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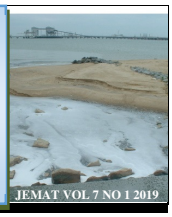

The Application of Crude Ginger Protease as an Inhibitive Assay for Heavy Metals

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ABSTRACT

In this work, a novel source of protease for the bioassay of heavy metals has been developed using proteases extracted from ginger. The result shows that the optimum protease activity was reached at 1 mg/mL protein concentration of the ginger protease. The optimum casein concentration toward crude ginger protease activity was 1.75 mg/mL. The most suitable pH for protease from crude ginger protease was within the range from pH 5.0 to 7.0. The proteases exhibited high protease activity in a broad range of temperature from 20 to 60 ºC. The optimum incubation time for the enzyme occurred at minute 30. Among the six heavy metals tested, only three heavy metals inhibited proteolytic activity of ginger crude with an inhibition more than 30% at 1 mg/L. The calculated LC_{50} for mercury, copper and silver were, 0.182, (95%, confidence interval (C.I.) 0.134 to 0.283), 0.071, (95% C.I. of 0.056 to 0.096), 0.054, (95% C.I. of 0.039 to 0.085), respectively. Data on the sensitivity of various proteases to heavy metals shows that the crude ginger protease is comparable in sensitivity for mercury to the achromopeptidase, bromelain, papain assays. The crude ginger protease assay for copper is comparable in sensitivity to the papain assay and appears to be more sensitive than the rest of the assays. For silver, the ginger protease was the most sensitive while other assay methods are either unable or be able to detect higher concentration of silver. The crude proteases extracted from ginger showed a good potential for the development of a rapid, sensitive, and economic inhibitive assay for the biomonitoring of mercury, copper, and silver in the environment.

INTRODUCTION

Water is amongst the most significant resources to man following air. The need of water to maintain everyday life is a significant issue because so many rivers these days are increasingly being contaminated with untreated waste materials from commercial and farming activities [1]. In Malaysia, as confirmed from recent surveys [2, 3, 4], water quality is getting close to to hazardous level as a result of intensely developed activities. Presently, commercial wastes are widespread in the makeup of the waste waters from massive industrial metropolitan areas causing the escalation of heavy metals which could eventually become released to the aquatic surroundings [5]. In line with Malaysian Department of Environment, these dangerous heavy metals play a role in extreme water quality issues in Malaysia [6].

The significance in overseeing and avoiding the continuing metals contamination is the vital thing to protect general public wellbeing. Heavy metal contamination have already been recognized to result in ill-effects to humans including

haematotoxic, neurotoxic and nephratoxic and every one of these illnesses are nearly untreatable as a result of permanent impact to biological systems [1]. As chronic polluters, heavy metals also build up in the human body leading to long term damage to tissues [2]. Hence, the buildup of toxic heavy metals in rivers have enticed researchers to create many strategies with contemplation on inexpensive, rapid and stability as an instrument to identify and monitor the existence of heavy metals in the environment [3–11].

To date various enzyme-based assays for heavy metal detections have been developed including molybdenum-reducing enzyme [12,13], urease [3] and proteases such as bromelain [14], papain [15], trypsin [11] coriander [16] and garlic [17]. Urease assay is regarded as the most sensitive assay to detect mercury contamination nevertheless its dual-sensitivity towards environmental ammonia present a challenge for its addition as an recognized method for the monitoring of heavy metals by health agencies globally [3]. Another sensitive assay for mercury is the Microtox© assay.

This assay is conducted at 15°C which prevent sites monitoring in countries such as Malaysia [3]. Newer bioassays using plant proteases such as papain, bromelain, Coriander protease and garlic protease exhibit broad ranges of pH and temperature stabilities. This makes the assays tolerant to fluctuation in sample pH and assay temperature. Nevertheless, for certain heavy metals, these assays cannot detect heavy metals at the level required by the Department of Environmental (DOE), Malaysia. Henceforth, there is a crucial need for screening of more sensitive proteases.

MATERIALS AND METHOD

Preparation of buffers

Citrate phosphate buffer from pH 5.0 to 6.0, sodium phosphate buffer from pH 6.0 to pH 7.5 and Tris buffer from pH 7.2 to pH 8.0. were prepared according to the methods of Dawson [18]. The final pH minor adjustment was carried out using NaOH (1 M) or HCl (1 M).

Preparation of dye-binding Bradford reagent

About 0.1 g of Coomassie Brilliant Blue G-250 from SIGMA was dissolved in a mixture of 50 mL of 95% ethanol and 100 mL of phosphoric acid of 85%. The dye was dissolved completely and stirred overnight and the volume was added up to 1 L. The solution was filtered through Whatman Filter Paper No. 1 and stored in a dark bottle. Alternatively, a commercial Bradford reagent from BIO-RAD was used according to manufacturer's recommendation.

Preparation of casein solution

Into 100 mL of deionized water, two grams of casein (SIGMA) was dissolved and the pH adjusted to pH 8.0 and stirred overnight at 60ºC. The casein stock solution was centrifuged at 10,000×g for 15 min. The protein concentration of casein in the clear supernatant was quantified using the Bradford dye-binding assay using crystalline BSA (SIGMA) as the standard. Casein (0.3 mg mL^{-1}) working solutions were prepared fresh daily.

Preparation of crude proteases from ginger

Fresh ginger was obtained from a local market. Fifty grams of ginger was soaked in 100 mL of 20 mM phosphate buffer pH 6.5 in a chiller at 4 ºC and homogenized by using an ice-cold steel blender in the same buffer. The supernatant (juice fraction) was recovered and centrifuged at 10,000 x g for 10 min. The clear supernatant was then filtered with a syringe filter (0.2 µm, cellulose acetate, Sartorius). The crude extract was then kept on ice at 0 ºC until further use.

Preparation of heavy metals solutions

Working solutions of heavy metals such as mercury (II), arsenic (v), cadmium (II), lead (II), copper (II), cobalt (II), iron (II), chromium (VI) and silver (I) sourced from MERCK (Merck, Darmstadt, Germany) Atomic Absorption Spectrometry standard were prepared fresh at concentrations from 0.1 to 10 mg/L and stored in acid-washed polypropylene containers.

Optimization of ginger protease activity

Various concentrations of ginger protease (0–0.5 mg/L) were tested by mixing suitable volumes with 50 µl of 100 mM phosphate buffer pH 6.5 and made up to 100 µl using deionized water. The mixture was incubated at room temperature for 20 min. Then 50 µl of casein working solution was added and mixed thoroughly. At time zero, a 20µl aliquot was withdrawn and mixed with Bradford dye-binding reagent (200 µl) in a microplate well and incubated for 5 min. The absorbance at 595 nm was then taken using a microplate reader (Stat Fax® 3200 Microplate Reader, Awareness Technology Inc., USA).

The remaining solution was incubated at room temperature for 30 min. Then, a 20 µl aliquot was taken and mixed with the Bradford assay and the absorbance taken as before. The optimum concentration of the substrate casein, crude concentration in the reaction mixture was fixed at 0.1 mg/Lwhilst for temperature and pH optimization studies, casein and crude concentrations were fixed at 0.3 mg/L and 0.1 mg/L respectively. One unit of activity is defined as the amount of casein (in mg) hydrolyzed per minute by 1 mg of protease under the specified assay conditions [19].

Inhibitive assay for heavy metals

The assay was conducted according to method of Shukor et al., [15]. The reaction mixture has a final volume of 200 µl. In an Eppendorf tube, to 120 µl of 50 mM phosphate buffer pH 6.0, 50 µl of enzyme was added followed by the addition of 20 µl of heavy metals. The heavy metals were replaced with deionized water in the control experiment. The final volume was made up using deionized water. This mixture was incubated at room temperature for 20 min. Then 30 µl of casein (0.3 mg/L stock solution) was added and thoroughly mixed. A 20 µl aliquot was withdrawn and then mixed in a microplate well with 200 µl of Bradford dye-binding reagent.

The mixture was incubated for 5 min for the time zero absorbance. After 60 min the remaining mixture was treated the same manner to the aliquot at time zero to obtain the final absorbance. The values for the Limits of Detection (LOD) and IC_{50} (inhibitory concentration, 50%), was calculated according to the method of Shukor et al [15] with the values generated using the PRISM (Prism version 5.00 for Windows) non-linear regression analysis software available from GraphPad, (GraphPad Software Inc., San Diego, CA). Means and standard errors were determined according to at least three independent experimental replicates.

RESULT AND DISCUSSION

Optimization of crude ginger concentration

To maximize the difference between initial and final absorbances, the optimum proteases activity was carried out by varying crude ginger concentration. The result shows that the optimum protease activity was reached at 1 mg/mL protein concentration of the ginger protease (**Fig 1**) which is very similar

to the optimum concentration of various proteases previously published [6,8,11,12,15–17]. Aside from the Bradford assay, the crude is usually assayed utilizing a number of techniques for example Lowry and Bichinconic acid with casein or azocasein as the substrate. In terms of straightforwardness, rapidity and stability, the Bradford dye binding assay demonstrated several advantages and appropriateness to this kind of inhibitive assay system in comparison to the other techniques mentioned. Applying this assay system, the activity of the crude follows in accordance to the capacity for the crude enzyme to hydrolyse casein to polypeptide with molecular weights of less than 2 kDa and at this size and smaller, the Bradford dye binding reagent will not be able to stain the polypeptide and the color stay brown. Too little crude enzyme will result in too little activity and the color remains brown.

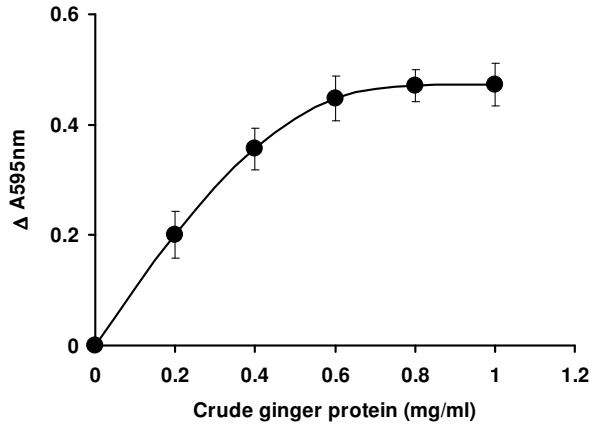

Fig 1. The effect of enzyme concentration on the activity of proteases from ginger. Data was mean ± standard deviation of the mean (n=3).

The effect of different concentration of casein toward crude ginger protease activity is presented in **Fig 2**. The optimized concentration was 1.75 mg/ml and above for the optimum protease activity while other proteases showed a much lesser requirement for casein at less than 0.5 mg/mL [6,8,11,12,15–17]. The optimized concentration should allow sufficient absorbance difference to be observed.

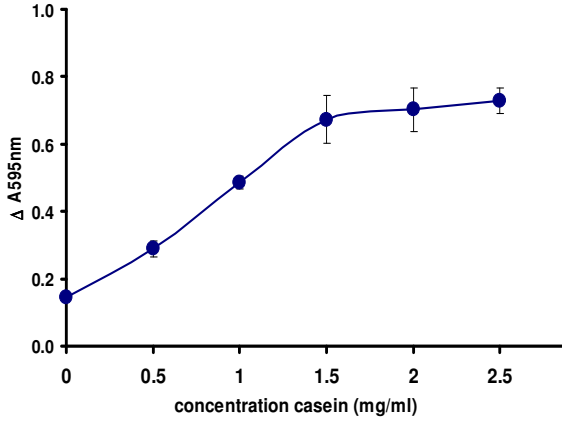

Fig 2. The effect of substrate casein toward proteases activity. Data was mean ± standard deviation of the mean (n=3).

The most suitable pH for protease from crude ginger protease was within the range from pH 5.0 to 7.0 as analyzed by ANOVA (**Fig 3**). Therefore, pH 6.0 of sodium phosphate buffer was selected through the entire experiment because the use of buffers having citrate species can behave as heavy metal chelating agent and avoid heavy metals from binding to the active sites of the enzyme. The catalytic activity of an enzyme is pH and temperature-sensitive given that the intermolecular bonds that keep proteins intact at their secondary and tertiary structures will be negatively affected by alterations in these factors. Essentially the most ideal pH value is the point in which the enzyme is in its most active (optimum pH).

Having a broad range for pH activity is advantageous in the course of field works, since strong variation in the sample's pH will not affect the activity of the enzyme and giving false positive signals as if the sample contains metal ions when in fact it did not. Therefore, combination of a broad range for optimum activity and the presence of a good buffer like phosphate will be very effective in the effective use of the bioassay system to prevent the pH of the samples from affecting the enzyme activity [6,8,11,12,15–17].

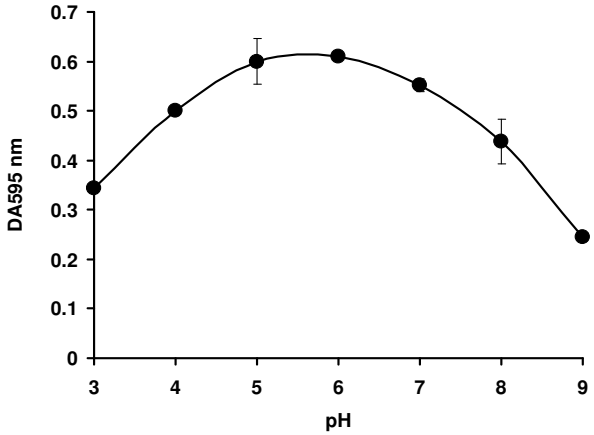

Fig 3. Effect of pH on the activity of proteases from ginger. Data was mean ± standard deviation (n=3).

The proteases exhibited high protease activity in a broad range of temperature from 20 to 60 ºC (**Fig 4**). Higher temperatures can cause denaturation of the tertiary and quaternary protein structure leading to loss of activity through thermal vibration and accelerated oxidation of sulfhydryl groups. Protease activity with a broad temperature range will be advantageous for field site trial monitoring in hot-climate countries and the optimized temperature was comparable with the other proteases assessed previously [6,8,11,12,15–17].

Temperature stability and optimum temperature for activity is leading problem for on-site biomonitoring using several commercial toxicity bioassay kits for examples Microtox™ based on *Vibrio fischery* which works strictly at 15ºC [3]. The stability of protease at increased temperatures will make this bioassay system inexpensive and straightforward with no use of costly refrigerated system.

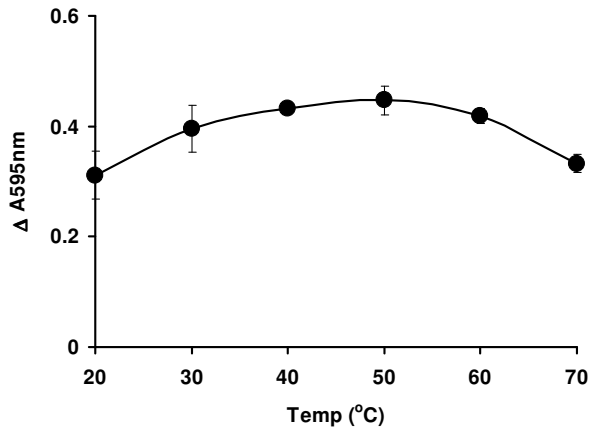

Fig 4. Effect of temperature on the activity of proteases from ginger. Data was mean± standard deviation (n=3).

The optimum incubation time for this enzyme occurred at minute 30 at least (**Fig 5**) which is shorter compared to other proteases previously reported that range from 45 to 90 min [6,8,11,12,15–17]. Hence, this assay is considered as a fast bioassay because the result can be obtained in less than an hour including incubation of the enzyme with heavy metals. As the purity of the crude is low, the removal of other contaminating proteins in the crude extract can probably improve the assay time further as exemplified in several previous studies [20,21].

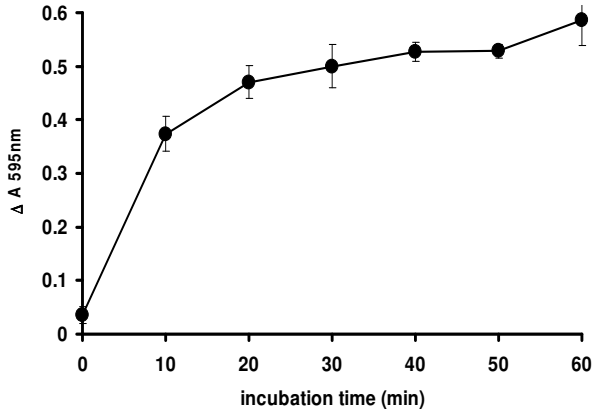

Fig 5. Effect of incubation time on activity of protease from ginger. Data was mean ± standard deviation (n=3).

Inhibition of proteases by heavy metals

Among the six heavy metals tested, only three heavy metals inhibited proteolytic activity of ginger crude with an inhibition more than 30% at 1 mg/L (**Fig 6**). The sensitivity of proteases toward mercury, copper and silver was determined based on the determination of LOD and the IC_{50}. The IC_{50} values for each of the heavy metals are summarized in **Table 1**. The corresponding correlation coefficient values of 0.95 above in all of the curves obtained suggesting a good relationship of data with theoretical model calculations.

The calculated LOD values for mercury was not able to detect mercury at the Maximum Permissible Limit for mercury in drinking water at 0.005 mg/L while the MPL level for both copper and silver are well higher than the LOD for these heavy metals suggesting the assay can be utilized to detect elevated levels of heavy metals at the concentration required by the DOE.

Data on the sensitivity of various proteases to heavy metals (**Table 2**) shows that the crude ginger protease is comparable in sensitivity for mercury to the achromopeptidase, bromelain, papain assays based on the overlap confidence interval [22] and is more sensitive to the rest of the assays. The crude ginger protease assay for copper is comparable in sensitivity to the papain assay and appears to be more sensitive than the rest of the assays. For silver, the ginger protease was the most sensitive while other assay methods are either unable or be able to detect higher concentration of silver.

Table 1. The summary of the IC_{50}, LOD and LOQ values for the three heavy metals that inhibits proteases from Crude ginger protease.

Heavy metals	R^2	IC_{50} (95% Confidence Interval)	LOD (mg/L)
Hg	0.9801	0.182, (0.134 to 0.283)	0.045, (0.021 to 0.102)
Cu	0.9933	0.071, (0.056 to 0.096)	0.006, (0.001 to 0.010)
Ag	0.9819	0.054, (0.039 to 0.085)	0.05, (0.01 to 0.012)

During inhibition of proteases by heavy metals, the substrate casein is not or partially degraded and this allows the resulting protein stainable by the Bradford reagent to form a blue solution [15]. The Bradford dye binds to the basic (arginine) and aromatic amino acids residues of protein in general [19]. It has been reported that the toxic effect of metal ions on enzymes was usually due to their binding to thiol groups present in or near the center of active sites, producing an irreversible inhibition. Hence, it can be assumed that the presence of metal ion inhibits the activity of the enzyme by binding to the cysteine group of the enzyme at the active site causing alteration of enzyme's structure and enzyme inactivation occurs as a result [19]. Consequently, crude ginger protease has the potential to be used in biomonitoring of heavy metals in the environment.

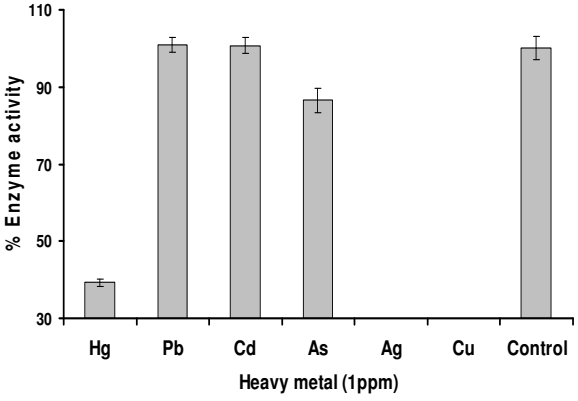

Fig 6. Effect of heavy metals on proteases activities. Data is mean ± standard deviation of mean (n=3).

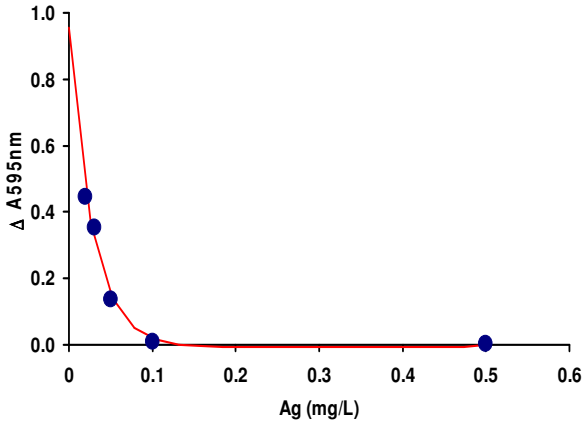

Fig 7. Nonlinear regression of the inhibition of crude ginger protease by silver using the one-phase exponential decay model. Data is mean ± standard deviation of mean (n=3).

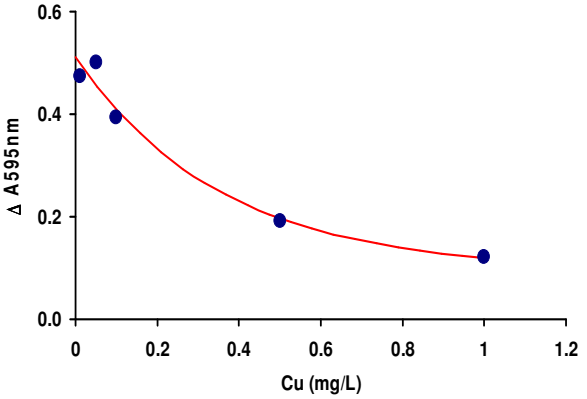

Fig 8. Nonlinear regression of the inhibition of crude ginger protease by silver using the one-phase exponential decay model. Data is mean ± standard deviation of mean (n=3).

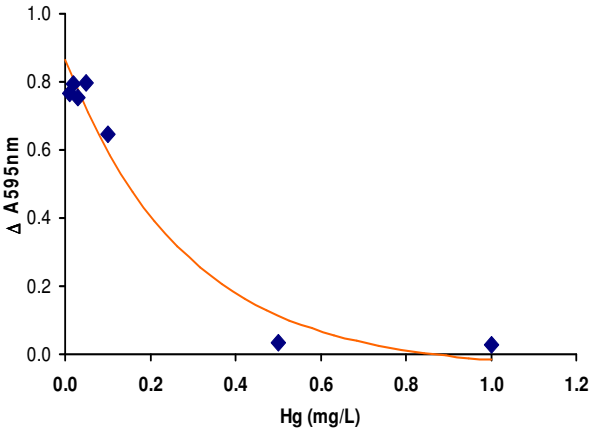

Fig 9. Nonlinear regression of the inhibition of crude ginger protease by silver using the one-phase exponential decay model. Data is mean ± standard deviation of mean (n=3).

Table 2. The summary of the IC_{50} value in comparison to other protease assays.

Heavy Metals	Crude ginger protease	Crude coriander [16]	Crude tomato [8]	Achromo-peptidase [7]	Trypsin [6]	Bromelain [20]	Papain [21]
Hg	0.134 to 0.283	3.21	-	-0.163 to 1.283	0.51 to 0.74	0.13 to 0.16	0.16 to 0.20
Cu	0.056 to 0.096	-	-	1.729 to 14.551	-	0.16 to 0.30	0.004 (LOQ)
Ag	0.039 to 0.085	-	-	-	-	-	0.21 to 0.29
Zn	-	0.727	1.407	3.131 to 7.089	0.05 to 0.07	-	1.51 to 2.63
Ni	-	-	-	-	-	-	-
Cd	-	-		4.697 to 7.622	-	-	0.1 (LOQ)
Cr	-	-	0.835	4.347 to 10.113	-		
Pb	-	-	0.707	17.534 to 27.146	-	-	0.89 to 1.23

CONCLUSIONS

In this work, a novel source of protease for the bioassay of heavy metals has been developed using proteases extracted from ginger. The result shows that the optimum protease activities were similar to previously published results using plant proteases. Among the six heavy metals tested, only three heavy metals inhibited proteolytic activity of ginger crude with an inhibition more than 30% at 1 mg/L. Data on the sensitivity of various proteases to heavy metals shows that the crude ginger protease is comparable in sensitivity for mercury to the achromopeptidase, bromelain, papain assays. The crude ginger protease assay for copper is comparable in sensitivity to the papain assay and appears to be more sensitive than the rest of the assays. For silver, the ginger protease was the most sensitive while other assay methods are either unable or be able to detect higher concentration of silver. The crude proteases extracted from ginger showed a good potential for the development of a rapid, sensitive, and economic inhibitive assay for the biomonitoring of mercury, copper, and silver in the environment. This assay has been shown to be simple, reproducible and rapid with good sensitivity.

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