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MEASUREMENT OF pH OF THE DIGESTIVE VACUOLE ISOLATED FROM THE *PLASMODIUM FALCIPARUM*-INFECTED ERYTHROCYTE BY DIGITONIN PERMEABILIZATION

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Keywords:

Plasmodium falciparum, Digestive vacuole, pH, FITC- dextran, Digitonin, Saponin

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ABSTRACT: The acidic pH of the digestive vacuole (DV) of *P. falciparum* is thought to play a major role in the mechanisms of digestion of host erythrocyte hemoglobin, detoxification of heme, and chloroquine (CQ) action and resistance. However, a definitive measurement of the DV pH has been technically difficult. A previous study by Abu Bakar (2015) measured the DV pH by using fluorescein isothiocyanate-dextran (FITC-dextran), a ratiometric pH indicator loaded into the malaria parasite's DV that had been isolated using saponin (DV_{sap}). To validate the FITC-dextran response to the DV_{sap} pH, pH of the DV isolated by digitonin permeabilization (DV_{digi}) was measured. The DV_{digi} pH of the CQ-sensitive parasite (D10) was 5.66 ± 0.07 that is approximately similar to that of the DV_{sap} pH (5.27 ± 0.03) estimated using the same probe. The DV_{digi} pH of the CQ-resistant parasite (Dd2) (5.62 ± 0.12) was not significantly different from the CQ-sensitive parasite's DV_{digi} pH ($P> 0.3$). Re-acidification of the DV_{digi} was also observed upon the addition of 2 mM ATP to the ATP-depleted medium. These data validate the use of FITC-dextran for quantitative DV pH analysis of the parasite isolated using either digitonin or saponin.

INTRODUCTION: The potential importance of the malaria parasite's DV pH in the degradation of hemoglobin and detoxification of heme to harmless hemozoin has long been discussed [1, 2, 3]. A study by Chugh *et al*. reported the presence of a collection of proteins in the DV that is required for the underlying mechanisms of hemoglobin digestion and hemozoin formation. This protein complex comprised parasite proteases such as histo aspartic protease, falciparum 2/2', and plasmepsin II and IV.

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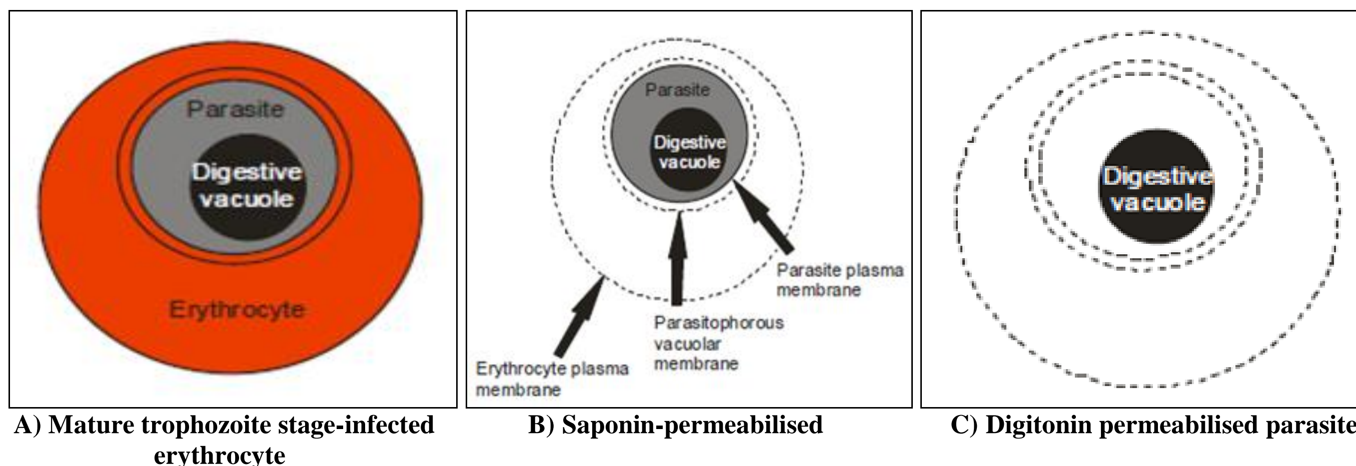
These proteolytic enzymes have pH optima ranging 4.5- 5.0, [5] suggesting an acidic environment of the DV. The proton-pumping V-type H^+-ATPase and H^+-pyrophosphatase have been implicated in the regulation of DV pH [6, 7] and localized at the DV membrane [6]. The disruption of pH regulation inside the DV has become the target of some antimalarial compounds [8]. Hence, a definitive measurement of DV pH is important due to the significance of pH in DV physiological function. Reproducibility between different laboratories is debatable when data obtained from single cell measurements and various pH-sensitive probes are compared **Table 1**. For example, in the studies of the mode of CQ action and resistance, some reported differences in the DV pH between CQ-sensitive and -resistant strains of *P. falciparum* [9, 10, 11], while others found no differences [12, 13, 14].

TABLE 1: DETERMINATION OF THE DV pH USING VARIOUS TECHNIQUES AND pH INDICATORS

Techniques	pH Indicators	References
Fluorescence microscopy, Photometry	Acridine orange, BCECF	9
Fluorescence microscopy	Acridine orange	10
Fluorescence microscopy	Acridine orange, DM-NERF	11
Spectrofluorometry	Fluorescein-dextran Oregon Green	12
Flow cytometry, GFP photobleaching	Enhanced GFP	13
Confocal microscopy, Live cell imaging	pHluorin, Lyso Sensor Blue SNARF-5F	14
Confocal microscopy, Live cell imaging	SNARF-1-dextran, LysoSensor Blue	15
Flow cytometry	FITC-dextran	1

A study by Abu Bakar *et al*., described a method for estimation of the DV pH using live cell imaging of the ratiometric SNARF-1-dextran fluorescence by confocal microscopy [15]. This measurement of pH dynamics in real time in different parts of single erythrocytes or between erythrocytes infected with live parasites offers some advantages over the earlier studies using conventional fluorescence methods.

The use of another ratiometric pH probe, FITC-dextran, for determining DV pH by flow cytometry on larger numbers of parasite-infected erythrocyte populations provided an alternative method [1]. This study used a technique to release the contents of the host erythrocyte and the parasitophorous vacuole by saponin permeabilization to provide substantial insight into the DV_{sap} pH **Fig. 1A-B**.



A) Mature trophozoite stage-infected erythrocyte **B) Saponin-permeabilised** **C) Digitonin permeabilised parasite**

FIG. 1: ILLUSTRATION OF DIFFERENT PARASITE PREPARATIONS. (A-B) The erythrocyte plasma membrane and the parasitophorous vacuolar membrane were permeabilized by saponin to release the contents of the host erythrocyte cytosol and the parasitophorous vacuole. (C) The parasite plasma membrane of saponin-isolated parasites was permeabilized by digitonin to release the content of the parasite cytosol.

To validate the result of FITC-dextran response to DV_{sap} pH, malaria parasite-infected erythrocytes treated with digitonin, a plant detergent that permeabilizes the parasite plasma membrane (PPM) were used in the present study **Fig. 1C**. Unlike saponin-permeabilization, digitonin permeabilization released the content of the parasite cytosol allowing the measurement of the fluorescence signal directly from a DV_{digi} -associated FITC-dextran by flow cytometry.

MATERIALS AND METHODS:

Culturing Parasites: CQ-sensitive (D10) and -resistant (Dd2) strains of *P. falciparum* were cultured using human erythrocytes, and pooled serum in complete RPMI 1640 culture medium (GIBCO BRL, Invitrogen) supplemented with Glutamax (4 mM, Invitrogen) and hypoxanthine (0.21 mM, Sigma) as described previously [1].

Blood samples were collected from informed consent healthy donors aged from 20-30 years who had no known diseases or blood-related diseases, took no medications, and were healthy by history and physical examination. They were recruited at School of Health Sciences, Universiti Sains Malaysia. Synchronized parasites were obtained by suspending infected erythrocytes in a 5% D-sorbitol solution (Sigma) [1]. Synchronized mature stage parasites were harvested to above 95% purity

using a magnetic separation system (VarioMACS, Miltenyi Biotec) [1].

Culturing Parasites in Resealed Erythrocytes: Resealed erythrocytes containing a pH probe were prepared by suspending washed erythrocytes in an ice-cold hemolysis buffer (5 mM sodium phosphate, 2 mM Mg-ATP, Sigma, pH 7.4) for 10 minutes in the presence of 50 µM FITC-dextran (10 kDa, Invitrogen) as reported previously [1, 16]. A volume of 220 µL resealing buffer A (5 mM sodium phosphate, 27.5 mM glucose, 100 mM KCl, 700 mM NaCl, pH 7.4) was added into the suspension to reseal the FITC-dextran-incorporated erythrocytes at 37 °C. After 20 min incubation, a volume of 8.5 mL resealing buffer B (10 mM sodium phosphate, 5.5 mM glucose, 20 mM KCl, 140 mM NaCl, pH 7.4) was added to the erythrocyte suspension.

After 20 min incubation, resealed erythrocytes were washed twice with resealing buffer B and once with RPMI medium. Synchronized and harvested mature stage parasites (3% parasitemia) were added to resealed erythrocytes at 2% hematocrit and grown under normal culture conditions [1]. Parasites grew and ingested the host erythrocyte cytosol incorporated with FITC-dextran, depositing it in the DV, thereby selectively labeling DV with FITC-dextran.

Permeabilizing the Erythrocyte Plasma Membrane, Parasitophorous Vacuolar Membrane (PVM) and Parasite Plasma Membrane (PPM): The plasma membrane of the host erythrocyte and the parasitophorous vacuole of trophozoite stage parasites was initially permeabilized by brief exposure (~10 sec) to saponin (0.025% final concentration w/v sapogenin, Sigma) using a modified method described previously [1, 6]. Saponin-permeabilized parasites were washed and resuspended in endocytosis medium (RPMI 1640, 25 mM HEPES, 0.02 mg/mL gentamycin, 0.125% albumax, 0.4 mM hypoxanthine, pH 7.4) at 37 °C. Saponin-permeabilized erythrocytes were then suspended in 2 ml ice-cold intracellular saline A (5 mM HEPES, 2 mM $MgCl_2$, 30 mM NaCl, 110 mM KCl, pH 7.3) and kept for 5-10 min before adding digitonin (0.01% final concentration w/v, Sigma) to permeabilize the PPM. Erythrocytes were gently mixed and returned to ice for a further 2 min, after which 1 ml of ice-cold intracellular saline A containing 1 mg/mL bovine serum albumin (BSA) was added. Digitonin-permeabilized parasites were immediately centrifuged and washed twice (15,800 × g, 1 min) with 1 ml of BSA-containing intra-cellular saline A and once with intracellular saline A without BSA. Digitonin - permeabilized erythrocytes were re-suspended in intracellular saline A and kept at 37 °C until being used.

Measuring DV_{digi} pH: Digitonin-permeabilized parasites were suspended in 20 mM solution of different buffers (MES pH 5.5 and 6.0; sodium phosphate pH 6.5, 7.0, 7.5 and 8.0; Tris pH 9.0) containing 150 mM NaCl. To equilibrate the DV_{digi} pH to that of the buffer pH, carbonyl cyanide m-chlorophenylhydrazone (CCCP, 10 µM final concentration, Sigma) was added before analysis by flow cytometry. Data were analyzed to generate a pH calibration curve of FITC-dextran and measure the DV_{digi} pH of the parasites. Kinetic analysis of re-acidification of the DV_{digi} following ATP addition (2 mM final concentration, Sigma) in the ATP-depleted medium was also performed.

Flow Cytometry Analysis: A 488 nm argon ion laser of a six-color FACSCanto™ II flow cytometer (Becton Dickinson) was used as the excitation source for FITC-dextran. Green and yellow fluorescence signals were collected using 530 nm (30 nm bandpass) and 585 nm (42 nm bandpass) filters, respectively. Digitonin-permeabilized parasites were selected based on their scatter profiles (10 000 events per sample). The FITC-dextran-labelled DV_{digi} of the parasites was monitored using an additional gate based on the ratio (R_{gy}) of green (I_g) / yellow (I_y) fluorescence intensities of FITC-dextran [1]. FCS Express Version 3 (De Novo Software) and FlowJo (Tree Star, Inc.) were used to analyze the data.

Statistical Analysis: Data were analyzed using Student's paired t-test for statistical significance and $P<0.05$ was considered significant. Values are given as the mean of three independent measurements ± SEM.

RESULTS AND DISCUSSION:

Characterization of FITC-dextran as a DV_{digi} pH Indicator: The steady-state pH of the parasite's DV remains controversial although

methods to quantify the DV pH have been refined over time. For instance, several studies showed CQ-sensitive (CQS) parasites had the DV pH between 5.2 and 5.5 [1, 12]. Other studies reported the DV pH of CQ-resistant (CQR) parasites was slightly higher relative to CQS parasites [9, 10, 11]. While other studies found no differences in the DV pH between CQR and CQS parasites [12, 13, 14].

In seeking to redress this matter, a recent study used FITC-dextran to measure DV pH by flow cytometry using PPM-enclosing parasites isolated from their host erythrocytes by saponin permeabilization [1]. This flow cytometry technique permitted rapid and robust sample processing for quantitative pH analysis from larger populations of mature stage parasites in which the DV was filled with highly pH-sensitive, FITC-dextran [17]. Saponin treatment has been shown to permeabilize both the plasma membranes of the host erythrocyte and the PVM of the parasite [6, 18]. To minimize the damage of the membrane, the parasites were briefly exposed to saponin (~10 seconds) [1]. An intact PPM was observed in more than 98% of the saponin-isolated parasites as they did not concentrate the Trypan blue dye. This parasite preparation rendered the plasma membrane of the host erythrocyte permeable to solutes such as glucose while leaving the PPM and the DV_{sap} membrane intact, which were able to generate and sustain a substantial proton gradient. Similar results were also described previously [6]. Another permeabilizing agent, streptolysin O (SLO) has been shown to remove hemoglobin through the permeabilization of the plasma membrane of the host erythrocyte while leaving the PVM or PPM intact [19]. However, SLO preferentially lyses uninfected erythrocytes rather than infected erythrocytes. Equinatoxin II (EqtII), a model of α-PFT from sea anemone has also been reported to form pores in sphingomyelin-containing membranes of uninfected and infected erythrocytes but does not disrupt the PPM [20]. This limits the use of SLO and EqtII for obtaining a substantial insight into the DV_{SLO} pH or the DV_{EqtII} pH.

In saponin-permeabilized parasites, the PPM remained intact and tested compounds added to the extracellular medium had to traverse this membrane to gain access to the DV_{sap} [1]. To obtain a substantial understanding of the DV pH, FITC-dextran fluorescence from the DV_{digi} of parasites isolated by digitonin permeabilization was prepared and examined in the present study. The PPM of the parasite was permeabilized by digitonin to release any fluorescent marker from the parasite's cytosol. This exposed the surface of the DV_{digi} directly to the extracellular solution and allowed the parasite-associated FITC-dextran to be quantitated directly from the DV_{digi}. This is due to the ability of digitonin that can immediately interact with cholesterol-containing membranes hence inducing membrane leakage [21]. To minimize the damage of the DV_{digi} membrane, prolonged exposure of the parasites to digitonin was avoided.

The determination of the FITC-dextran fluorescence intensities for the parasite population at different pH values was carried out by suspending digitonin-permeabilized erythrocytes in buffers of different pH. An ionophore, CCCP was added to equilibrate all parasite compartments with the pH of the buffers. The population of digitonin-permeabilized erythrocytes was first identified based on the scatter profile and gated as "parasite scatter" **Fig. 2Ai**. This gate of the parasite-infected erythrocytes was established by comparing samples of non-labeled or FITC-dextran labeled uninfected cells (data not shown). Fluorescence peaks in green and yellow channels were displayed in the histograms of the parasite scatter-gated population of the digitonin-permeabilized erythrocytes **Fig. 2Aii-iii**. The higher intensity represents fluorescence from the intact FITC-dextran labeled DV_{digi} of the parasites, while the lower intensity might represent debris or parasites with non-labeled DV_{digi} that had infected non-resealed erythrocytes presence during the inoculation procedure. The peak of higher fluorescence from green and yellow channels was gated (double arrows) **Fig. 2Aii-iii** and the geometric mean of the fluorescence intensities in the green (I_g) and yellow (I_y) channels were used to calculate the ratio (R_{gy}), which background intensities were subtracted from each event. Based on the relationship between pH and R_{gy}, the calibration curve for FITC-dextran was constructed in **Fig. 2B**.

FITC-dextran arising from the DV_{digi} showed a characteristic sigmoidal response to pH changes similar to the FITC-dextran response obtained using the DV_{sap} previously investigated by Abu Bakar [1]. An increase in R_{gy} values with increasing

pH indicated the FITC-dextran acid-base transition with the inflection point corresponding to a pKa of ~5.8. Another information derived from the curve was the range of pH (~5.0-7.0) when the change in the FITC-dextran signal is linear. These results suggest that FITC-dextran can be used to measure DV pH (at pH values <6) that cannot be measured by SNARF-1-dextran, a ratiometric pH indicator with a pKa of >7.0 [15]. A similar pKa value was obtained from the pH calibration curve of FITC-dextran in saponin-permeabilized erythrocytes [1]. These results validate that the R_{gy} measurement can differentiate between FITC-dextran located in the DV_{digi} or DV_{sap} and that located in a neutral or alkaline environment.

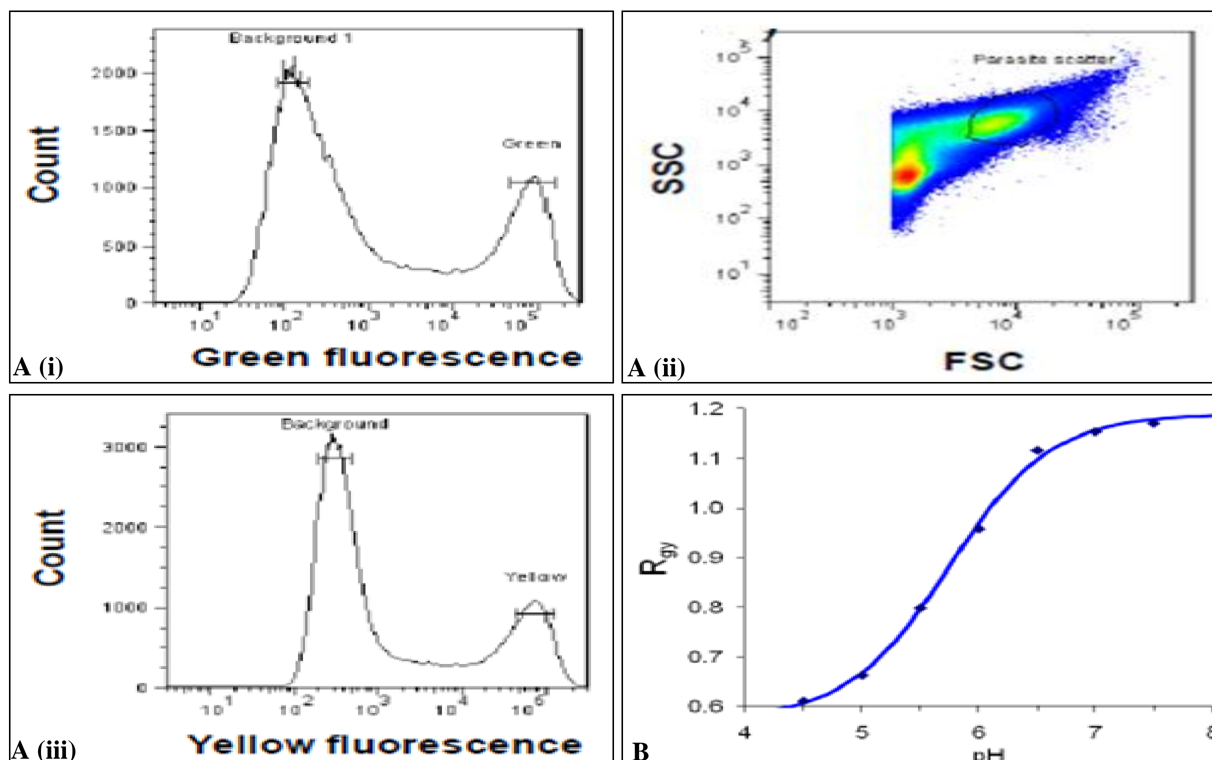


FIG. 2: CHARACTERIZATION OF FITC-DEXTRAN AS A pH INDICATOR IN DIGITONIN-PERMEABILIZED ERYTHROCYTES. (A) Digitonin-permeabilized erythrocytes in which the DV was preloaded with FITC-dextran were suspended in buffers of different pH (with CCCP) and analyzed by flow cytometry. (Ai) The parasitized population was gated as a 'parasite scatter.' (Aii) The green and (Aiii) yellow intensity distributions of the gated population are displayed in the histograms. (B) A pH calibration curve was generated using FITC-dextran in digitonin-permeabilized erythrocytes.

Measurement of the DV_{digi} pH: Fig. 3A shows normalized distributions of the R_{gy} values using digitonin-permeabilized erythrocytes suspended in CCCP-containing buffers of different pH. The DV_{digi} of CQS parasites (D10) suspended in a medium containing ATP (2 mM) showed a R_{gy} value of 0.85 ± 0.07 (n = 6, three experiments), which corresponds to a pH value of 5.66 ± 0.07 (**Fig. 3B**, purple curve). This pH value was 0.16 and 0.39 pH unit higher than that reported previously by Hayward *et al.*, (2006) (5.50 ± 0.14) [12] and Abu Bakar (2015) (5.27 ± 0.03) [1], respectively. The R_{gy} distribution of the DV_{digi} shifted to the right (**Fig. 3B**, red curve) when the CQS parasites were suspended in an ATP-depleted medium.

This indicated an alkalinization of the DV_{digi} (pH = 7.10 ± 0.10, R_{gy} = 1.15 ± 0.07) (n = 6, three experiments), but this effect was rapidly reversed within ~5 minu when ATP was restored to the medium (**Fig. 3B**, indicated by the horizontal arrow towards the left, purple curve). Treatments with specific V-type H^+-ATPase inhibitors such as concanamycin A and bafilomycin A1 caused a pronounced alkalinization of the permeabilized DV [6] suggesting a reduced concentration of ATP hence lacking the fuel required to pump H^+ ions both into the DV and out of the parasite [22]. Furthermore, the absence of glucose also contributes to the alkalinization of the DV. Saliba *et al.,* (2003) revealed the H^+ pump was dependent on glucose to sufficiently supply ATP [6].

According to Jida *et al*., (2016) the accumulation of Fluo-CQ, a pH-sensitive fluorescent chloroquine analog in the DV did not occur in the glucose-free medium. This was due to the lack of energy input to acidify the DV, which is necessary for the accumulation and trapping of Fluo-CQ in the DV. The acidification of DV was observed through the strong fluorescence signal of Fluo-CQ [23].

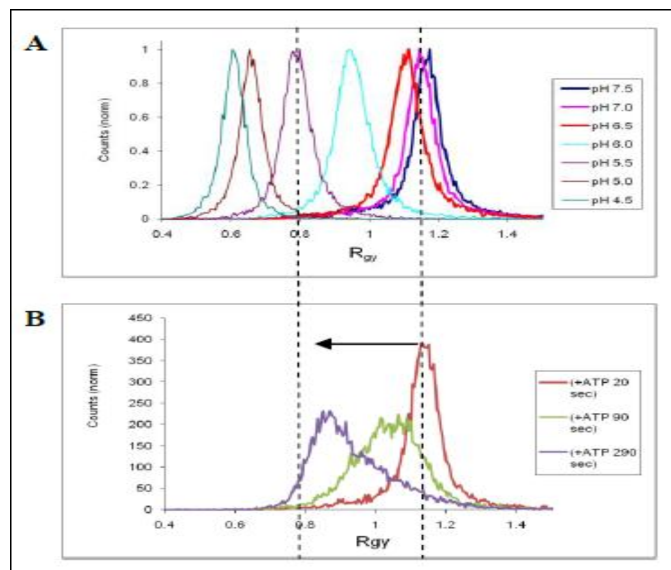


FIG. 3: (A) NORMALIZED DISTRIBUTION OF THE FITC-DEXTRAN FLUORESCENCE RATIOS (R_{gy}) OF THE DIGITONIN - PERMEABILIZED GATED CELL POPULATIONS SUSPENDED IN BUFFERS OF DIFFERENT pH (WITH CCCP). A SHIFT OF THE R_{gy} DISTRIBUTION WITH pH WAS OBSERVED. THE VERTICAL DASHED LINES CORRESPOND TO pH 5.5 (LEFT) AND 7.0 (RIGHT). (B) THE ALKALIZATION OF THE DV IN NO ATP-CONTAINING MEDIUM AND THE RE-ACIDIFICATION OF THE DV UPON THE RESTORATION OF ATP (2 mm) WERE OBSERVED AS SHOWN BY THE SHIFT OF THE R_{gy} DISTRIBUTION OF THE CELL POPULATION TO THE LEFT (INDICATED BY THE ARROW).

Because FITC-dextran is a ratiometric pH probe, determinations are therefore independent of the concentration of the probe, the effect of bleaching and the shift of focus during measurements [12, 14]. Thus, differences in fluorescence intensities should not affect pH determinations. The apparent variations in the DV pH values might be due to the use of cells undergoing different treatment conditions to elucidate a substantial understanding of the DV pH [1, 12, 14]. In the course of the present study, the optimal level of FITC-dextran (50 µM) was used during the loading step. This is because overloading the probe into the host erythrocyte might result in the concentration of the probe being too close to the DV's buffering capacity [6, 12]. One may argue that the invasive protocol used in the present study could affect the steady-state DV pH value compared to other non-invasive methods such as the use of *P. falciparum* expressing a pH-sensitive green fluorescent protein (GFP) in the DV [13, 14]. However, we did not observe significant differences in the DV pH values of the digitonin-permeabilized FITC-dextran-loaded parasites concerning the transfected parasites expressing pHluorin [14] or chimeras of plasmepsin II [13]. Meanwhile, the measured average of cytosolic pH in *P. falciparum* trophozoites was 7.16 ± 0.18 using the pHluorin [24] that is approximately the same in the parasites perfused with fluorescent pH indicator, 2', 7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) [10] indicates that both invasive and non-invasive methods do not significantly affect the pH regulation between DV and cytosolic parasites.

In the present study, the CQR parasite (Dd2) was also used in an attempt to compare the DV pH value with that of the CQS parasite (D10). When CQR parasites were suspended in an ATP-containing medium, the DV_{digi} showed a pH value of 5.62 ± 0.12 (n = 6, three experiments, data not shown). This DV pH of CQR parasites was not significantly different from that of the DV_{digi} pH value of CQS parasites (P > 0.3), consistent with the pH determinations made by other studies [12, 13, 14]. The sensitivity of the flow cytometry to quantify the fluorescence intensity of single cells makes this technique applicable to measure pH of parasite intracellular compartments such as the DV [1, 13]. Using a conventional spectrofluorometer, measurement of such populations is not easy to perform due to the hemoglobin absorption and the light scattering generated by cells [6, 12]. Single-cell live imaging by confocal microscopy has the advantage of allowing a direct pH estimation of specific parasite compartments [13, 14, 15], but generating statistics of identified populations is tedious and time-consuming.

CONCLUSION: Overall, the present study validates the use of FITC-dextran as a probe suitable for quantitative pH measurements under the conditions employed using either digitonin- or saponin-isolated parasites. The DV_{digi} and DV_{sap} showed similar behaviors and pKa values of FITC-dextran (~5.8). No difference was also observed in the DV pH of CQS and CQR parasites.

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CONFLICT OF INTEREST: The authors declare no conflict of interest.

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