READERS' OPINION AND DISCUSSION

Outstanding insecurities concerning the use of an Ov16-based ELISA in the Amazonia onchocerciasis focus

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*In a recent issue of Memórias do Instituto Oswaldo Cruz, published in Rio de Janeiro in February 2014 (*109*: 87-92), Adami et al. have published a survey reporting* Mansonella *parasite prevalence in the Amazon Region. This report makes a useful contribution to the existing knowledge of filarial parasite distribution within the Amazon area, parasite prevalence rates in relation to age and occupation and provides observations on the possible clinical impact of* Mansonella ozzardi. *Their publication also provides an account of what appears to be a novel ELISA that has recently been used in the Simuliidae and Onchocerciasis Laboratory of the Oswaldo Cruz Institute, Rio de Janeiro, Brazil. We are concerned that the publication of this ELISA may have created an excessively positive impression of the effectiveness of the onchocerciasis recrudescence serological surveillance tools that are presently available for use in the Amazonia onchocerciasis focus. In this letter we have, thus, sought to highlight some of the limitations of this ELISA and suggest how continuing insecurities concerning the detection of antibodies to* Onchocerca volvulus *within the Amazonia onchocerciasis focus might be minimised.*

Key words: Amazonia - ELISA - Ov16 - onchocerciasis - *Mansonella ozzardi* - recrudescence

In a recent issue of Memórias do Instituto Oswaldo Cruz, published in Rio de Janeiro in February 2014 (*109*: 87-92) [Adami et al. (2014), first available on-line in advance of publication in October 2013] a survey reporting *Mansonella* parasite prevalence in the Amazon Region has been made. This report makes a useful contribution to the existing knowledge of filarial parasite distribution within the Amazon area, parasite prevalence rates in relation to age and occupation and provides observations on the possible clinical impact of *Mansonella ozzardi*. Their publication also provides an account of what appears to be a novel ELISA that has recently been used in the Simuliidae and Onchocerciasis Laboratory of the Oswaldo Cruz Institute, Rio de Janeiro, Brazil. We are concerned that the publication of this ELISA could potentially adversely influence the work of the Onchocerciasis Elimination Program for the Americas (OEPA) in the Amazonia onchocerciasis focus. Our concerns about this ELISA are outlined below.

The Amazonia onchocerciasis focus is the last of the Latin American onchocerciasis foci where transmission is still thought to be ongoing and is the last onchocerciasis focus where OEPA expects to eliminate onchocerciasis (Eberhard 2013, WHO/PAHO 2013). Eliminating the disease from this focus presents numerous special challenges including coping with problems arising from the co-existence of *M. ozzardi* and *Onchocerca volvulus* parasites within the site, but is nevertheless still expected to occur by 2019 (WHO/PAHO 2013). The detection of antibodies to *O. volvulus* in children is an important step in OEPA's published recrudescence surveillance guidelines (Cupp 2012) and has already played a key role in disease monitoring at other Latin American foci (Convit et al. 2013, Rodríguez-Pérez et al. 2013). Presently, OEPA guidelines only recommend Ov16-based serological surveillance be used for this function [Lobos et al. (1991), Lipner et al. (2006) and Lindblade et al. (2007), all in Cupp (2012)], for which *M. ozzardi* cross-reaction problems are known to exist (Lobos et al. 1991). Although alternative serological monitoring tools could perform the same function, these alternatives also suffer from *M. ozzardi* cross-reaction problems (Cabrera et al. 1989, Shelley et al. 2001, Post et al. 2003). The novel *M. ozzardi*-cross-reaction-free *O. volvulus* ELISA that Adami et al. (2014) have published thus seems, ostensibly, like a timely development for the OEPA committee and to fit well with OEPA-published guidelines on recrudescence monitoring (Cupp 2012). We, however, feel that the description of this ELISA is potentially misleading and that outstanding limitations of the assay were not made sufficiently clear. We have, therefore, decided to raise some of our concerns about the Adami et al. (2014) paper here. Our hope is that in doing this World Health Organization time and resources may avoid being misspent.

Our most serious concern with the publication of this (Ov10, Ov11, Ov16) ELISA is that no sensitivity data have been presented with it. In the methodology section of Adami et al. (2014) the authors state that each ELISA

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plate contained "a reference positive (n = 3, strongly reacting plasma from onchocerciasis patients)". Whether the authors deemed that their data set of sera positive for antibodies to *O. volvulus* is insufficiently large or insufficiently representative of what is found in the field as to have any value for sensitivity calculations is unclear. What is clear, however, is that they have published no sensitivity data (with or without predictive values) and that because of this the rest of the ELISA data are of only very questionable value. Certainly, with the information provided by Adami et al. (2014), the ELISA's potential value cannot be properly measured against the ELISAs described in Lobos et al. (1991) and Bradley et al. (1993) or other important serological surveillance tools like the Lipner et al. (2006) assay which has been used for recrudescence monitoring in other Latin American onchocerciasis foci.

Our second concern with the Adami et al. (2014) paper is that its findings appear to be in direct conflict with findings that Adami et al. (2008) paper. The two papers both report *O. volvulus* ELISA cross-reactivity data from ELISAs that seem to be based on the same *O. volvulus* protein cocktail containing the Ov10, Ov11 and Ov16 protein antigens. The results reported from the two studies are, however, very different. In the Adami et al. (2008) paper an *O. volvulus* ELISA is described as having cross-reacted with 40% of the tested *M. ozzardi* positive sera from Vila Antimary, whereas the *O. volvulus* ELISA reported in the Adami et al. (2014) is said to have cross-reacted with none of the *M. ozzardi* positive sera samples taken from the very same site. While the authors offer an explanation that their change of the Ov29 protein for an Ov16 protein might be the reason why they have not observed the cross reactivity problems that were reported in Shelley et al. (2001), they make no explicit reference to why the same ELISA cocktail they used in 2014 produced such radically different results in 2008 (Adami et al. 2014). If the authors have developed a new protocol to resolve the problems they first encountered with the Ov10, Ov11 and Ov16 protein cocktail that they reported in 2008, this should have been made clearer in their 2014 paper. Similarly, if the Adami et al. (2008) report of ELISA cross-reactivity was erroneous, this should have been made clear in the Adami et al. (2014) paper.

Our final concern with the Adami et al. (2014) paper that we would like to highlight relates to its filarial parasite identifications. Adami et al. (2008) reported the existence of typical and atypical *M. ozzardi* parasites in the same area in which Adami et al. (2014) have tested their novel ELISA and, indeed, in the Adami et al. (2014) paper it is recorded that the novel ELISA was tested on both types of parasite. While similar reports of morphologically atypical *M. ozzardi* in Peru have previously been shown to be molecularly identical to typical forms (Marcos et al. 2012), the parasites at the Adami et al. (2014) study site have not yet been shown to be equally homogenous. Given that the authors present a picture of a diverse, transient, fluctuating population of *M. ozzardi* in their study area, it is disappointing that they chose not to clarify that the parasites that are presently in their study area are indeed the same parasites that Shelley et al. (2001) reported causing ELISA cross-reactivity problems. There are many existent polymerase chain reaction (PCR)-based filarial parasite identification techniques that they could have easily been adapted to assist with this - see, for example, Morales-Hojas et al. (2001), Post et al. (2009) or Tang et al. (2010).

In light of the outstanding issues relating to *O. volvulus* ELISA cross-reactivity with *M. ozzardi*, we recommend that before a serological tool is chosen for recrudescence monitoring in the Amazonia onchocerciasis focus, its specificity and sensitivity should be established and compared against all existing alternatives on samples obtained from within the focus. We recommend that the data generated for each of these tools should be compared directly, with microscopy and PCR evaluations of skin biopsies and blood samples taken from the same individuals. We additionally recommend that if OEPA has not already modified their recrudescence surveillance guidance (Cupp 2012) to take account of potential *M. ozzardi* ELISA cross-reactivity issues inside the Amazonia onchocerciasis focus, they should consider modifying their serological survey to include a PCR assay of ELISA positive blood samples found at the site. We recommend that a PCR [like that described in Tang et al. (2010)] should be performed on all *O. volvulus* positive blood samples before skin biopsies are performed, at least until such times as ELISA *M. ozzardi* cross-reactivity can be properly resolved. Such a modification could minimise the impact of false positives on disease control and planning strategies and could also avoid the trauma caused by unnecessary skin biopsies. We also believe that such a modification could help clarify which tested people may have actually been "exposed" to onchocerciasis.

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REPLY

The seroepidemiology and the use of an ELISA test with a cocktail of Ov-recombinant proteins in the Brazilian Amazon

In the letter entitled "Outstanding insecurities concerning the use of an Ov16-based ELISA in the Amazonia onchocerciasis focus" Luz et al. criticised the use of an ELISA with a cocktail of recombinant proteins from *Onchocerca volvulus* in an endemic area for *Mansonella ozzardi*. Here we advocate the secure use of the test and provide a critical review of what has been done in the last few years. Additionally, we explain why our system is reliable and that in this particular area studied, no cross-reactions between *M. ozzardi* infected individuals and *O. volvulus* recombinant proteins were detected.

Undoubtedly, originality with total scientific rigor as a form of contribution to scientific knowledge was one of the objectives of our scientific paper.

We also try to avoid the simple repetition of ideas and attempt to breed new topics and ideas, to enable the discussion of relevant concepts to the scientific community. We believe using an adequate method is fundamental to the understanding of any scientific paper, be it focused on a novel scientific idea or not. This is why the article published by Adami et al. (2014) successfully surpassed the rigorous scrutiny of the editors and revisors of the Memórias do Instituto Oswaldo Cruz scientific journal. We also believe that any divergence of ideas and concepts relating to a scientific publication should be discussed with strict compliance to cordiality and ethical values for the utmost benefit to science and the readers of the journal.

In their critical review of the article, Luz et al. (2014) argue about what seems to be "a novel ELISA that has recently been used in the Simuliidae and Onchocerciasis Laboratory (LSO) of the Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil". At first sight, we thought there was some kind of misunderstanding, because we believed that Luz et al. (2014) had background in research about immunology and public health and would be able to understand the tool and the employment of an ELISA assay in *O. volvulus* non or endemic areas.

In areas under vector or chemotherapeutic control, onchocerciasis diagnosis may be problematic, as a drastic reduction in the microfilaria load in the skin can be present (Bradley & Unnasch 1996). The traditional skin snip method may fail in these cases and, as the parasite has a long prepatent period, one of the questions is that after control programmes the sensitivity of the method is reduced and a recrudescence would not be detected. Besides, the method is painful, invasive, expensive and rejected by children in endemic areas (Weil et al. 2000). Thus, an immunological assay would be useful as a seroepidemiological tool for population studies, rather than for diagnosis of onchocerciasis in individuals (Ramachandran 1993, Bradley & Unnasch 1996, Rodríguez-Pérez et al. 2011). Indeed, the latter authors state that "the test must be extremely specific but, within reasonable limits, need not be optimally sensitive". At this point it was identified that the antigens Ov7 (identical to Ov10), Ov11 and Ov29 were recognised by variable proportions of sera from persons with prepatent as well as patent infections. In 1993, Bradley et al. used these three antigens as a cocktail in an ELISA system and the results were encouraging although some variations were detected in serum samples from different geographical areas. The test supplied controversial results in a *M. ozzardi* endemic area in Brazil and it was clear that cross reactions with this parasite - that may occurs sympatrically with *O. volvulus* in the Amazonian focus - could represent a problem (Shelley et al. 2001, Shelley 2002). Thus, it became clear that the priority should be given to specificity since cross reactivity could lead to the

* All papers undertaken by the LSO resulting from either research, collections and reference services are done with total independence and without conflict of interest with relation to their partners or supporters (Brazilian Health Ministry, OEPA) among others.

erroneous conclusion that either onchocerciasis is still present or that ivermectin is not effective (Rodríguez-Pérez et al. 2003). A recombinant prepatent antigen of *O. volvulus* named Ov16 is proclaimed by Onchocerciasis Elimination Program for the Americas (OEPA) and used to measure the prevalence of IgG4 antibodies as a serosurvey in children in areas where transmission is thought to be interrupted in the Americas region (Convit et al. 2013). Instead, the ELISA based on detection of antibody responses to several *O. volvulus* specific antigens is still being used to monitor onchocerciasis control measures and/or elimination programs by different research groups in the world (Andrews et al. 2008, Burbelo et al. 2009, Rodríguez-Pérez et al. 2011, Garms et al. 2013, Baum et al. 2014, Katabarwa et al. 2014).

The work performed at LSO was performed out of the Amazonian onchocerciasis focus, into an area where there was a suspicion of the existence of an *Onchocerca*-like microfilaria, but at that time, there was no proof of this parasite (Adami 2009). In the search for it, we collected 526 skin snips samples from scapula and iliac crest from some consenting volunteers of the communities, which is the "gold standard" method for the screening of *O. volvulus* microfilarias (Bradley & Unnasch 1996) and none of them was found positive during parasitological examination (Adami 2009). These results were corroborated by the ELISA's results, but were suppressed in the article for editorial reasons and may have contributed to some doubts. For instance, it is important to remember that definite proof of infection with *O. volvulus* occurs by demonstrating microfilarias in skin snips samples or adult worms in the nodules excised. Indeed, some errors may arise when the snip samples contain microfilaria other than *O. volvulus* - such as *Mansonella* spp, which occur in certain areas of the Brazilian Amazon (Moraes 1991). Nevertheless, the skin snip method may be insensitive in light infection and in low transmission areas, while molecular tools - which are dependent on the skin snipping method, such as the ones based on the polymerase chain reaction - are unable to detect prepatent infections, which can represent a critical delay in the detection of a recrudescence of transmission in areas under control programs (Rodríguez-Pérez et al. 2011, Golden et al. 2013).

Indeed, sensitivity, by definition, is related to the "ability of a test to correctly identify those individuals with a particular disease". We find it hard to understand why Luz et al. (2014) were so apprehensive with the non-presentation of data about the test's sensitivity, when they note that "no sensitivity data have been presented with it". In an *O. volvulus* free area and in all tests - including skin snips - with negative results, it was not possible to obtain a result for sensitivity. But, as for the definition, "the specificity of a clinical test refers to the ability of the test to correctly identify those patients without the disease" (Lalkhen & McCluskey 2008), it was possible to perform the calculations and present the result reported in the article. Still, the cocktail was primarily employed by Rodríguez-Pérez et al. (2003) and reactivity was found with *Dracunculus medinensis* and *Wuchereria bancrofti*. Even so, according to the results obtained they postulated that as these filarial species do not occur in the Mexican onchocerciasis-endemic areas, they assumed the test was 100% specific.

Indeed, when Luz et al. (2014) declare that "our *O. volvulus* sera positive sample set is insufficiently representative", we understand that they do not know that positive and negative controls are used in every test carried out. Indeed, the controls are used to provide that the test is working properly and not in the calculation of the cut-off in the system adopted and more: the number of controls used is dependent on the protocol employed by each laboratory (Voller et al. 1978, Egwang et al. 1994).

We cannot agree with Luz et al. (2014) when they assert that our ELISA's potential value cannot be properly measured against ELISA's described in Lobos et al. (1991) and Bradley et al. (1993) and, for sure, that comparison was not the LSO group's intention. But, on the contrary of what is declared by Luz et al. in their report that "the cocktail failed in Brazil because of cross reactivity of this species (referring to *O. volvulus*) with *M. ozzardi*" (Tang et al. 2010), Lobo (1991) did not see reactivity with *M. ozzardi* as a problem, as the only reactive serum sample was of an individual from an area where coinfection with *O. volvulus* was a possibility. Still, Shelley et al. (2001) considered and used an ELISA test with a cocktail of recombinant proteins and faced the possibility of a cross reactivity in the area where the samples were collected. Unfortunately, in this work the methodology is unclear, as they do not provide any data on the number of positive and negative controls used, the protocol employed and the cut-off adopted.

Furthermore, Luz et al. (2014) insist that in our article we described a "novel ELISA" and it seems that the group are not aware of the many instances in the literature in which this test was employed. For instance, the cocktail was used in its primary form as Ov10, Ov11 and Ov29 by Guderian et al. (1997), in Ecuador, by Bradley et al. (1998) and Shelley et al. (2001) and because of the cross reactions in some areas, the latter antigen was replaced in the cocktail by Ov16 in the studies of Rodríguez-Pérez et al. (2003) and in the Oaxaca focus in Mexico (Rodríguez-Pérez et al. 2008).

In their last noted concern, Luz et al. (2014) refer to the existence of typical and atypical microfilarial forms of *M. ozzardi* in the study area and note that a similar form was found in Peru. We would like to emphasise that I have carried out the morphological identification of the atypical microfilarias found in Peru (Arrospide et al. 2012) in close collaboration with local researchers and of course we do not know if the atypical forms found in Brazil have the same characteristics. But, for instance, according to Shelley (2002) only *M. ozzardi* was present in the reported area and that was confirmed by a study of parasite DNA by Morales-Hojas et al. (2001). Yet, in the article of Tang et al. (2010), the group of Dr. Luz report a test for distinguishing sympatric filarial species in Brazil, including *Mansonella perstans*, but until now, neither their group nor any others that suggest the existence of this filarial species in Brazil have been able to prove or show any consistent epidemiological data related to it.

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