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Comparison of the MAKLER & HINRICHS (1993) Technique Versus Application of Hepes Lysis Solvent in Determining the Activities of *Plasmodium Lactate Dehydrogenase* (pLDH) in *Plasmodium berghei*- Infected Erythrocytes

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Abstract

Plasmodium lactate dehydrogenase (pLDH) is a clinically valuable diagnostic indicator for malaria disease. In this preliminary report, there are some considerable interest in measurements of pLDH assay. All these years, pLDH was evaluated according to method which was established by Makler and Hinrichs in 1993. This method has became very popular among malaria researches . In this study , we tried to describe an alternative way or method for measuring pLDH assay. The method was used Hepes lysis to disrupt the parasitized erythrocyte membrane and thus released the pLDH in the solution. Measurement was done based on the intensity of chromatographic changes in color through ELISA reader at 650nm. The Hepes lysis technique was recently introduced in few years ago. Both Makler (1993) and Hepes lysis techniques have demonstrated their ability and efficiency for assessing the pLDh assay . Based on the results, Makler and Hinrich (1993) technique have shown that pLDH assay activities were higher than Hepes lysis technique significantly (2 way ANOVA). The results were also supported by the microscopic view on both techniques .The experiment were conducted at 10% parasitemia and 30% parasitemia. Further investigation are needed in order to create and have more robust lactate sensing format and simple device with fast and precise respond.

Keywords: Plasmodium lactate dehydrogenase , Makler and Hinrich (1993), Hepes lysis , pLDH assay

1. Introduction

Plasmodium lactate dehydrogenase (pLDH) is an essential metabolic enzyme that responsible for energy production in the parasite and parasite development. It is present in all malarial parasites of man and other animal host. pLDH catalyzes dehydrogenation of lactate and generates pyruvate by using NAD^+ as a cofactor. For more efficient and reliable test for pLDH activity, it can easily be differentiated from host lactate dehydrogenase (LDH) with 3-acetylpyridine adenine dinucleotide (APAD), an analogue of nicotinamide adenine dinucleotide (NAD) (Makler et. al 1993). pLDH can utilize APAD at 200 fold more rapidly and effectively than host LDH isoforms.

The pLDH converts $APAD^{+}$ to $APADH/H^{+}$ and in turn reduces colorless Nitro Blue Tetrazolium (NBT) to blue formazan that regenerates APAD for another cycle of reaction with pLDH. It is apparent that this colormetric assay is very versatile, having the ability to follow drug resistance, monitoring drug therapy and diagnose malaria.

The early method of assessing activity of pLDH was introduced by Makler et.al 1993. This technique has been widely used by many researchers with some modifications. The assay has been proven able to detect the presence of *Plasmodium falciparum* from *in vitro* cultures at parasitemia levels of 0.02% (Makler and Hinrichs, 1993). In recent years, there were some researchers working on pLDH by using Hepes lysis to hemolyzed red blood cells. The technique by While Makler et.al 1993 hemolyzed the red blood cells by four freeze-thaw cycles during which the samples were frozen at -20^{o}C and thawed in a 37^{o}C water bath. These two methods have different steps or procedures in order to obtained the pLDH and determined the formation of APADH at 650nm using a multiwavelength Elisa plate reader. Details of both techniques as explained in material and method section. The aim of this study is to compare the efficiency, precision, sensitivity and ease to evaluate pLDH activities between the two techniques. It is clear that both techniques hold a prominent position as the available sources of method to detect the presence of pLDH activities. It's also a reliable nonmicroscopic screening assay for any plasmodium species at a different level of sensitivity. Previous studies have showed a correlation between levels of parasitemia and the activity of parasite LDH (IMR, 2000; Vander Jagt et.al, 1981& Xu, X.L. et.al, 2007).

In this report, the *Plasmodium berghei* (*P. berghei*)– rodent model has been developed and used in this study to allow the determination of pLDH activities from both techniques. The significance of using malaria rodent instead of cultured human malaria is because it is easy and safe to handle and manipulate any stage of the life cycle in the laboratory (C.J.Janse, 1995). Hence, rodent malaria are still valuable models in several crucial areas of malaria research. The life cycle of *P.berghei* has similarities with *Plasmodium vivax* (*P.vivax*) which provide the first justification for the use of rodent in malaria research and definitely for in this study.

2. Material and method

Malarial Parasites

The *Plasmodium berghei* (NK-65) was obtained from swiss mice that reared in Universiti Kebangsaan Malaysia animal house. The *P. berghei* was used to test for the pLDH activities. Parasites are maintained thorough weekly blood passage in mice. The mice were treated and the experimental works were approval by Universiti Kebangsaan Malaysia Animal Ethics Committee (FSKB/BIOMED/2009/ZAKIAH/20-AUGUST/275-AUGUST-2009-DECEMBER-2009).

Detection of Parasitemia

Parasitemia was determined by light microscopy (1000 x magnification) using Giemsa-stained thin smears. The percentage of parasitemia in mice was monitored until it reached up to required percentage parasitemia which are 10% and 30 %. Then the blood will be collected by orbital sinus or heart puncture. The collected blood was pooled in EDTA tube. Then it will readily to be used for further steps.

Plasmodium Lactate Dehydrogenase Bioassay

The two techniques which are Makler et.al 1993 with some modifications and Hepes Lysis were conducted to determine the activities of pLDH respectively.

According to Makler et. al 1993, the blood samples which consisted of plasma or hemolyzed red blood cells would have gone through freeze and thaw cycle for at least 4 times. The blood samples were frozen at -20^{o}C and thawed in 37^{0} C water bath. Then all aliquots were transferred into 96- well, flat bottomed microtiter plate containing Malstat reagent (20mg of Sodium L-lactate, 5.5mg of basic Tris Buffer (Tris –C) and 3.7 mg of APAD dissolved in 1 ml of distilled water) and NBT-PES mixture (1.6mg of Nitroblue Tetrazolium (NBT) and 0.1 mg of Phenazine ethosulphate (PES) dissolved in 1 ml of distilled water). Absorbance was measured at 650nm using an ELISA plate reader.

In this study, the temperature for freezing was done at -40^{0}C for red blood cells to be completely lysed. The above procedures are directly assayed without incubation process. For incubation process, the samples were incubated in a candle jar for 48h at 37^{0}C, and were subsequently cooled at -40^{o}C to lyse the red blood cells. For the positive control, parasitized red blood cells were used whereas non parasitized red blood cells were prepared for the negative control.

For the technique that used Hepes Lysis, the blood samples were centrifuged at 10 000xg to obtain supernatant samples containing pLDH. The supernatants were then transferred into 96-well microtiter plate containing Malstat reagent and followed by addition of NBT-PES mixture. Then measurements of pLDH were assayed at 650nm using Elisa plate reader. As with the Makler technique, some samples were incubated and some were directly assayed.

3. Results

The min activities of pLDH for both techniques were analysed with 2 ways ANOVA test. Study had shown that activities of pLDH were significantly difference according to percentage level of parasitemia and technique used.

The pLDH activities by Makler technique (1.16) were higher than for the Hepes Lysis technique (0.800) (Fig 1). Both are significantly different with $F(1, 20) = 27.042$, ($p<0.05$). Infected cells with parasitemia at 10% were shown significantly different from normal cells or non parasitized cells (0% parasitemia) by both techniques . Based on Hepes lysis technique, the mean activity of pLDH in non parasitized cells (1.130 ± 0.031) were significantly lower than parasitized cells at 10% parasitemia(1.930 ± 0.027). The Makler technique gave lower mean activity of pLDH in non parasitized cells (1.560 ± 0.040) compared to parasitized cells at 10% parasitemia (2.72 ± 0.040). In comparison, the mean activity of pLDH in non parasitized cells (1.130 ± 0.031) and parasitized cells at 10% parasitemia (1.930 ± 0.027) were significantly lower by Hepes lysis technique than non parasitized (1.560 ± 0.040) and parasitized cells (10% parasitemia)(2.721 ± 0.040) by Makler technique respectively . Briefly, the results from the above study demonstrated that Makler technique was more efficient and reliable than Hepes lysis technique.

The above results were supported by the microscopic view on both techniques. . By doing freeze and thaw cycling, the red blood cells were almost completely lysed, whereas, Hepes Lysis still

showed some red blood cells that did not lyse. The hemolysed parasitized cells will release the pLDH in homogenates or supernatant. The more the cells lysed the bigger chance of parasitized cells to be lysed as well.

In this study, densities of infected cells do play a major role to achieve high activity of pLDH assay. As we have seen, the higher parasitemia , the more infected cells containing pLDH to be released. The 30% parasitemia indicated that 30 out of 100 red blood cells were infected with plasmodium .Likewise, 10% parasitemia indicated that 10 of 100 red blood cells were infected.

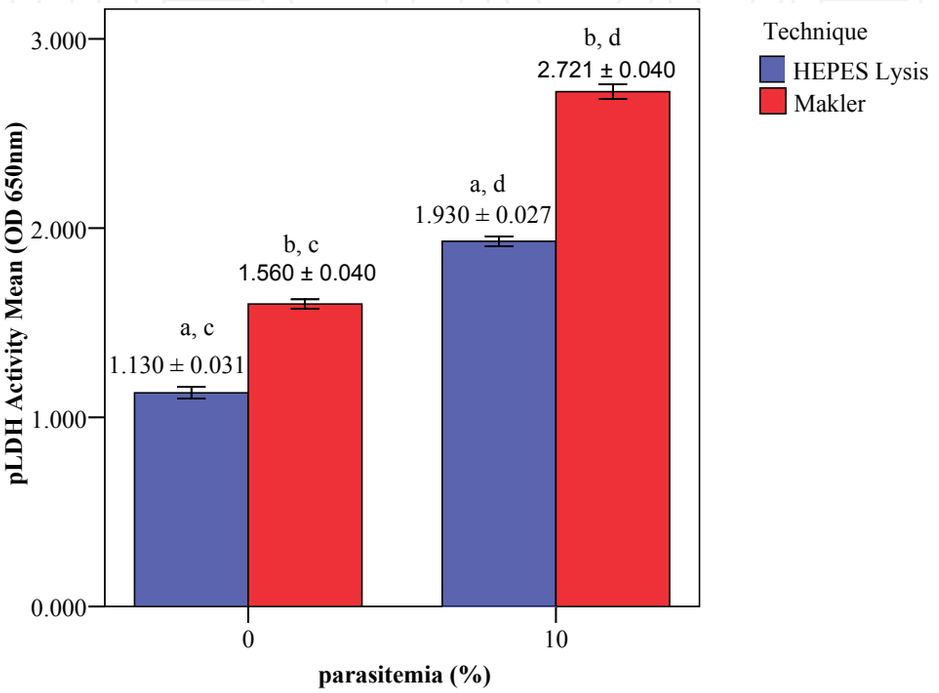

Fig 1. Determination Mean Activities of pLDH Between Makler (1993) Technique And Hepes Lysis

a = Min activity of pLDH in normal cells (0% parasitemia) which are significantly different compared to infected cells (10% parasitemia) by Hepes lysis technique.
b = Min activity of pLDH in normal cells (0% parasitemia) which are significantly different compared to infected cells (10% parasitemia) by Makler technique.
c = Min activity of pLDH in normal cells (0% parasitemia) by Hepes lysis technique which are significantly lower compared to min activity of pLDH in normal cells (0% parasitemia) by Makler technique .
d= Min activity of pLDH in infected cells (10% parasitemia) by Hepes lysis technique which are significantly lower compared to min activity of pLDH in infected cells (10% parasitemia) by Makler technique .

4. Discussion

Initially pLDH assay was developed to detect the presence of Plasmodium in the erythrocytes of malaria patient. The assay uses APAD (3-acetyl pyridine adenine dinucleotide) which is an analog to NAD (β- Nicotinamide adenine dinucleotide) with higher oxidation potential than NAD. It can substitute for NAD as a hydrogen accepting cofactor in many dehydrogenase reactions ; e.g : lactate dehydrogenase from *Toxoplasma* , *Clonorchis* , *Plasmodium* as well as mammalian dehydrogenase. It can also act as a proton acceptor in various transhydrogenation reactions with

NADH or NADPH. In *Plasmodium*, the lactate dehydrogenase works efficiently 200 times higher than human lactate dehydrogenase with APAD.

The assay development was introduced and established by Makler and Hinrichs in 1993. For many years, the assay was used in diagnosing malaria infection. In recent years, it has become popular as a validated target for antimalarial agent development in drug screening. In the meantime, there is an alternative method of pLDH assay by using Hepes Lysis solvent. It was used by some researchers but the performance is still under investigation. Basically, the principal for both methods were lysed the membrane erythrocyte. By doing so, pLDH would be released and allow to be measured by Elisa reader based on colorimetric changes. From these studies both methods have shown significant results. The negative control which was normal cells or non parasitized cells showed lower activities. However, the findings showed that Makler and Hinrichs (1993) technique exhibited higher activities than Hepes Lysis.

According to Makler and Hinrichs technique (1993) , thaw and freeze cycle process will disrupted the structure of erythrocyte membrane . Disruption will lead to cells hemolysate . For parasitized erythrocyte , hemolysate process assisted in releasing the pLDH. The more the parasitized cells lysed , the more the pLDH would released . The lysate erythrocyte turned out to be small debris and scattered in it's surrounding.

By using Hepes lysis solvent , erythrocyte was lysed chemically. Hepes lysis was alkaline solution with pH 8. For normal cells the pH ranging between 7.34 to 7.45. Zeidler and Kim (1977) , reported that alkaline condition will disrupt the stability of the cell membrane protein and eventually lysed. Most of the erythrocytes were not lysed. The morphology of treated erythrocyte were seen similar as in control cells or untreated cells. Probably the Hepes lysis need to be more alkaline to work efficiently.

Based on the overall results, erythrocytes that were treated with Makler and Hinrichs technique (1993) showed significantly higher activities than erythrocytes treated with Hepes Lysis. The results were supported by microscopic view .The activities of pLDH will increased in relation to the higher numbers of parasites. Comparison of pLDH activities were conducted by Makler and Hinrichs (1993) technique at 30% and 10% parasitemia respectively .The parasitized erythrocytes at 30% parasitemia presented significantly higher pLDH activity than 10% parasitemia and 0% parasitemia respectively.

Regarding the morphology of erythrocyte infected-*Plasmodium berghei* , the erythrocytes as the host may had more than one parasites reside in it. The size of the infected erythrocytes is bigger than normal cells. The features were similar to *Plasmodium vivax* .The colour were light purplish red. The Plasmodium could be found in various stages, mostly, were ring and trofozoite form.

On the other hand , the pLDH assay showed higher activities in cells without incubation process compared to cells treated by incubation process. This might be due to the contamination of the cells which occured during the incubation period.

5. Summary and conclusion

In this study, Makler and Hinrichs (1993) technique showed to be more efficient than Hepes lysis solvent to quantitate the activities of *Plasmodium Lactate dehydrogenase* .

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