Preclinical Evaluation of Caprylic Acid-Fractionated IgG Antivenom for the Treatment of Taipan (Oxyuranus scutellatus) Envenoming in Papua New Guinea

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Abstract

Background: Snake bite is a common medical emergency in Papua New Guinea (PNG). The taipan, Oxyuranus scutellatus, inflicts a large number of bites that, in the absence of antivenom therapy, result in high mortality. Parenteral administration of antivenoms manufactured in Australia is the current treatment of choice for these envenomings. However, the price of these products is high and has increased over the last 25 years; consequently the country can no longer afford all the antivenom it needs. This situation prompted an international collaborative project aimed at generating a new, low-cost antivenom against O. scutellatus for PNG.

Methodology/Principal Findings: A new monospecific equine whole IgG antivenom, obtained by caprylic acid fractionation of plasma, was prepared by immunising horses with the venom of O. scutellatus from PNG. This antivenom was compared with the currently used F(ab')² monospecific taipan antivenom manufactured by CSL Limited, Australia. The comparison included physicochemical properties and the preclinical assessment of the neutralisation of lethal neurotoxicity and the myotoxic, coagulant and phospholipase A₂ activities of the venom of O. scutellatus from PNG. The F(ab')² antivenom had a higher protein concentration than whole IgG antivenom. Both antivenoms effectively neutralised, and had similar potency, against the lethal neurotoxic effect (both by intraperitoneal and intravenous routes of injection), myotoxicity, and phospholipase A₂ activity of O. scutellatus venom. However, the whole IgG antivenom showed a higher potency than the F(ab')² antivenom in the neutralisation of the coagulant activity of O. scutellatus venom from PNG.

Conclusions/Significance: The new whole IgG taipan antivenom described in this study compares favourably with the currently used F(ab')² antivenom, both in terms of physicochemical characteristics and neutralising potency. Therefore, it should be considered as a promising low-cost candidate for the treatment of envenomings by O. scutellatus in PNG, and is ready to be tested in clinical trials.

Citation: Vargas M, Segura A, Herrera M, Villalta M, Estrada R, et al. (2011) Preclinical Evaluation of Caprylic Acid-Fractionated IgG Antivenom for the Treatment of Taipan (Oxyuranus scutellatus) Envenoming in Papua New Guinea. PLoS Negl Trop Dis 5(5): e1144. doi:10.1371/journal.pntd.0001144

Editor: David G. Lalloo, Liverpool School of Tropical Medicine, United Kingdom

Received October 15, 2010; Accepted February 27, 2011; Published May 17, 2011

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Funding: This study was supported by Viceministerio de Investigación, Universidad de Costa Rica (project 741-A9-003); the PNG Office of Higher Education, CTP Limited (Milne Bay Estates), and the Australian Venom Research Unit (University of Melbourne), which is funded by the Australian Government Department of Health and Ageing, the Australia Pacific Science Foundation and Snowy Nominees. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Mariángela Vargas, Alvaro Segura, María Herrera, Mauren Villalta, Ricardo Estrada, Maykel Cerdas, Guillermo León and José María Gutiérrez work at Instituto Clodomiro Picado, Universidad de Costa Rica, where one of the antivenoms tested in this study was produced.

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Introduction

Envenoming by snake bite is a common medical emergency in Papua New Guinea (PNG) [1–3]. Despite incomplete epidemiological data, studies in PNG show that the incidence of snake bite ranges from under five cases per 100,000 people per year in the mountains of Goilala and Hiri (Central Province) and in Madang, to 526–561 cases per 100,000 people per year in the coastal Kairuku lowlands [1,2,4]. A mortality rate of 7.9 deaths per 100,000 people per year in Central Province was reported for the period 1987–1992 [2]. At Port Moresby General Hospital (PMGH) only envenomed snakebite patients are admitted, and most of these are sent to the Intensive Care Unit (ICU). A study of snakebite admissions to the PMGH ICU between 1992 and 2001 revealed case fatality rates of 8.2% for adults and 14.6% for children [5]. More recently, case fatality rates of 14.5% for adults and 23.9% for children have been reported from the ICU of the same hospital [3].

Throughout PNG three species of elapid snakes are responsible for nearly all systemic envenomings: Acanthophis laevis (smooth-scaled death adder), Micropechis ikaheka (New Guinea small-eyed snake), and Oxyuranus scutellatus (Papuan taipan). A very small
**Author Summary**

Snake bite envenomings represent an important public health hazard in Papua New Guinea (PNG). In the southern lowlands of the country the majority of envenomings are inflicted by the taipan, Oxyuranus scutellatus. The only currently effective treatment for these envenomings is the administration of antivenoms manufactured in Australia. However, the price of these products in PNG is very high and has steadily increased over the last 25 years, leading to chronic antivenom shortages in this country. As a response to this situation, an international partnership between PNG, Australia and Costa Rica was initiated, with the aim of generating a new, low-cost antivenom for the treatment of PNG taipan envenomings. Horses were immunised with the venom of *O. scutellatus* from PNG and whole IgG was purified from the plasma of these animals by caprylic acid precipitation of non-immunoglobulin proteins. The new antivenom, manufactured by Instituto Clodomiro Picado (Costa Rica), was compared with the currently available F(ab’)_2 antivenom manufactured by CSL Limited (Australia). Both were effective in the neutralisation of the most relevant toxic effects induced by this venom, although the whole IgG antivenom showed a higher efficacy than the F(ab’)_2 antivenom in the neutralisation of the coagulant activity.

number of envenomings are caused by other *Acanthophis* species, Pseudechis papuanus (Papuan blacksnake) and Pseudonaja textilis (New Guinea brownsnake) [3]. For many years the Papuan taipan has been regarded as a separate subspecies (*Oxyuranus s. cannii*) to Australian populations (*Oxyuranus s. scutellatus*). However, recent taxonomic and biogeographical studies have shown that, despite some perceived morphological differences, molecular genetic analysis reveals no significant differentiation between the two populations [6,7]. On this basis, *O. scutellatus* is now considered a single species with both Australian and New Guinean populations. In southern PNG and neighbouring southern Papua, up to 95% of life-threatening snake bites are caused by *O. scutellatus* (Fig 1). The effects of taipan bite include mild local effects and severe systemic manifestations characterised by coagulopathy with spontaneous haemorrhage, myotoxicity, irreversible flaccid paralysis, acute kidney injury and cardiac disturbances [2,3,8–10]. The neurotoxic manifestations of taipan bite are dominated by the effects of extremely potent, destructive, presynaptic phospholipase A2 toxins, resulting in physical damage to nerve terminals [11,12]. Only the early (within 4-6 hours) administration of suitable antivenom can prevent or reduce this presynaptic damage; consequently, when treatment is delayed, severe paralysis occurs, requiring endotracheal intubation and mechanical ventilation until neuromuscular synapses have regenerated [2,13].

Intravenous administration of either taipan monospecific antivenom or polyvalent antivenom prepared in Australia by CSL Limited (CSL), Melbourne, Victoria, Australia (batch B0548-06301; expiry date March 2012),

- (a) Monospecific taipan antivenom manufactured by CSL Limited (CSL), Melbourne, Victoria, Australia (batch B0548-06301; expiry date March 2012),
- (b) Monospecific taipan antivenom manufactured by Instituto Clodomiro Picado (ICP) (batch 4511209 ICP; expiry date November 2012).

**Antivenom Production**

CSL monospecific taipan antivenom is prepared from the plasma of horses immunised with the venom of *O. scutellatus* from Australia. It is manufactured using a protocol based on pepsin digestion and ammonium sulphate fractionation of plasma, and therefore consists of F(ab’)_2 fragments [26]. ICP monospecific taipan antivenom is raised in horses immunised with the venom of *O. scutellatus* from PNG. Immunisation was performed in a group of three horses which had not previously been used for antibody production, by using Freund’s complete and incomplete adjuvants during the first two injections, respectively, followed by subsequent inoculations of venom dissolved in PBS. All injections were performed by the subcutaneous route in a single anatomical site.
When a satisfactory neutralising titre was reached, the animals were bled from the jugular vein, with the blood being collected in 6 L plastic bags containing sodium citrate as anticoagulant. After sedimentation of blood cells, the plasma was separated and the immunoglobulins purified by caprylic acid precipitation (5% final concentration of caprylic acid and one hr stirring) [19]. After filtration in 8 μm pore filter paper, the filtrate was diafiltered and then formulated to contain 7.5 g/L NaCl, 1.6 g/L phenol, pH 7.2
The antivenom solution was sterile filtered using 0.22 μm pore membranes and glass vials were filled with 40 mL of antivenom. The resultant antivenom met all the requirements of the quality control protocol at Instituto Clodomiro Picado.

Physicochemical analysis of antivenoms

Total protein concentration was determined by the Biuret test [27], and electrophoretic analysis was performed by SDS-PAGE, under non-reducing conditions, using an acrylamide concentration of 7.5% [28]. Gels were stained with Coomassie Brilliant Blue R-250. Phenol concentration was determined according to a modification of the method of Lacoste et al. [29]. Caprylic acid concentration was quantified by HPLC according to Herrera et al. [30]. The content of antibody monomers was assessed by FPLC gel filtration in a Superdex 200 10/300 GL column using 0.15 M NaCl, 20 mM Tris, pH 7.5, as eluent. Turbidity was determined using a turbidimeter (La Motte, model 2020, Chesterton, MD), and expressed as nephelometric turbidity units (NTU).

Table 1. Physicochemical characteristics of antivenoms.

| Characteristic | ICP IgG antivenom | CSL F(ab')2 antivenom |
|---------------|-------------------|-----------------------|
| Protein (g/L) | 45.9±0.9          | 144.6±0.4             |
| Phenol (g/L)  | 1.6±0.04          | 2.12±0.03             |
| pH            | 7.2               | 6.4                   |
| Caprylic acid (mg/L) | 31.1±1 | –                     |
| Monomer content (%) | 93±1.0 | –                     |
| Turbidity (NTU) | 25                | 23                    |

Variation of venom activities upon incubation

Pre-incubation of PNG O. scutellatus venom at 37°C for 30 min resulted in a significant loss of coagulant activity. Thus, it was considered that degradation of the procoagulant toxins may be occurring. On this basis, all of the venom activities assessed in this study (LD50, MCC, MMD and PLA2) were determined for venom solutions (a) immediately after dissolution in PBS and (b) after 30 min of incubation at 37°C. Lethality (LD50) by both i.p. and i.v.
routes, myotoxicity (MMD) and PLA₂ activity were not affected by incubation at 37°C for 30 min. In contrast, the coagulant activity of O. scutellatus venom was reduced upon incubation at 37°C for 30 min. We therefore altered the experimental design of the ED-MCC assay by limiting incubation of venom and antivenom to not more than 3 minutes, in order to avoid the loss of activity associated with incubation.

Statistical analysis

All descriptive statistic calculations and the Mann Whitney U test used to determine the significance of the differences between the median values of two non-parametric experimental groups in the neutralisation tests were performed using the InStat statistics program.

Results

Characteristics of antivenoms

The physicochemical characteristics of the two antivenoms are summarised in Table 1. The CSL F(ab')₂ antivenom had a 3.15 fold higher total protein concentration than ICP whole IgG antivenom. When examined using SDS-PAGE run under non-reducing conditions, the CSL antivenom, manufactured by pepsin digestion and ammonium sulphate fractionation, presented predominant bands in a molecular mass range corresponding to 90% IgG or F(ab')₂ monomers, as judged by gel filtration analysis (Table 1; Fig 3), thus showing a very low protein aggregate profile of antivenoms

Toxic and enzymatic activities of venom and neutralising profile of antivenoms

Toxic and enzymatic activities of O. scutellatus venom. The venom of O. scutellatus is highly toxic for CD-1 mice, with LD₅₀ values of 0.04±0.01 μg/mouse (2.3±0.6 μg/kg) and 0.08±0.01 μg/mouse (4.2±0.5 μg/kg) for the i.p. and the i.v. routes, respectively. Lethality was associated with neurotoxicity, as mice showed evidence of limb and respiratory paralysis. Venom also caused myotoxicity in mice, with a MMD of 1 μg per mouse, corresponding to the dose that increased 4 times the plasma CK activity of 187±6 U/L. Mice receiving doses higher than 1 μg died before 3 hr. O. scutellatus venom induced coagulation of human plasma, with higher activity in conditions in which CaCl₂ was added to citrated plasma before the addition of the venom, as previously reported [35,36]. Without addition of calcium, the MCC was 0.76±0.20 μg/mL, whereas when CaCl₂ was added to plasma immediately before the venom, the MCC was 0.33±0.13 μg/mL (p<0.05). The PLA₂ activity of this venom corresponds to 297±7 μEq fatty acid released per mg protein per min (Table 2). Neutralisation by antivenoms. The two antivenoms were effective at neutralising the four activities tested. No significant differences were observed in the ED₅₀ of either product against the lethal and PLA₂ activities of PNG O. scutellatus venom as well as in the ED against myotoxic effect (Table 2). In contrast with lethal, PLA₂ and myotoxic activities, there was a significant difference in the value of ED against in vitro coagulant activity of the venom of O. scutellatus from PNG, with the ICP antivenom having 5.2 times and 2.9 times the potency of the CSL antivenom, in experiments performed with calcium and without calcium, respectively (Table 2). In order to ascertain whether this difference is due to antigenic variations between the procoagulants of the venom from the Australian and Papuan populations of O. scutellatus, the neutralisation of coagulant activity of the venom of Australian O. scutellatus, which is used in the immunizing mixture of the CSL antivenom, was investigated. The MCC of this venom was 1.3±0.6 μg/mL (in experiments where calcium was added to
Table 2. Toxic activities of *O. scutellatus* venom and neutralisation by antivenoms.

| Effect                        | Activity | ICP IgG antivenom (Batch 4511209 ICP) | CSL F(ab')2 antivenom (Batch B0548-06301) |
|-------------------------------|----------|---------------------------------------|-------------------------------------------|
|                               |          | mg Venom/mL Antivenom* | Neutralising Units* | mg Venom/mL Antivenom* | Neutralising Units* |
| Lethality (i.p.) (LD50)*       | 0.04±0.01 µg | 4.50 (3.18–6.41) | 18,000 | 5.65 (3.89–8.77) | 18,645 |
| Lethality (i.v.) (LD50)*       | 0.08±0.01 µg | 4.35 (3.05–5.29) | 17,400 | 5.81 (4.08–7.04) | 19,173 |
| Coagulant (MCC) without calcium| 0.76±0.20 µg/mL | 2.43±0.29* | 0.84±0.04* | |
| Coagulant (MCC) with calcium   | 0.33±0.13 µg/mL | 2.37±0.08* | 0.45±0.17* | |
| Myotoxic (MMD)                | 1 µg     | 4.0 | 4.0 | |
| PLA2 (µEq/mg/min)             | 297.7    | 1.47±0.29 | 1.0±0.38 |

*Lethal activity was determined for the i.p. and i.v. routes in CD-1 mice. Median Lethal Dose (LD50) is expressed as µg venom/mouse (mean ± SD).

*Coagulant activity, expressed as the Minimum Coagulant Concentration (MCC), either in citrated plasma or in citrated plasma to which CaCl2 was added immediately before the test. Results are the mean ± S.D.

Myotoxic activity, expressed as the Minimum Myotoxic Dose (MMD).

Neutralisation is expressed as either ED50 (lethality and PLA2) or ED (coagulant and myotoxic effects) (see Materials and Method for details). Results are presented as mg venom neutralised per mL antivenom. For lethality, the 95% confidence limits are included in parenthesis. For the other effects, results are presented as mean ± S.D. (n=4–6). Challenge doses of venom correspond to 4 LD50s (lethality), 2 MCCs (coagulant), 1 MMD (myotoxicity), and 1.5 µg venom (PLA2 activity).

CSL taipan antivenom is labelled as containing at least 12,000 Neutralising Units of antivenom, where 1 Unit = 0.01 mg venom neutralised (i.e.: 12,000 units neutralise 120 mg venom). Actual neutralising unit values for each antivenom are given here for comparison and are calculated from fill volumes of each product. p<0.05 when the two antivenoms are compared.

doi:10.1371/journal.pntd.0001144.t002

plasma) and 4.1±0.6 µg/mL (without added calcium) (p<0.05). Both antivenoms effectively neutralized this activity, with ICP antivenom showing a higher potency. The EDs in conditions where calcium was added were 10.0±2.5 mg venom neutralized per mL (ICP antivenom) and 4.1±1.4 mg venom/mL antivenom (CSL antivenom) (p<0.05). On the other hand, when calcium was not added to plasma, the values of EDs were 4.9±0.4 mg venom/mL antivenom (ICP antivenom) and 1.5±0.2 mg venom/mL antivenom (CSL antivenom) (p<0.05). When comparing the neutralizing ability of the antivenoms against all effects studied, since the CSL antivenom contains 3.15 times the protein of the ICP antivenom, a lesser amount of antivenom protein is required to achieve neutralisation of the various effects in the case of ICP antivenom.

**Discussion**

In the present work, a new, whole IgG monospecific antivenom, obtained by caprylic acid precipitation, was prepared against the venom of Papua New Guinean *O. scutellatus*, the most medically important venomous snake in the southern halves of both PNG and Indonesian Papua. It was shown that, in standard WHO-endorsed preclinical neutralisation assays, against venom of *O. scutellatus* from PNG, this new antivenom compares very favourably with the F(ab')2 taipan antivenom currently in use in Australia and PNG.

The venoms of *Oxyuranus* spp. are among the most toxic ever reported [39], and our LD50 data on mice confirm the high toxicity of *O. scutellatus* from PNG. This high toxicity is due predominantly to the presence of a number of neurotoxins, in particular the presynaptic PLA2 trimer, taipoxin (“cannitoxin”) which destroys nerve terminals and also binds to skeletal muscle, leading to myolysis [11,40–42]. A number of mononeric neurotoxic and myotoxic PLA2, a 52 kDa multimeric voltage-dependent calcium channel blocker, taicatoxin, and a 6.7 kDa post-synaptic α-neurotoxin of 6.7 kDa, α-oxotoxin 1, have also been characterized [43–47]. The dominance of destructive presynaptic neurotoxicity in the clinical syndrome of *O. scutellatus* envenoming has important implications for the treatment of taipan bites, since anticholinesterases do not improve neurotransmission, and more importantly antivenom cannot reverse established neurotoxic manifestations secondary to physical damage to nerve terminals [2,48]. Both antivenoms tested in this work are effective in the neutralisation of the lethal effect of Papuan *O. scutellatus* venom after pre-incubation, thus evidencing their capacity to neutralize the neurotoxins present in this venom.

Disruption of the integrity of skeletal muscle fibre plasma membranes, with rapid impairment of the ability of this membrane to regulate its permeability to ions and macromolecules, is induced by the myotoxic PLA2s, taipoxin and OS2, from the venom of *O. scutellatus* [42,49–50]. Both of the tested antivenoms effectively neutralised myotoxicity due to *O. scutellatus* venom. Since the neurotoxic and myotoxic actions of taipoxin, and similar presynaptically-acting neurotoxins, depend on PLA2 enzymatic activity of these toxins [51,52], the effectiveness of the two antivenoms to neutralize PLA2 activity of *O. scutellatus* venom is compatible with the neutralisation of these toxic activities.

Coagulopathy occurs in a majority of patients envenomed by *O. scutellatus* in PNG [8]. This is due to the procoagulant effect of serine protease inhibitors that are potent prothrombin activators [53–55]. In agreement with previous studies [35–36], Papuan *O. scutellatus* venom showed higher in *vitro* coagulant activity on plasma in conditions where CaCl2 was added to plasma immediately before venom. Regardless of whether the experiments were performed with or without the addition of calcium, ICP antivenom showed a higher potency for the neutralisation of coagulant effect. Interestingly, ICP antivenom was also more effective in the neutralisation of coagulant activity induced by venom from Australian *O. scutellatus*. These observations suggest that the differences shown by these antivenoms regarding neutralisation of coagulant effect are not likely to be due to antigenic variations in the procoagulant enzymes of these two populations of taipan, but instead to a higher antibody titre against these enzymes of both venoms in ICP antivenom. The basis of this finding remains to be elucidated, but may have to do with differences in the immunization schemes employed in the production of these antivenoms.
antivenoms. It is recommended that the analysis of the neutralisation of coagulant activity by Australian venoms by antivenoms should be performed in conditions where calcium is added to citrated plasma before the addition of venom, for reasons previously described [35–36]. Clinical studies carried out in PNG demonstrated that CSL taipan antivenom was effective at restoring blood coagulability within 6–12 hr in 93% of patients treated [8]. A future clinical trial will determine whether this difference in the neutralisation of in vitro coagulant activity between these antivenoms will translate into differences in their in vivo clinical efficacy at restoring blood coagulability in envenomed patients.

An unexpected observation during this study was the partial loss of in vitro coagulant activity of the venom upon incubation at 37°C for 30 min. Whether this observation is due to proteolytic degradation or alteration in the quaternary structure of the procoagulant present in this venom, or to a physiologically suboptimal environment in the in vitro coagulant activity assay remains unknown. Notably, serine proteinase inhibitors had to be used in the isolation of the prothrombin activator [54] and an apparent in vivo inactivation of the coagulant activity of O. scutellatus venom was described in a monkey model of envenoming [56]. Consequently, we modified the coagulant activity neutralisation protocol in order to avoid the incubation of venom at 37°C for 30 min. Although this modification departs from the conventional way to assess neutralization by antivenoms, i.e. incubation of venom-antivenom mixtures for 30 min, a shorter incubation time, such as the one adapted in this study, also allows a proper testing of neutralization of this activity, since the binding of antibodies to antigens is a very rapid phenomenon. Moreover, both antivenoms were able to neutralize coagulant activity in these circumstances.

There were two important considerations in embarking upon a project that is designed to develop an alternative to a currently available product of established efficacy. These were:

- (a). Antivenom price.

From 1987 to 2007 the cost of CSL polyvalent and taipan antivenoms to the Papua Guinea Department of Health increased drastically, leading to a 40% decline in product availability [57,58]. As a consequence, these antivenoms have become increasingly unaffordable to a health system already under enormous stress, leading to chronic antivenom shortages and negative patient outcomes [16]. The high prices and relative scarcity have led to a flourishing black market, where stolen antivenoms are resold by private pharmacies and unlicensed wholesalers [17]. We have focused on the need to produce an effective antivenom with a fill volume (40–50 mL) sufficient to neutralize the average “milked” venom yield (120 mg) of healthy, adult O. scutellatus, at the lowest sustainable price, as a means of restoring access to affordable antivenom supplies.

- (b). Local capacity-building.

PNG currently lacks the capacity to produce its own antivenoms or vaccines. Our successful collaborative development of a potent experimental Papuan taipan antivenom demonstrates the relevance of international partnership for approaching public health issues. This project has allowed the development of PNG capacity for venomous snake husbandry, and production of venoms for immunization and quality control. Further efforts will be aimed at strengthening other local capacities in PNG which, in the long term, may lead to the sustainable manufacture of antivenoms in this country.

In conclusion, a new low-cost whole IgG antivenom, obtained by caprylic acid fractionation of horse plasma, was prepared against the venom of O. scutellatus from PNG. The antivenom has a satisfactory preclinical profile in the neutralisation of lethal, PLA2, myotox and coagulant effects of O. scutellatus venom, comparable to that of the F(ab)2 antivenom currently in use in PNG. These two antivenoms will be compared further in a randomised, non-inferiority, controlled trial in PNG in order to determine the clinical efficacy and safety profiles of both products.

Acknowledgments

The authors thank the staff of the Industrial Division of Instituto Clodomiro Picado for their support in the preparation of the antivenom, and Professor David A. Warrell for critical revision of the manuscript and for valuable suggestions and insights. We also thank Jasper Gabagabu for his excellent husbandry of our captive Papuan taipans; Mark O’Shea, Wolfgang and Catharine Wüster, Timothy Bosalidi, Ben Bal and Ronelle Welton for assisting in the capture of taipan snakes under difficult, dangerous field conditions; the University of PNG (Professor Ross Hynes, Professor Sir Isis Kevau and Professor Mathias Saburi) for infrastructure resources and support; past and present PNG Ministers for Health & HIV/AIDS, the Hon. Sir Peter Barter MP, and the Hon. Sasa Zibe MP.; past and present Health Secretaries, Dr Nicholas Mann and Dr Clement Malau; past Deputy-Health Secretary Dr Timothy Pyakalyia; and especially the late Sir Philip Willmott-Sharp and Lady Brenda Willmott-Sharp who have extensively supported work by one of us (DJW) in PNG for many years.

Author Contributions

Conceived and designed the experiments: M Vargas A Segura M Herrera M Villalta R Estrada KD Winkel G León JM Gutiérrez DJ Williams. Performed the experiments: M Vargas A Segura M Herrera M Villalta R Estrada M Cerdas G León DJ Williams. Analyzed the data: M Vargas A Segura M Herrera M Villalta O Paiva T Matainaho SD Jensen KD Winkel G León JM Gutiérrez DJ Williams. Wrote the paper: O Paiva SD Jensen KD Winkel G León JM Gutiérrez DJ Williams.

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PLOS NEGLECTED TROPICAL DISEASES, 2011, 5 (5)

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