA for both *L. kmetyi* serovar *malaysia* strain Bejo-Iso9 and *L. borgpetersenii* serovar *hardjobovis* strain 117123 antigens. For all analyses, *p*-values less than 0.05 were considered as statistically significant.

Performance comparison between the tests: A true positive leptospiral infection and true negative leptospiral infection groups were defined assuming that MAT is 100% sensitive and specific. The perfomances of ELISA-Bejo-Iso9, ELISA-117123 and CUSABIO® commercial ELISA were compared and represented by means of descriptive statistics.

RESULTS

Development of in-house ELISA-Bejo-Iso9 (ELISA-Bejo-Iso9): In the ROC curve analysis, it showed that the AUC for serum dilutions 1:50, 1:100 and 1:200 were more than 0.95 for both ELISA-Bejo-Iso9 and ELISA-117123 (Figure 1 and 2). Even though all three serum dilutions had *p*-value less than 0.001, serum dilution of 1:100 had higher AUC and smaller confidence interval (CI) compared than 1:50 and 1:100 for both assays (Table 1 and 2). Hence, the optimal serum dilution was 1:100 for both ELISA-Bejo-Iso9 and ELISA-117123.

Performance comparison between in-house ELISA-Bejo-Iso9, ELISA-117123 and commercial ELISA (CUSABIO®): The ELISA-Bejo-Iso9 had 53.75% sensitivity and 98.75% specificity with 97.73% positive predictive value (PPV) and 68.10% negative predictive value (NPV). The ELISA-117123 had 73.25% sensitivity and 98.75% specificity with 98.33% PPV and 79.00% NPV. The CUSABIO® commercial ELISA had 80.00% sensitivity and 97.50% specificity with 96.97% PPV and 82.98% NPV. The CUSABIO® commercial ELISA had better perfomance compared than both in-house ELISA.
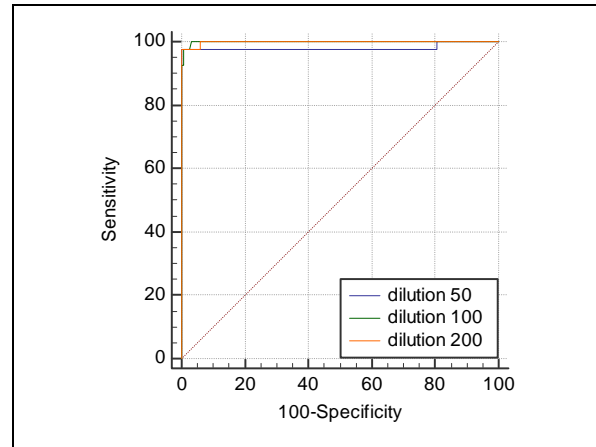

Figure 1. Receiver operating characteristic (ROC) curve plots for three different serum dilutions. The ROC was constructed from 160 serum samples tested using ELISA-Bejo-Iso9
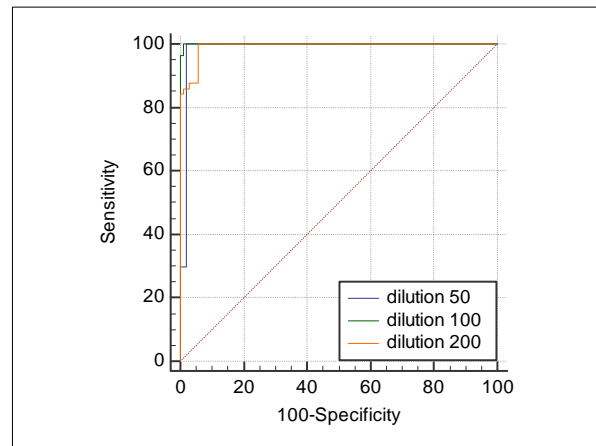

Figure 2. Receiver operating characteristic (ROC) curve plots for three different serum dilutions. The ROC was constructed from 160 serum samples tested using ELISA-117123

DISCUSSION

Leptospirosis is under-reported in many countries due to a lack of diagnostic techniques. The diagnosis must rely on the epidemiological history and clinical signs (Bourhy *et al*., 2013). However, most of leptospiral infections in livestock particularly in cattle are subclinical (Wynwood *et al.,* 2015). Laboratory diagnosis and confirmation are essentials to provide information on leptospiral infection in cattle and to take rapid measures in case of an outbreak. Unfortunately, only few tests possess both high sensitivity and specificity (Levett, 2001).

Table 1. Area under the curve (AUC) generated from ROC analysis at three different serum dilutions for ELISA-Bejo-Iso9

Dilution	Cut-off value	Area Under the Curve	95% Confidence Interval (CI)		Difference between lower and upper bound	*p*-value
			Lower Bound	Upper Bound		
1:50	0.580	0.980	0.945	0.996	0.051	<0.001
1:100	0.543	0.999	0.975	1.000	0.025	<0.001
1:200	0.484	0.998	0.972	1.000	0.028	<0.001

Table 2. Area under the curve (AUC) generated from ROC analysis at three different serum dilutions for ELISA-117123

Dilution	Cut-off value	Area under the curve	95% Confidence Interval (CI)		Difference between lower and upper bound	*p*-value
			Lower bound	Upper bound		
1:50	0.616	0.986	0.954	0.998	0.044	<0.001
1:100	0.568	1.000	0.977	1.000	0.023	<0.001
1:200	0.441	0.992	0.963	1.000	0.037	<0.001

Therefore, an in-house ELISA using local strain Bejo-Iso9 (ELISA-Bejo-Iso9) was developed in this study to determine its potential in diagnosing leptospirosis. The most convenient method to quantify the diagnostic accuracy of a test is to express its performance by area under the curve (AUC) measurements of the receiver operating characteristic (ROC) curve plots. Receiver operating characteristic (ROC) is a quantitative and descriptive expression of how close the ROC curve to a perfect one (AUC = 1) (Greiner *et al.,* 2000). Higher of the values of AUC was indicated a higher predictive capability of in-house IgG ELISA in diagnosing leptospirosis. Three dilutions (1:50, 1:100 and 1:200) were selected to determine the positive cut-off point for ELISA-Bejo-Iso9 and ELISA-117123. Although the diagnostic performance for all dilutions were high (based on AUC and CI), the 1:100 dilution had better diagnostic performance than 1:50 and 1:200. Thus, positive cut-off point was determined from dilution 1:100 for both assays. Cut-off points 0.543 for ELISA-Bejo-Iso9 and 0.568 for ELISA-117123 were selected in this study as positive cut-off point. In previous study, the positive cut-off point selected was 0.55 from dilution 1:80 by using *Leptospira* local strain IMR/75 (Tan *et al.,* 2014).

The sensitivity and specificity of commercial ELISA were higher compared than ELISA-Bejo-Iso9 and ELISA-117123. The sensitivity and specificity of ELISA-117123 were almost the same as CUSABIO® commercial ELISA. However, ELISA-Bejo-Iso9 had low sensitivity, but it had high specificity. This situation could be because of strain 117123 was reference strain isolated from cattle while strain Bejo-Iso9 was local strain isolated from environment. The PPV of CUSABIO® commercial ELISA and both in-house ELISAs were more than 95.00% which means among cattle that had a positive ELISAs test, the probability of disease was more than 95.00%. However, the NPV for both in-house ELISAs were less than 80.00%, while NPV for commercial ELISA was more than 80.00%. This situation may indicate that, among cattle that had a negative in-house ELISAs test, the probability of being disease-free was less than 80.00%.

In the previous study by Bourhy *et al.* (2013) showed that in-house ELISA had the applicability to screen leptospirosis in human with 94.00% sensitivity and 99.00% specificity. Besides that, Tan *et al.* (2014) also showed the similar findings, using in-house ELISA with a 90.38% sensitivity and 87.72% specificity, hence, may be suitable to use for the serological diagnosis of leptospirosis in human. However, a study by Honarmand *et. al.* (2010) showed in-house ELISA was more specific (87.1% sensitivity and 91.0% specificity) and commercial ELISA was more sensitive (100% sensitivity and 42.9% specificity) to diagnose leptospirosis, thus, those results will support findings in this present study.

CONCLUSION

As the leptospirosis is endemic in Malaysia, a very specific test is needed because it is very important not to misdiagnose. The specificity of ELISA-Bejo-Iso9 was higher than CUSABIO® commercial ELISA. Therefore, it was suggested that, the test method to be used as an alternatif serological diagnosis method for detection of leptospiral infection in cattle.

CONFLICT OF INTEREST

We declared that we have no conflict of interest.

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