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Evaluation of diagnostic tests for *Wuchereria bancrofti* infection in Brazilian schoolchildren

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**ABSTRACT**

**Introduction**: Since the launch of the Global Programme to Eliminate Lymphatic Filariasis, more than 70% of the endemic countries have implemented mass drug administration (MDA) to interrupt disease transmission. The monitoring of filarial infection in sentinel populations, particularly schoolchildren, is recommended to assess the impact of MDA. A key issue is choosing the appropriate tools for these initial assessments (to define the best intervention) and for monitoring transmission. **Methods**: This study compared the pre-MDA performance of five diagnostic methods, namely, thick film test, Knott’s technique, filtration, Og4C3-ELISA, and the AD12-ICT card test, in schoolchildren from Brazil. Venous and capillary blood samples were collected between 11 pm and 1 am. The microfilarial loads were analyzed with a negative binomial regression, and the prevalence and associated 95% confidence intervals were estimated for all methods. The accuracies of the AD12-ICT card and Og4C3-ELISA tests were assessed against the combination of parasitological test results. **Results**: A total of 805 schoolchildren were examined. The overall and stratified prevalence by age group and gender detected by Og4C3-ELISA and AD12-ICT were markedly higher than the prevalence estimated by the parasitological methods. The sensitivity of the AD12-ICT card and Og4C3-ELISA tests was approximately 100%, and the positive likelihood ratios were above 6. The specificity of the Og4C3-ELISA was higher than that of the AD12-ICT at different prevalence levels. **Conclusions**: The ICT card test should be the recommended tool for monitoring school-age populations living in areas with ongoing or completed MDA.

**Keywords**: Filariasis diagnosis. ELISA. Membrane filtration. Laboratory tests.
Og4C3-ELISA, and the AD12-ICT card test, in children from an endemic area of Brazil prior to the initiation of an MDA program.

METHODS

Study setting

The study was conducted in the three neighborhoods of the City of Olinda, Metropolitan Region of State Pernambuco, Brazil, with the highest prevalence of microfilaremia, according to a survey conducted in 1998 (unpublished data), between 2007 and 2010. The city has an area of 41,659km² and a population of 377,779, as reported in the last census\(^1\), and is considered one of the remaining foci of filariasis in Brazil\(^1\)\(^1\)\(^2\).

Children between the ages of 4 and 15 years who attended five public schools in these neighborhoods (Alto da Conquista, Alto da Bondade, and Sapucaia) participated in this study.

Data collection

Parents or guardians were informed of the main study objectives, and consent was sought for the children's participation. After obtaining written consent, each child's data (name, address, age, and gender) were obtained using a standardized form. The blood samples were obtained between 23:00 and 1:00. Initially, capillary blood samples were obtained by finger prick to perform the thick blood film (~60µL of blood) and the AD12-ICT card (100µL) tests. A venous blood sample (7mL) was then collected to perform the filtration, Knott's concentration, and Og4C3-ELISA tests. This sample was partitioned into tubes containing ethylenediamine tetraacetic acid (EDTA), tubes containing 2% formalin, and dry tubes to perform the filtration, Knott's concentration, and Og4C3-ELISA tests. This sample was processed on the following day.

Laboratory procedures

I) Parasitological methods: A) Thick blood film (TBF)
test: The smear was left to dry overnight at room temperature. The slides were then dehemoglobinized in water, fixed in methanol for 3 min, stained according to previously described procedures, and read with an optical microscope\(^13\). B) Knott's concentration method: Venous blood (~1mL) was drawn into a tube containing 9mL of 2% formalin and processed according to the methodology described by Knott\(^14\). C) Filtration technique: Blood (~1mL) was filtered through a polycarbonate membrane with a width of 13mm and pore size of 3µm, as described by Dennis and Kaen\(^15\). These three parasitological tests rely on the microscopic visualization of microfilariae. Thus, their specificity, although not their sensitivity, can be considered to be very high. II) Immunological methods: A) AD12-ICT card test: This test detects circulating filarial antigen (CFA) using the monoclonal antibody AD12, which recognizes a 200-kDa filarial antigen from either adult worms or microfilariae\(^16\). The test was performed according to the manufacturer's instructions and read by trained technicians in the field after 10 min. The visualization of the two lines (test and control) was interpreted as a positive result. B) Og4C3-ELISA: The test was performed as recommended by the manufacturer (TropBio®, Townsville, Australia). Samples with an antigen concentration ≥128mL units (UA) were interpreted as positive\(^17\). This test detects CFA using a monoclonal antibody against antigens of the bovine parasite Onchocerca gibsoni, which has no known cross-reactivity with other human helminthes. The test provides quantitative results that enable the observation of variations in antigenemia levels after treatment\(^18\)\(^-\)\(^24\).

Data analysis

The reading and interpretation of all laboratory tests were performed by observers who were blinded to the other test results. Data entry and analysis were performed using EpilInfo and R software. Mean microfilarial densities were estimated and compared with a negative binomial regression\(^25\). The parasite load, determined by the three parasitological techniques (thick smear, Knott's concentration, and membrane filtration), and antigenemia, determined by the Og4C3-ELISA in terms of unit antigens (UA), were stratified by age group and gender. Antigenemia was compared between groups with a Kruskal-Wallis test. The mean antigenemia was quoted for descriptive purposes only, as most subjects were negative and, therefore, the median values were zero.

The infection prevalence identified by each technique was stratified by age group and gender. Statistical comparisons between groups were made using chi-square (χ²) and Kruskal-Wallis tests for qualitative and continuous results, respectively. A p-value of 0.05 was considered statistically significant.

The sensitivity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (LR+), negative likelihood ratio (LR-), and their respective 95% confidence intervals (CIs) for the AD12-ICT card test and Og4C3-ELISA were estimated against the gold standard of the combination of the parasitological techniques (thick blood film, Knott's concentration, and filtration). The LR+ measures how many times more likely it is that a positive result is obtained for individuals with the disease than for individuals without the disease, whereas the LR- reveals how many times more likely it is that a negative result is obtained for individuals with the disease than for individuals without the disease. Values of LR+ above 10 and LR- below 0.1 provide strong evidence to confirm or rule out the diagnosis of disease, respectively\(^26\).

The parasitological tests are assumed to have 100% specificity but are of unknown sensitivity, as many individuals can harbor adult worms without demonstrating microfilaremia\(^12\). Consequently, the sensitivity of the ICT card test and the Og4C3-ELISA can be estimated, but not their specificity. Because the specificity cannot be directly evaluated, we estimated the specificities of the Og4C3-ELISA and AD12-ICT card tests as a function of the prevalence of filariasis using the equation derived by Staquet et al\(^27\). This calculation allows for the comparison of the specificities of the Og4C3-ELISA and AD12-ICT card tests for different prevalences\(^27\) of samples with viable worms (adult or microfilaria) or circulating antigen.
The performances of the AD12-ICT card and thick film tests were compared according to sections 2.2 and 3.2.1 of Hayen et al., with the filtration test used as the reference standard. There are eight possible combinations of the three tests, but we considered only the four cells in which the filtration test is positive. Due to the high specificity of filtration, these cells can reliably be considered to be truly positive. The Table 1 was adapted from Table 2 of Hayen et al. The true positive fractions in the AD12-ICT card and thick blood film tests can be estimated as \( \frac{r+t}{n} \) and \( \frac{r+s}{n} \), respectively, and the McNemar test can be used to assess the difference between these values.

Ethical considerations

This study was approved by the Ethics Committee of the Aggeu Magalhães Research Center (CAEE: 0069.0.095.000-06). All of the participants with a positive result in any of the tests were treated and underwent clinical and laboratory examinations.

| TABLE 1 - Contingency table of two tests in a paired design among those positive for the reference standard (filtration). Test N is a ‘new’ test, e.g., the AD12-ICT card test, and test E is an existing test, e.g., the thick blood film test. Adapted from Table 2 of Hayen et al. |
|----------|----------|----------|
|          | positive | negative |
| Test E   |          |          |
| positive | r        | s        |
| negative | t        | u        |
| Total    | r+t      | s+u      | n |

AD12-ICT: immunochromatographic test using the monoclonal antibody AD12. Note: \( n = r + s + t + u \), where \( n \): number of people with the disease; \( r \): true positive; \( s \): false negative; \( t \): false positive, and \( u \): true negative.

RESULTS

A total of 805 schoolchildren participated in the study and agreed to be tested via finger prick. Overall, 783 children were tested by the thick blood film test and 797 by the AD12-ICT card test. Consent for taking venous blood samples was obtained from only 554 participants, all of whom were examined by Knott’s concentration method, 546 by the filtration technique, and 545 by the Og4C3-ELISA test. In 487 schoolchildren, filarial infection was assessed with all laboratory tests.

The microfilaria (MF) loads ranged from 0 to 216 MF/60μL, 0 to 1,423 MF/mL, and 0 to 1,834 MF/mL according to the thick blood film, Knott’s concentration, and filtration tests, respectively. No statistically significant variation in MF load was observed between age groups or genders when assessed by any of these three methods (Table 2). The mean values of filarial antigenemia also did not differ according to age group or gender (Table 2).

The estimated prevalence of filarial infection according to both filarial antigen tests (overall and stratified by age group and gender) was similar and markedly higher than the prevalence estimated by parasitological methods. This difference in estimates was more pronounced in the younger age group (4-9 years), in whom the prevalence of filarial infection estimated by the ICT and Og4C3-ELISA tests was approximately five times higher than that obtained by parasitological methods (thick blood film, Knott’s concentration, or filtration test) (Table 3).

The MF prevalence estimated by either Knott’s (4.6%; 95%CI: 3.1-6.8) or the filtration (5.2%; 95%CI: 3.5-7.5%) method was slightly higher than the prevalence estimated by the thick film method. The prevalence of filariasis according to the parasitological methods was significantly higher in the 10-15-year-old age group than in the 4-9-year-old age group, whereas no difference between age groups was found by the immunological methods (AD12-ICT card test and Og4C3-ELISA) (Table 3). The prevalence of filarial infection was higher in boys than in girls according to all techniques, although a statistically significant difference in the prevalence between the groups was observed only with the AD12-ICT card test (Table 3).

The comparative analysis of the performance of the AD12-ICT card test and Og4C3-ELISA against the gold standard (the combination of all parasitological test results) showed that both immunological tests exhibited 100% sensitivity. The PPVs were approximately 30%, and the NPVs were close to 100%. The LR+ was 6.89 and 7.89 for the AD12-ICT card test and Og4C3-ELISA, respectively. Both LR- values were zero (Table 4).

Figure 1 shows the curves comparing the specificities of the AD12-ICT and Og4C3-ELISA tests over the feasible range of values of infection prevalence (3% to 20%) based on the method of Staub et al. Although the exact values are unknown, we can conclude from these ranges that a) both tests have relatively high specificities (84.4% [95% CI: 80.8-87.4] for the AD12-ICT and 86.5% [CI 95%: 83.0-89.3] for the Og4C3-ELISA), and b) the Og4C3-ELISA has a slightly higher specificity than the AD12-ICT test.

The true positive fractions (TPFs) of the AD12-ICT card and thick blood film tests, calculated with the Hayen formula, were 100% (27+0/27) and 85.2% (23+4/27), respectively. This difference of 14.8% between the TPF values was statistically significant, suggesting that for each 1,000 schoolchildren with the infection, the AD12-ICT card test was able to detect 148 more cases than the thick blood film test. The TPFs for the AD12-ICT card test and Og4C3-ELISA were both 100%. Accordingly, no difference could be found in the ability of the tests to detect cases of filarial infection.
### Table 2 - Means of microfilaria density and antigenemia measured by the thick film, Knott concentration, membrane filtration, and Og4C3-ELISA tests according to age group and gender.

<table>
<thead>
<tr>
<th></th>
<th>Thick film</th>
<th>Knott</th>
<th>Filtration</th>
<th>Og4C3-ELISA (UA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>examined</td>
<td>mean*</td>
<td>min-max</td>
<td>examined</td>
</tr>
<tr>
<td></td>
<td>(n)</td>
<td>(95%CI)</td>
<td>(mf/60µ)</td>
<td>(n)</td>
</tr>
<tr>
<td>Total</td>
<td>776</td>
<td>1.39</td>
<td>(0.55-3.53)</td>
<td>0-216</td>
</tr>
<tr>
<td>Age group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-9</td>
<td>407</td>
<td>2.82</td>
<td>(0.45-17.7)</td>
<td>0-150</td>
</tr>
<tr>
<td>10-15</td>
<td>360</td>
<td>0.76</td>
<td>(0.22-2.67)</td>
<td>0-216</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>393</td>
<td>0.99</td>
<td>(0.15-6.36)</td>
<td>0-152</td>
</tr>
<tr>
<td>female</td>
<td>383</td>
<td>1.40</td>
<td>(0.38-5.18)</td>
<td>0-216</td>
</tr>
</tbody>
</table>

**Og4C3-ELISA**: enzyme-linked immunosorbent assay with the 4C3 monoclonal antibody targeting *Onchocerca gibsoni*; 95% CI: 95% Confidence Interval; LR χ²: Likelihood ratio chi square; n: number of children examined; pos: number of positive results with the technique used; * Determined by a negative binomial regression model.

### Discussion

This study assessed the accuracy of tests in detecting bancroftian filariasis in a large and representative sample of schoolchildren from an endemic area in northeastern Brazil before the initiation of MDA. The performance evaluation of these tests in children is critical to evaluate the impact of the clinical and epidemiological characteristics of these tests in children. It is of particular importance, as this demographic group has been recommended as a sentinel population by the WHO in whom to assess MDA efficacy.

The prevalence of filariasis estimated by the filarial antigen tests (AD12-ICT card test and Og4C3-ELISA) was far higher than that estimated by parasitological methods. The difference between the results of these methods was as much as fivefold in the group aged below 10 years. The comparative evaluation of the performance of the thick film test in relation to the AD12-ICT card test using the Hayen et al. formula also showed that in each group of 1,000 schoolchildren with filarial infection, the AD12-ICT card test was able to detect approximately 150 more cases than the thick film test. These results are in agreement with previous studies conducted in the school-age population and reinforce the usefulness of the AD12-ICT card test in screening for filarial infection in children for the assessment of MDA efficacy. However, it is worth noting that both the AD12-ICT and Og4C3-ELISA tests may yield positive results after treatment even when there is no viable infection, which could overestimate prevalence estimates. Conversely, because this study population was tested before MDA, it is probable that any prevalence excess due to false-positive test results after treatment may not be of low magnitude. Additionally, the data drew attention to the low sensitivity of the parasitological methods, particularly the thick blood film test, in the screening of filarial infection at younger ages. Several studies have shown that the sensitivity of both parasitological and antigen tests decreases with the age of the subjects, with younger children having significantly lower sensitivity than adults. This decrease may be due to the fact that children have lower levels of microfilarial infection, which may not be detected by parasitological methods. However, the greater sensitivity of the antigen tests can be explained by their ability to detect low-level microfilaria and amicrofilaric cases that are typically more common in children than adults. The greater sensitivity of the antigen tests may be particularly important in areas undergoing MDA intervention, where the clinical and epidemiological characteristics of the population may influence test performance due to changes in the prevalence of infection. This evaluation of the clinical and epidemiological characteristics of the population undergoing MDA intervention is critical in evaluating the impact of the MDA on the prevalence of filariasis in children. Therefore, the use of antigen tests as a screening tool for MDA intervention is recommended by the WHO and is being evaluated by the UNICEF as a sentinel population.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Thick film Knott Filtration (60 µL)</th>
<th>AD12-ICT card</th>
<th>Og4C3-ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (pos)</td>
<td>% (95%CI)</td>
<td>N (pos)</td>
</tr>
<tr>
<td>Total</td>
<td>776 (24)</td>
<td>3.1 (2.0-4.6)</td>
<td>541 (27)</td>
</tr>
<tr>
<td>Age group (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-9</td>
<td>407 (8)</td>
<td>2.0 (0.9-4.0)</td>
<td>306 (7)</td>
</tr>
<tr>
<td>10-15</td>
<td>369 (16)</td>
<td>4.4 (2.1-7.3)</td>
<td>252 (12)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>393 (15)</td>
<td>3.8 (2.2-6.3)</td>
<td>281 (17)</td>
</tr>
<tr>
<td>Female</td>
<td>383 (9)</td>
<td>2.3 (1.1-4.6)</td>
<td>288 (10)</td>
</tr>
</tbody>
</table>

χ² = 3.87; χ² = 8.91; χ² = 6.61; χ² = 1.85; χ² = 2.37; p-value = 0.049; p-value = 0.003; p-value = 0.010; p-value = 0.173; p-value = 0.124

The analysis according to age showed that the prevalence was significantly higher in the 10-15-year-old age group than in the 4-9-year-old age group, as evaluated by all parasitological methods. However, no difference in the prevalence of antigenemia between age groups according to the ICT and Og4C3 tests was observed. Moreover, the prevalence of infection estimated by the antigen tests (ICT and Og4C3) was approximately seven times higher than that estimated by the parasitological methods in the younger age group (4-9 years). In the older group (10-15 years), although the prevalence estimated by the antigen tests was again higher than that estimated by the parasitological methods, this difference was less pronounced. These results demonstrate the lower sensitivity of the parasitological methods in the younger age groups compared with the older population, as a larger proportion of individuals in the prepatent stages or who have microfilaricemia infections (with adult worms but with no or few reproducing females) are usually found in this population, as previously described.

The performance evaluation of the AD12-ICT card test and Og4C3-ELISA against the gold standard of the combination of the parasitological test results demonstrated 100% sensitivity for both tests. Additionally, the values of LR+ and LR- were compatible with the high accuracy of these tests in the diagnosis of infection in this study population, even when considering that these measures may be distorted due to the imperfection of the gold standard due to the combination of parasitological results. The LR+ for both tests may represent an underestimate because subjects with an microfilaricemia infection could have been misclassified as free of disease according to the gold standard. Similarly, the low PPVs of the AD12-ICT card and Og4C3-ELISA tests can also be explained by the gold standard, which is assumed to have a lower sensitivity than the antigen-based tests (AD12 and OG4C3 tests).

In regions with ongoing elimination programs, it is important to use high-sensitivity tests to ascertain the actual status of transmission in the population with reasonable significant. Similarly, we found no significant differences in antigenemia according to age or gender. Thus, any variation in test accuracy across these subgroups of this particular population may be limited.

The prevalence estimated by Knott’s test and the filtration test was, in general, slightly higher than that estimated by the thick film test. This finding implies that the former tools are more appropriate for filariasis screening of school-age populations before antifilarial treatment. Knott’s technique is more laborious than the filtration technique but is usually less expensive and it showed a similar performance in detecting microfilaria (15.3 vs. 12.0 MF/mL). Thus, it may be preferred in areas with limited financial resources. A further advantage of this test is its ability to differentiate species of filaria, which makes it useful in other endemic areas where co-infection with other filarial parasites occurs.

Onchocerca gibsoni.
TABLE 4 - Sensitivity, positive predictive value, negative predictive value, accuracy, and likelihood ratio of the ICT card and Og4C3-ELISA tests compared with those of the combination of all parasitological tests (gold standard).

<table>
<thead>
<tr>
<th>Diagnostic performance</th>
<th>AD12-ICT test (n=546)</th>
<th>Og4C3-ELISA (n=542)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity % (95% CI)</td>
<td>100.0 (85.4-100.0)</td>
<td>100.0 (85.4-100.0)</td>
</tr>
<tr>
<td>PPV % (95% CI)</td>
<td>27.8 (19.8-37.7)</td>
<td>30.8 (22-41.3)</td>
</tr>
<tr>
<td>NPV % (95% CI)</td>
<td>100.0 (98.9-100.0)</td>
<td>94.6 (98.9-100.0)</td>
</tr>
<tr>
<td>Likelihood positive (95% CI)</td>
<td>6.9 (5.6-8.5)</td>
<td>7.9 (6.3-9.9)</td>
</tr>
<tr>
<td>Likelihood negative</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Accuracy % (95% CI)</td>
<td>86.2 (83.0-88.9)</td>
<td>88.0 (84.9-90.5)</td>
</tr>
</tbody>
</table>

AD12-ICT: immunochromatographic test with the monoclonal antibody AD12; Og4C3-ELISA: enzyme-linked immunosorbent assay with the 4C3 monoclonal antibody, targeting Onchocerca gibsoni; 95%CI: 95% confidence interval; PPV: positive predictive value; NPV: negative predictive value.

FIGURE 1 - Estimated specificity of the AD12-ICT card test and Og4C3-ELISA according to the prevalence of microfilaria determined by the combination of all parasitological tests.

The comparative evaluation demonstrated the high specificity of the AD12-ICT and Og4C3-ELISA tests in the study population, although a slightly higher specificity of the Og4C3 test was observed when the different levels of prevalence were taken into account. Similar results were also found by Gass et al.40, who demonstrated that the Og4C3-ELISA test showed a greater specificity. These results suggest that the Og4C3-ELISA test is more appropriate for assessing the interruption of filarial transmission in areas with ongoing elimination programs because it yields more accurate negative results, in addition to providing quantitative results that enable the monitoring of MDA effectiveness19. Furthermore, the Og4C3-ELISA test can be performed using filter paper, which facilitates the attainment of samples in field studies as well as in venous blood samples. However, there is a need for better standardization of the technique, as well as better quality control of the kits by the manufacturer, particularly with regard to the plates and reagents.

Based on these results, we conclude that for an initial assessment of possible transmission of *W. bancrofti*, the ICT card test is the ideal tool for surveys and for mapping infection in the school-age population. The high accuracy of the AD12-ICT card test, in addition to other features such as rapidity and the simplicity of execution, makes this test the method of choice for the screening of filarial infection in this population prior to the initiation of antifilarial treatment.

Due to their low sensitivity, the parasitological methods, particularly the thick film technique, are very limited in their usefulness for the estimation of the burden of infection in this population even before treatment. The ICT card test is considered the most suitable test due to its high sensitivity and, as previously mentioned, its lower specificity in populations that have already been treated16,46-48. Finally, in areas with ongoing or completed MDA, the interruption of transmission, as assessed by the tracking of incident cases of filariasis by the ICT card test in the school-age population without prior antifilarial treatment, is the preferred approach.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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