

## Detection of malaria sporozoites by standard ELISA and VecTest™ dipstick assay in field-collected anopheline mosquitoes from a malaria endemic site in Ghana

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### Summary

We compared the VecTest™ dipstick assay for detection of *Plasmodium* sporozoites in *Anopheles* vectors of malaria with standard circumsporozoite (CS) microplate ELISA for detection of *Plasmodium falciparum* circumsporozoite protein (PfCSP) in *Anopheles* mosquitoes. Mosquitoes were collected from a malaria endemic site (Kassena Nankana district) in northern Ghana. Of 2620 randomly sampled mosquitoes tested, the standard CS-ELISA gave a sporozoite rate of 10.8% compared with 11.2% by VecTest™, which was not statistically different ( $P = 0.66$ ). Visual reading of the CS-ELISA results gave a sporozoite rate of 13.4%, which was higher than the other tests ( $P > 0.05$ ). To allow a more objective evaluation of the sensitivity of the dipstick, an additional 136 known CS-ELISA-positive specimens were analysed. The prevalence of the test (including the additional samples) was 14.6% and 14.7% for CS-ELISA and dipstick, respectively ( $P > 0.05$ ). The estimated prevalence by visual assessment of the CS-ELISA results was 17.5%. The relative specificity and sensitivity of the VecTest™ dipstick and visually read ELISA were estimated based on the CS-ELISA as a gold standard. The specificities of the dipstick and visual ELISA were high, 98.0% and 96.6%, respectively. However, the sensitivities of the two assays were 88.8% for VecTest and 100% for visual ELISA ( $P < 0.01$ ). Concordance between VecTest and CS-ELISA was good ( $\kappa = 0.86$ ). Similarly, there was a good concordance between the dipstick and the visually read ELISA ( $\kappa = 0.88$ ). Extrapolating from PfCSP controls (titrated quantities of *P. falciparum* sporozoites), mean sporozoite loads of CS-ELISA-positive *An. gambiae* ( $286 \pm 28.05$ ) and *An. funestus* ( $236 \pm 19.32$ ) were determined ( $P = 0.146$ ). The visual dipstick grades showed high correlation with sporozoite load. The more intense the dipstick colour, the higher the mean sporozoite load (+ = 108, ++ = 207, +++ = 290,  $r = 0.99$ ,  $r^2 = 1$ ). The VecTest dipstick offers practical advantages for field workers needing rapid and accurate means of detection of sporozoites in mosquitoes.

**Keywords** *Anopheles*, *Plasmodium falciparum*, malaria vectors, dipstick assay, circumsporozoite protein

### Introduction

Malaria remains a leading cause of morbidity and mortality worldwide with an estimated 500 million cases and 2.5 million deaths annually (Stauffer & Kamat 2003). The disease also continues to be a significant health threat to travellers and military troops deployed to areas where malaria is endemic. *Anopheles gambiae* and *An. funestus* transmit the *Plasmodium* parasites in sub-Saharan Africa among the human population. Determination of risk of malaria transmission requires quick and accurate methods of assessing transmission intensity, which is the product of

man-biting rates and rate of infectivity of vectors, especially when targeting vector control.

To determine the infection status of suspected mosquito vector species, microscopic examination and dissection are accurate but time-consuming. However, more rapid test kits and automated procedures are now available. These include the standard circumsporozoite (CS) protein ELISA for malaria parasite detection in mosquitoes, considered to be the 'gold standard' although it takes 4–6 h to run, requires equipment, electric power supply, refrigerated storage of reagents and specialized personnel. A new rapid dipstick

immuno-chromatographic assay (Vec-Test<sup>TM</sup> Malaria) that detects specific peptide epitopes (Pf, Pv210 and Pv247) of CS protein of two species of *Plasmodium* sporozoites in mosquitoes has also been developed (Ryan *et al.* 2001). Whereas the dissection technique is limited by inadequate sensitivity and therefore less suitable as a tool for evaluation of vaccines and new drugs, the CS-ELISA is more sensitive but less field applicable. However, the 15 min VecTest<sup>TM</sup> assay is a promising one-step procedure that is read visually. This study was conducted to compare the VecTest<sup>TM</sup> dipstick with the CS ELISA, which is currently being used for detecting *Plasmodium* sporozoites in field collected mosquitoes from a malaria endemic field site, Kassena Nankana district (KND) in Ghana.

## Materials and methods

### Study area

The study was conducted in KND, located 10°30' to 11°00'N, 1°00' to 1°30'W and covering about 1674 km<sup>2</sup> of Sahelian savannah in Ghana. The population of 140 000 live mostly in mud houses with thatched roofs grouped to form multi-family compounds. Most inhabitants are engaged in subsistence farming of millet, groundnut and livestock. The average annual rainfall of 850 mm occurs mostly in the wet months of May–September, followed by a long dry season. A large reservoir in the middle of the district and many small water impoundment schemes provide water for irrigation and livestock throughout the year. The irrigation canals and small dams also serve as breeding sites for *Anopheles* mosquitoes.

### Study design

We randomly selected 2620 *Anopheles* mosquitoes from a pool of 21 200 specimens collected from the study area, and tested them by CS-ELISA and VecTest dipstick for the presence of CS protein. Both tests were performed blindly. CS-ELISA results were read as optical densities (OD) using spectrophotometer and also by visual assessment. To allow a more objective evaluation of the sensitivity of the dipstick, an additional 136 known CS-ELISA-positive specimens were analysed. The *P. falciparum* sporozoite loads for positive CS-ELISA results were determined. The three test results (CS-ELISA OD, visually read CS-ELISA and VecTest) were then compared using prevalence rates, sporozoite rates, sporozoite loads, sensitivity and specificity of the tests with CS-ELISA as gold standard.

### Mosquito collection

Mosquitoes were collected weekly by all night human landing catches during the wet season (June–September, 2001) from randomly selected compounds (WHO 1975). The captured mosquitoes were sorted into various genera and the anophelines were identified to species level using morphological keys (Gilles & De Meillon 1968). The head and thorax of individual female *Anopheles* mosquitoes were removed and put in 1.5 ml micro-centrifuge tubes with perforated cups and kept dry with dessicant in zip-lock bags until used.

### Circumsporozoite enzyme-linked immunosorbent assay

The head and thorax of individual female *Anopheles* mosquitoes were homogenized in 250 µl of grinding buffer (PBS, pH 7.4 containing 0.5% NP-40 and 0.5% casein) using a glass pestle. CS protein micro-plate ELISA using 50 µl/well of the homogenate was done in 96-well microtitre plates coated with anti-*P. falciparum* monoclonal antibodies at 22–25 °C for 30 min (Wirtz *et al.* 1987). Captured CS antigen was revealed by monoclonal antibody (MoAb) horseradish peroxidase conjugate incubated for 1 h. Addition of ABTS [2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonate)] substrate gave a green colour reaction for positive results which were read by visual assessment of the colour reactions, and OD measured within 30 min using spectrophotometer (Multiskan Ascent, Model 354; ThermoLabsystems, Finland) at 414 nm. Sample positivity was determined by titration of PfCSP-positive control antigen using cut-off OD values equivalent to 12 pg of PfCSP or 50 sporozoites (Collins *et al.* 1988). The PfCSP concentrations of test samples were determined by extrapolation from a graph of PfCSP OD versus protein concentration in the controls, and the corresponding sporozoite loads estimated.

### VecTest dipstick assay

Aliquots (150 µl) of mosquito triturates were transferred into the wells of micro-titre plates (Sero-Wel; Bibby Sterilin Ltd, Stone Staffs, UK) for testing. VecTest dipstick strips (MAS<sup>TM</sup>; Camarillo, CA, USA) were placed separately in the test wells of the micro-titre plate and allowed to develop for 15 min at room temperature (22–25 °C). Positive dipstick results showed a horizontal reddish purple line in addition to a control line on each test strip in a distinct detection zone. The degree of positivity (+, ++ and +++) was assessed by comparing the colour intensity of a positive test to that of the positive control line. Negative results showed only the positive control line.

## Results

### Mosquito species tested

A total of 2756 *Anopheles* mosquitoes were analysed by CS-ELISA and VecTest. *Anopheles gambiae* s.l. (1641) and *An. funestus* (1038) made up 97.2% of the samples. *Anopheles pharoensis* (73) and *An. rufipes* (4) constituted the remaining 2.8%.

### Plasmodium sporozoite rates in *Anopheles* mosquitoes

In 2620 randomly selected *Anopheles* mosquitoes that were tested, standard CS-ELISA detected an overall sporozoite rate of 10.8% (284/2620) and VecTest detected 11.2% (295/2620) ( $P = 0.66$ ). Visual reading of the CS-ELISA results gave a sporozoite rate of 13.4% (350/2620), which was statistically higher than the sporozoite rate estimates of the other tests ( $P < 0.05$ ). Table 1 shows the *P. falciparum* sporozoite rates in the different mosquito vector species. As shown, *An. funestus* gave the highest sporozoite rates compared with *An. gambiae* by all three tests and the differences were highly significant ( $P < 0.001$ ). None of the other *Anopheles* mosquito species tested positive by the tests.

**Table 1** *Plasmodium falciparum* sporozoite rates of *Anopheles gambiae* and *An. funestus* determined by CS-ELISA, VecTest dipstick and visual ELISA

Test	<i>An. gambiae</i>		<i>An. funestus</i>	
	Number tested	Number positive (%)	Number tested	Number positive (%)
CS-ELISA	1574	150 (9.5)	971	134 (13.8)
Dipstick	1574	154 (9.8)	971	141 (14.5)
Visual ELISA	1574	183 (11.6)	971	167 (17.2)

### Sensitivity, specificity and prevalence of *Plasmodium falciparum* in *Anopheles* mosquitoes as determined by circumsporozoite enzyme-linked immunosorbent assay, VecTest and visual-ELISA

A total of 2756 mosquito specimens including 136 known CS-ELISA positives were analysed to evaluate the sensitivity of VecTest. This gave a dipstick test prevalence of 14.7% (405/2756), a CS-ELISA prevalence of 14.6% (402/2756) and a visual-ELISA prevalence of 17.5% (482/2756). The overall sensitivity and specificity of the tests are summarized in Table 2. As shown, both the dipstick and visual-ELISA were more than 96% specific compared with CS-ELISA as a gold standard test. However, the relative sensitivity of the dipstick (88.8%) was significantly lower than that of visual-ELISA (100%) ( $P < 0.01$ ). Nevertheless, the agreement between the VecTest and CS-ELISA was good, with a kappa ( $\kappa$ ) value of 0.86.

Tables 3 and 4 summarize data on the ability of the three tests to detect *Plasmodium* sporozoites in *An. gambiae* and *An. funestus*, respectively. The dipstick recorded a higher sensitivity in detecting infections in *An. gambiae* (90.91%) than in *An. funestus* 86.53% although the difference was not statistically significant ( $P = 0.16$ ). Likewise, the specificity of the dipstick was higher for *An. gambiae* (98.5%) than *An. funestus* (96.92%) although the difference was not statistically significant ( $P > 0.05$ ).

Against the CS-ELISA as a gold standard test, both dipstick and visual-ELISA gave some false positive results. As shown in Table 2, 48 of the 2354 CS-ELISA negative specimens (2.0%) tested positive by dipstick. The false positive rate for visual-ELISA (3.4%) was higher but not significantly different ( $P > 0.05$ ). Although the dipstick assay failed to detect antigen in 45 of the 402 (11.2%) CS-ELISA positives, visual-ELISA had no false negatives. Despite the observed discrepancies between dipstick and

Diagnostic test	Number tested	Number positive	Number negative	Relative specificity (%)	Relative sensitivity (%)
CS-ELISA	2756	402 (45)* (0)†	2354 (48)‡ (80)§	100	100
Dipstick	2756	405 (5)† (48)¶	2351	98.0	88.8
Visual ELISA	2756	482 (82)* (80)¶	2274	96.6	100

**Table 2** Sensitivity and specificity of the CS-ELISA, VecTest™ dipstick and visual ELISA assays in detecting *Plasmodium falciparum* circumsporozoite (CS) antigen in mosquitoes

\* Number of samples that tested negative by dipstick ELISA.

† Number of samples that tested negative by visual ELISA.

‡ Number of samples that tested positive by dipstick ELISA.

§ Number of samples that tested positive by visual ELISA.

¶ Number of samples that tested negative by ELISA.

**Table 3** Sensitivity and specificity of the CS-ELISA, VecTest™ dipstick and visual ELISA assays in detecting *Plasmodium falciparum* sporozoites in *Anopheles gambiae*

Diagnostic test	Number tested	Number positive	Number negative	Relative specificity (%)	Relative sensitivity (%)
CS-ELISA	1641	209 (19)* (0)†	1432 (22)‡ (40)§	100	100
Dipstick	1641	212 (1)† (22)¶	1429	98.46	90.91
Visual ELISA	1641	249 (40)* (40)¶	1392	97.21	100

\* Number of samples that tested negative by dipstick ELISA.

† Number of samples that tested negative by visual ELISA.

‡ Number of samples that tested positive by dipstick ELISA.

§ Number of samples that tested positive by visual ELISA.

¶ Number of samples that tested negative by ELISA.

**Table 4** Sensitivity and specificity of the CS-ELISA, VecTest™ dipstick and visual ELISA assays in detecting *Plasmodium falciparum* sporozoites in *Anopheles funestus*

Diagnostic test	Number tested	Number positive	Number negative	Relative specificity (%)	Relative sensitivity (%)
CS-ELISA	1038	193 (26)* (0)†	845 (26)‡ (40)§	100	100
Dipstick	1038	193 (5)† (26)¶	845	96.92	86.53
Visual ELISA	1038	233 (37)* (40)¶	805	95.27	100

\* Number of samples that tested negative by dipstick ELISA.

† Number of samples that tested negative by visual ELISA.

‡ Number of samples that tested positive by dipstick ELISA.

§ Number of samples that tested positive by visual ELISA.

¶ Number of samples that tested negative by ELISA.

visual-ELISA, the results revealed strong concordance with a kappa index of 0.88.

#### Influence of *P. falciparum* sporozoite load on the specificity and sensitivity of VecTest

Sporozoite loads in 144 *An. gambiae* and 307 *An. funestus* ( $n = 451$ ) were estimated to assess the influence of sporozoite load on dipstick reactivity. The mean sporozoite loads ( $\pm$ SE) of CS-ELISA-positive *An. gambiae* ( $286 \pm 28.05$ ) and *An. funestus* ( $236 \pm 19.32$ ) were not significantly different ( $P = 0.146$ ). Similarly, the mean sporozoite loads ( $\pm$ SE) of dipstick-positive *An. gambiae* ( $206 \pm 28.21$ ) and *An. funestus* ( $179 \pm 18.15$ ) were statistically not significantly different ( $P = 0.408$ ). The different dipstick grades (+, ++ and +++) based on visual assessment of colour intensity, also showed corresponding differences in sporozoite load. The more intense the dipstick colour, the higher the mean sporozoite load ( $+ = 108$ ,  $++ = 207$ ,  $+++ = 290$ ) ( $r = 0.99$ ,  $r^2 = 1$ ). Visual-ELISA colour grading showed a similar trend with sporozoite load.

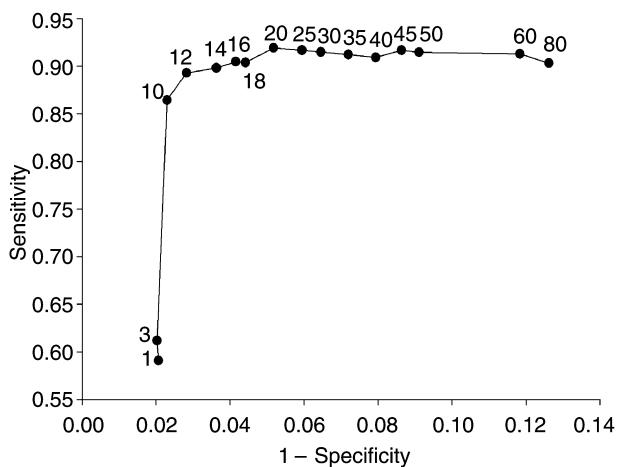
Of the specimens analysed for sporozoite loads, 78/451 (17.3%) were positive by CS-ELISA using OD cut-off at 50 sporozoites, compared with 108/451 (24.0%) by dipstick.

The dipstick detected antigen in 30 of the CS-ELISA negative specimens (373), giving a false positive rate of 8.0%. There were no dipstick false negatives. A higher percentage (80%) of the false positive results occurred among weak positive samples graded (+). Specimen graded (++) had a lower rate of false positives (20%), whereas specimen graded (+++) had no false positives.

Figure 1 shows the receiver-operating curve describing changes in sensitivity and specificity of the CS-ELISA at different sporozoite load cut-offs. As shown, the sensitivity of the CS-ELISA rises sharply, and remains high at sporozoite loads  $>12$ , whilst there is loss in specificity when the sporozoite load cut-off is raised further. At a cut-off of 50 sporozoites, the mean OD (1.118) was over 40 times higher than the mean background OD (0.028). Using 12.5 sporozoites as threshold, the cut-off OD (0.259) was nine times the background OD and the false positive rate reduced to 2.8% (10/353).

#### Discussion

The VecTest dipstick for detection of *P. falciparum* sporozoites in mosquitoes has been evaluated in Kenya, Peru and Thailand, and was found highly concordant with the gold standard CS-ELISA (Ryan *et al.* 2001).



**Figure 1** The receiver-operating curve describing changes in sensitivity and specificity of the circumsporozoite enzyme-linked immunosorbent assay (CS-ELISA) at different sporozoite load cut-offs. The numbers beside each point indicate the cutoff, as calculated sporozoite load, corresponding to the sensitivity and specificity at that point.

The objective of this study was to further evaluate the VecTest dipstick in a new geographical area in Ghana and determine its usefulness in detecting *P. falciparum* sporozoites in field caught mosquitoes. This will facilitate the decision to use the dipstick in planning and monitoring malaria intervention strategies such as vector control, use of chemotherapy and vaccines.

Similar to the observations by Ryan *et al.* (2001), this study showed high concordance ( $\kappa = 0.86$ ) between VecTest dipstick and CS-ELISA in detecting *P. falciparum* sporozoites in field caught *Anopheles* mosquitoes. The lack of statistical differences ( $P = 0.66$ ) between sporozoite rate estimates by VecTest dipstick and CS-ELISA in this study is therefore explicable. In agreement with Ryan *et al.* (2002), the relative specificity (98.0%) of the dipstick compared with CS-ELISA as a gold standard was high using a cut-off limit of 50 sporozoites. However, with this cut-off limit, the relative sensitivity of the dipstick (88.8%) is the lowest so far reported. Adjusting the cut-off limit to a higher or lower level changes the number of false negatives and false positives either way, thereby affecting the sensitivity and specificity of the test (Ryan *et al.* 2002). They therefore suggested that concentration should be on the accuracy of the test, which exceeded 97%, irrespective of the cut-off used. In this study, the accuracy, which is the number of true positives and true negatives divided by the total number of tests performed, was 93.4% using the cut-off limit of 50 sporozoites. However, the accuracy improved to 97.8% when the cut-off was lowered to 12.5

sporozoites. Nevertheless, this cut-off limit may offer practical disadvantages as false negatives or false positives may be more acceptable depending on the entomological objectives such as incrimination of mosquitoes as vectors and rapid surveys of malaria vectors (Ryan *et al.* 2002).

The high measure of agreement ( $\kappa = 0.88$ ) between dipstick and visual ELISA indicates that the latter could be used to detect sporozoites in mosquitoes in endemic areas without the use of expensive spectrophotometric equipment. This is in agreement with Bockarie *et al.* (1993) who reported that visual assessment of sporozoite ELISA results is as reliable as a plate reader in determining infection rates in field-samples of *Anopheles*. Unlike the dipstick, the visual-ELISA had no false negatives in this study. In addition, earlier studies revealed very few false negatives suggesting a superior performance of visual-ELISA (Beier & Koros 1991; Bockarie *et al.* 1993). However, the ELISA is more difficult to perform.

Wirtz *et al.* 1987 reported that ELISA techniques are capable of quantifying the levels of CS-antigen or sporozoite load in individual mosquitoes. This was collaborated by the observation in this study that the higher the CS-ELISA colour intensity, the higher the mean sporozoite load. Likewise, the dipstick colour grading showed a similar trend with sporozoite load, suggesting that the test could be adapted for sporozoite load estimation. The results show that although most false positive results occurred among weak positive samples (+), strong positive results (+++) were more accurate.

In conclusion, this quick and easy dipstick test offers practical advantages for field workers in rapidly and accurately detecting sporozoites in mosquitoes. However, the observation that, rarely, specimen with loads as high as 400 sporozoites could test negative by dipstick remains inexplicable, and further investigation is required.

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