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Amebocyte Lysate Asian Horseshoe Crab for Bacterial Endotoxin Test from the Estuary Waters of Banyuasin, South Sumatra

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Abstract. Horseshoe crab blood contains amebocyte lysate which is very important in the process of detecting bacterial endotoxins. When endotoxin enters the bloodstream, the inflammatory response of our immune system causes damage to blood vessels. The purpose of this study was to determine the ability of Asian Horseshoe Crab (AHC) amebocyte lysate from the Estuary waters of Banyuasin, South Sumatra to detect endotoxin bacteria. A sampling of horseshoe crabs was carried out in March 2021. The 28 individual horseshoe crabs were identified using morphometrics and 10 ml of blood was taken from each individual. Afterward, the horseshoe crabs were released back into their habitat. The bacterial endotoxin test (BET) is based on the qualitative method (Gel Clot) and quantitative method (Chromogenic). The bacterial endotoxin concentrations used were 1 EU/ml, 0.5 EU/ml, 0.25 EU/ml and 0.125 EU/ml. The identification species showed 2 species of AHC, namely *Carcinoscorpius rotundicauda* and *Tachypleus gigas*. Out of 28 individuals for amebocyte lysate assay resulted 4 individuals were able to detect the presence of endotoxin bacteria using the Gel Clot method. It is characterized by the presence of a weak coagulant with a maximum endurance of 12 seconds of incubation. The Chromogenic method can only detect one individual of *T. gigas* with a very strong correlation (r = 0.905) within 60 minutes of incubation. The results showed that the amebocyte lysate of AHC from the Estuary waters of Banyuasin, South Sumatra could detect the presence of bacterial endotoxin.

Keywords: Amebocyte lysate, Bacterial Endotoxin, Chromogenic, Gel Clot, South Sumatra

INTRODUCTION

Asian horseshoe crab (AHC) is one of the marine animals that is included as a rare animal. According to the government regulation of the Republic of Indonesia NO.7/1999, the AHC species *Tachypleus tridentatus*, *Tachypleus gigas,* and *Carcinocsorpius rotundicauda* are included in the protected marine biota whereas referring to the IUCN IUCN red list of threatened species they are classified as data deficient respectively [1–3]. AHC species *T. gigas* and *C. rotundicauda* were found in the waters of the Banyuasin estuary, South Sumatra because this area has extensive mangrove plants and is suitable for horseshoe crab habitat [4–7].

The horseshoe crab has many benefits for human life. Ecologically, horseshoe crabs can be used as bait to catch fish named Sembilang fish. In Thailand and China, the horseshoe crab is also used as a food ingredient [8]. Horseshoe crab also has benefits in the health sector. The blood of horseshoe crab can detect endotoxin bacteria so that it is processed into an amebocyte lysate reagent to detect endotoxin bacteria. In the United States, foodstuffs and drugs

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approved by the U.S. Food and Drug Administration must be tested first using amebocyte lysate to detect bacterial contamination or not [9].

Tests for bacterial endotoxin detection using horseshoe crab blood can be carried out using two methods, namely qualitative methods (Gel Clot) and quantitative methods (Turbidimetric and Chromogenic). The Gel Clot method is a commonly used method to detect endotoxin bacteria with test results in the form of coagulation (positive) or non-coagulation (negative). The Turbidimetric method is a method that utilizes turbidity and the Chromogenic utilizes color as the result of an endotoxin test, whose value can be read by absorbance [10]. The purpose of this study was to determine the ability of amebocyte lysate to detect endotoxin bacteria using AHC blood from Estuary water of Banyuasin waters, South Sumatra.

MATERIALS AND METHODS

A sampling of horseshoe crab was carried out in March 2021 in the Estuary waters of Banyuasin, South Sumatra. The processing of AHC blood was carried out at the Laboratory of Marine Bioecology, Marine Science Department, Faculty of Mathematics and Natural Sciences, Sriwijaya University, and at the Laboratory of Applied Genetics Engineering and Protein Design, Indonesian Institute of Sciences Biotechnology Research Center, Bogor. Determination of the location of the AHC is done by following the fishermen who catch shrimp, crabs, or demersal fish because the AHCs are the discarded catch by the fishermen [6]. After that, it was identified based on morphology[11]. AHC blood samples were taken using a 10 ml syringe and returned to their habitat.

AHC blood was added with anticoagulant solution (3% NaCl, 100 mM Dextrose, 47 mM Citric Acid, and 10 mM EDTA) [12, 13]. After that, it was centrifuged as much as 3000 RPM for 10 minutes and separated between the pellet and the supernatant. The pellet was then lysed using 4 ml of Tris HCl pH 8 [14] (Figure 1).

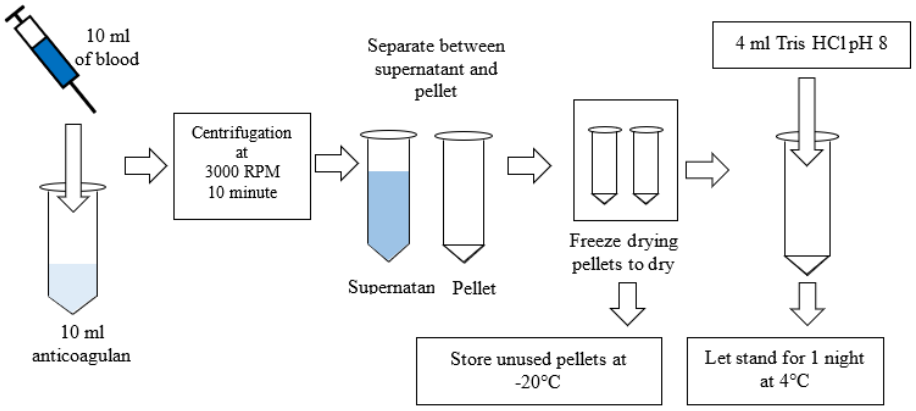


FIGURE 1. Blood component collection and separation scheme

Samples were tested using Gel Clot and Chromogenic methods. For the Gel Clot method, blood samples are mixed with bacterial endotoxin concentration (1 EU/ml, 0.5 EU/ml, 0.25 EU/ml and 0.125 EU/ml) with ratio is 1:1 [12]. After that, it was homogenized and incubated for 60 minutes at 37°C. The results were declared positive if coagulation was formed and negative if no coagulation was formed (Figure 2). For the Chromogenic method, blood samples, p-Nitroaniline, and CSE control standard endotoxin solution) were mixed with a volume of 25 µl each into a microplate [14] with three repetitions. Samples were incubated for 60 minutes at 37°C and the absorbance value was measured every 5 minutes at a wavelength of 405 nm using an ELISA Reader (Figure 3). The Gel Clot method was analyzed descriptively and the Chromogenic method was analyzed using the Standard Deviation formula [15]. Chromogenic method data analysis also refers to the endotoxin standard curve [16].

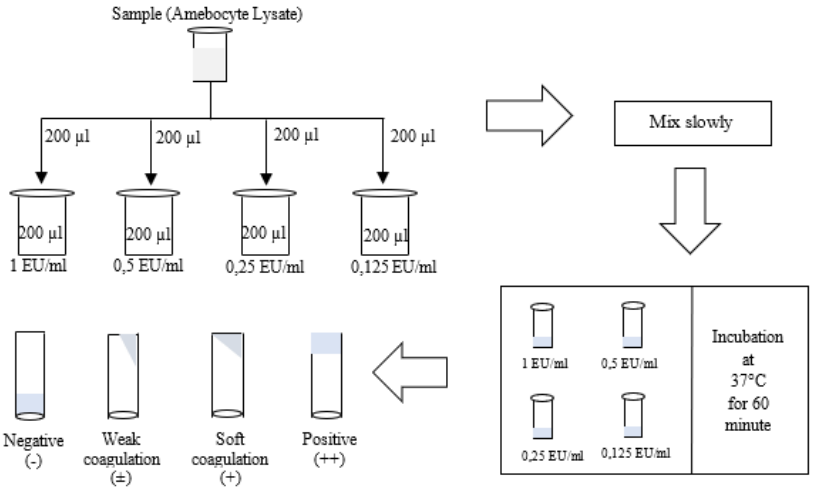


FIGURE 2. Endotoxin test Gel Clot method scheme

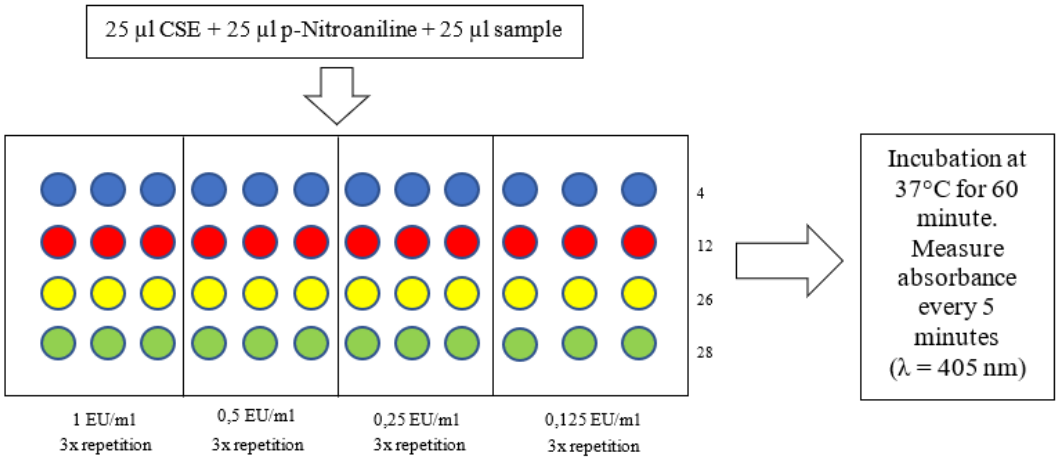


FIGURE 3. Endotoxin test Chromogenic method scheme

RESULT AND DISCUSSION

Distribution and Characteristics of Asian Horseshoe Crab in Estuary Waters of Banyuasin

The Estuary waters of Banyuasin are an area with ecosystems that support the life of AHC [4, 5, 7, 17]. These waters are dominated by mangrove ecosystems. The map of the distribution of AHC found in the Estuary waters of Banyuasin can be seen in Figure 4.

AHC was found in the Makarti Jaya area at stations 1 and 2 (local people call it the Legok area) and Carat Cape (station 3). Research [2, 3] states that these waters are a habitat for AHC in Banyuasin waters. The bathymetry of the Banyuasin estuary area is between 1-20 m. At stations 1 and 2, the depth is 2 m and at station 3 the depth is 1-5 m. At station 1, 6 AHC were found, at station 2, 6 AHC were found, and at station 3, 16 AHC were found. The number of AHC found can be seen in Table 1.

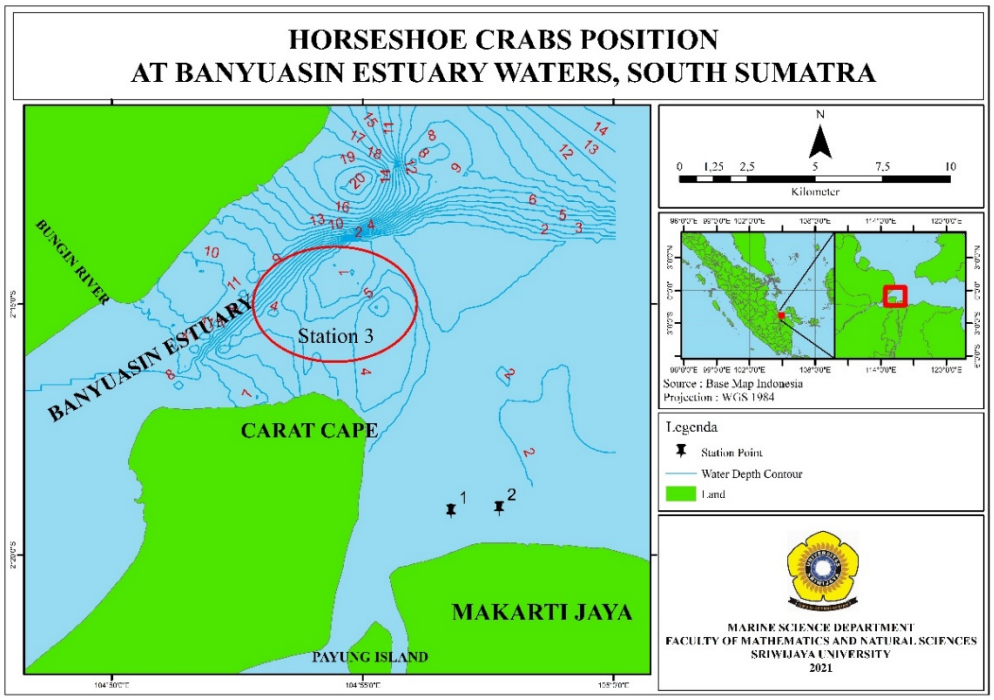


FIGURE 4. The discovery location of the horseshoe crabs in the Estuary Banyuasin waters, South Sumatra

TABLE 1. Number of Asian Horseshoe Crabs found in Banyuasin Estuary Waters, South Sumatra

Species	Sex	Station			Total	%
		1	2	3		
C. rotundicauda	Male	2	3	0	5	43
	Female	3	3	1	7	
T. gigas	Male	0	0	14	14	57
	Female	1	0	1	2	
Total		6	6	16	28	100

TABLE 2. Morphometric Asian Horseshoe Crabs found in the Banyuasin Estuary

Species	Sex	Average Morphology of Horseshoe Crabs				
		Weight (g)	Width (cm)	Body Length (cm)	Telson (cm)	Total Length (cm)
C. rotundicauda	Male	104.00	11.13	10.90	12.25	23.20
T. gigas	Male	364.14	16.75	16.29	17.29	33.57
C. rotundicauda	Female	253.86	13.71	13.93	16.00	30.17
T. gigas	Female	770.00	21.00	20.75	23.25	44.00
	Mean	373.00	15.65	15.47	17.20	32.73
Mean of Male		234.07	13.94	13.59	14.77	28.39
Mean of Female		511.93	17.36	17.34	19.63	37.08

Based on Table 1 and Figure 4, it can be observed that the species of *T. gigas* was more commonly found in the Carat Cape area (station 3) and the type of *C. rotundicauda* was more commonly found in the area of Makarti Jaya (station 1,2). *T. gigas* was more found (57%) than *C. rotundicauda* (43%).

Based on the morphometrics (Table 2), the female AHC (511.93) was heavier than the male (234.07) in both *C. rotundicauda* and *T. gigas*. Overall, female AHC has a larger body size than male AHC. If we look more closely, *T. gigas* has a larger body size (length and weight) than *C. rotundicauda.*

This indicates that the female *T. gigas* has a larger body size than the male *T. gigas*, female and male *C. rotundicauda*, with an average weight of 770.00 g and a length of 44.00 cm. The female horseshoe crab has a larger size than the male horseshoe crab because the female horseshoe crab has a greater molting growth and is influenced by the number of eggs contained in the female prosomal cavity [18].

The Carat Cape area is a horseshoe crab habitat that is more suitable for the type of *T. gigas*, characterized by the majority of this species being found in the Station 3 area. *T. gigas* lives in habitats with a wider range of salinity with sandy or muddy sand as the substrate [19, 20]. Meanwhile, *C. rotundicauda* was found mostly in the waters of Makarti Jaya, which has a high mangrove habitat. *C. rotundicauda* is often called mangrove horseshoe crab because it lives in muddy and brackish water mangrove habitats [17, 19].

Qualitative Method Test Results (Gel Clot)

The test results using the Gel Clot method showed that 4 samples (14%) of amebocyte lysate from 28 samples could detect the presence of endotoxin bacteria. The reacted amebocyte lysate samples came from both male and female *C. rotundicauda* species, and only male *T. gigas*. The test results were the formation of weak coagulants in samples number 4, 12, 26, and 28 with bacterial endotoxin concentrations of 1 - 0.125 EU/ml. The presence of soft coagulant formation in sample number 26 with bacterial endotoxin concentration of 1 EU/ml. The coagulation resistance in the incubator is 6-12 seconds (Figure 5 and Table 3).

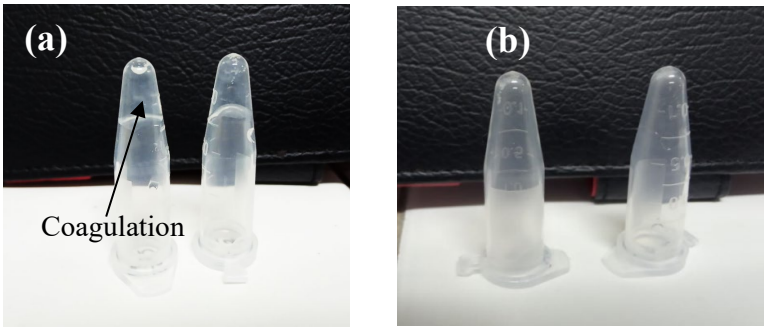


FIGURE 5. Test Results using the Gel Clot method
(a) positive (coagulation formed), and (b) negative (no coagulation formed)

TABLE 3. Gel Clot Test results that can detect endotoxin bacteria

Sample Number/ Station	Species/ Sex	Weight (g)	Length (cm)	Form a Coagulation (60 minute)				Coagulation Resistance (second)			
				Endotoxin Concentration (EU/ml)				Endotoxin Concentration (EU/ml)			
				1	0.5	0.25	0.125	1	0.5	0.25	0.125
4/1	CR/F	250	33.5	-	±	±	±	-	7	6	6
12/2	CR/M	140	29	-	±	±	-	-	6	7	-
26/3	TG/M	325	34.5	+	±	-	-	12	7	-	-
28/3	TG/M	285	33	-	±	±	-	-	7	6	-

Note:
CR = *Carcinoscorpius rotundicauda* (++) = Strong Coagulation
TG = *Tachypleus gigas* (+) = Soft Coagulation
F = Female (±) = Weak Coagulation
M = Male (–) = Not Coagulate

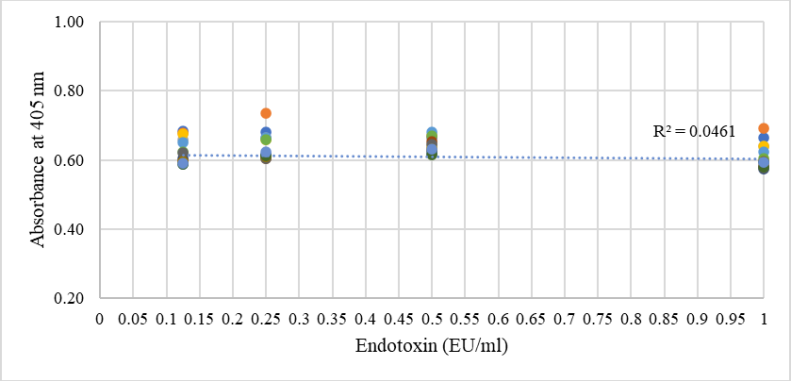
Sample number 4 is female *C. rotundicauda*, which has a wider detection ability of bacterial endotoxins with concentrations of 0.5 EU/ml to 0.125 EU/ml compared to the other 3 samples, so it is assumed to have a better quality of amebocytes than other samples. Sample number 26, namely male *T. gigas*, was only able to detect bacteria at a higher concentration of 1 EU/ml to 0.5 EU/ml.

Sample number 26 formed soft coagulation when reacted with bacterial endotoxin, namely at a concentration of 1 EU/ml with a maximum coagulation resistance of 12 seconds. AHC blood samples numbers 4, 12, and 28 have a weak form of coagulation with a fairly short coagulation resistance, which is 6-7 seconds.

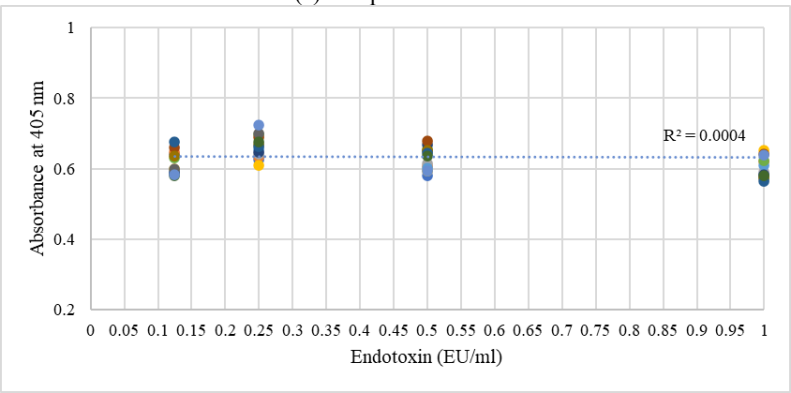
Based on the data from Table 3, it is assumed that each AHC blood sample has a different sensitivity of amebocyte lysate to detect bacterial endotoxin. Amebocyte lysate has a function as the body's defense system for horseshoe crab [21]. There are substances in horseshoe crab blood that can trigger the formation of coagulation, namely coagulated proteins, and four serine protease zymogens (factor B, factor C, factor G, and pro clotting enzymes) [9].

Quantitative Method Test Results (Chromogenic)

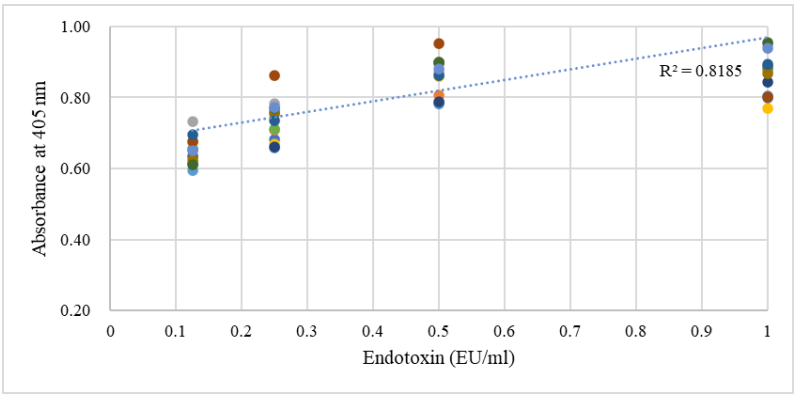
The results of the test using the Chromogenic method only 1 sample (3.6%) of amebocyte lysate from 28 samples that can detect the presence of endotoxin bacteria. Based on the test results in Figure 6, the highest correlation value between the number of endotoxin bacteria and the absorbance value was found in sample number 26 (*Tachypleus gigas* male), with a value of r = 0.905 (R^2 = 0.8185). In sample number 26, the absorbance measurement results have a value that is directly proportional to the concentration of the number of endotoxin bacteria. While the correlation values in the other three samples have very low values, namely sample number 4 with r = 0.215 (R^2 = 0.0461), sample number 12 with r = 0.020 (R^2 = 0.0004), and sample number 28 with a value of r = 0.150 (R^2 = 0.0224).



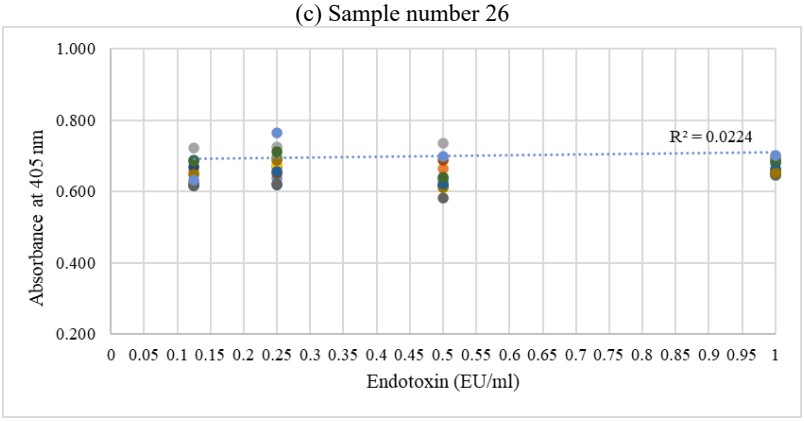
(a) Sample number 4



(b) Sample number 12



(c) Sample number 26



(d) Sample number 28

FIGURE 6. Endotoxin test activity (1 EU/ml, 0,5 EU/ml, 0,25 EU/ml, and 0,125 EU/ml) on Asian horseshoe crabs blood samples by Chromogenic method for 60 minutes on samples: (a) number 4, (b) number 12, (c) number 26, and (d) number 28

Based on the results of the Gel Clot method, samples 4, 12, and 28 had weak coagulants and did not react positively to bacterial endotoxins at a concentration of 1 EU/ml, but could detect them at lower concentrations. If it is associated with the results of the Chromogenic test in Figure 6, samples 4, 12, and 28 have no significant difference in absorbance values for each bacterial endotoxin concentration (from 1 EU/ml to 0.125 EU/ml) and have a low correlation value. The test results of the Gel Clot method sample 26 had soft coagulants and reacted positively to bacterial endotoxin concentration of 1 EU/ml. Likewise, the results of the Chromogenic test, sample 26 has a high correlation value.

This is assumed because the concentration of protease enzymes in the blood that plays an important role in the formation of coagulation has a low concentration, so the results of the Gel Clot and Chromogenic test methods on samples 4, 12, and 28 are not optimal and unstable. The amebocyte samples tested in this study came from AHC blood directly (not using LAL Reagent), which is assumed to contain other substances in the blood that may affect the test results.

The quality of amebocyte lysate is influenced by various factors, such as water quality parameters, sample handling methods, and seasonal changes. Seasonal changes are assumed to be the main factor in changes in the quality of amebocyte lysate in AHC blood. Blood samples taken in the dry season had a higher concentration of amebocyte lysate than samples taken in the rainy season. Based on the data from Table 3, the AHC sample was taken at the beginning of March (Transition Season I), so it is assumed that the concentration of amebocyte lysate in AHC blood in this season is low. AHC does a spawning cycle in the summer [22], which is in the dry season from June-August [23]. It is assumed that during the spawning season, amebocyte lysate in AHC blood has a high concentration.

CONCLUSION

Morphometrically, 2 species of Asian horseshoe crab (AHC) from Banyuasin, South Sumatra were found, namely *Carcinoscorpius rotundicauda* and *Tachypleus gigas*. The results of the bacterial endotoxin detection test (BET) using the Gel Clot method, which is 4 samples from 28 blood samples can form weak coagulation with a duration of 6-12 seconds in types *C. rotundicauda* and *T. gigas*. The Chromogenic method can detect bacteria from one blood sample (T. *gigas*) with a correlation value of r = 0.905. The small number of blood samples that can detect bacterial endotoxins can be influenced by the content of other substances in the blood. This indicates that the amebocyte lysate of AHC blood from Banyuasin, South Sumatra can detect the presence of endotoxin bacteria.

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