SENSITIVE AND SPECIFIC SERODIAGNOSIS OF ONCHOCERCIASIS WITH RECOMBINANT HYBRID PROTEINS

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Abstract. Onchocerciasis remains a major health hazard in many tropical countries. However, the existing tools for diagnosis of the disease have limitations, particularly regarding the detection of low level or early infections. To design an optimized reagent, we exploited the high antibody reactivity of patient sera against the *Onchocerca volvulus* proteins Ov20 and Ov33, which have been described as highly sensitive and specific immunodiagnostic reagents for producing hybrid proteins. The construct OvH2 was composed of Ov20 fused to Ov33, while OvH3 consisted of the C-terminus of Ov20 linked to Ov33. When these constructs were tested with sera from patients with onchocerciasis and control sera, OvH2 showed a sensitivity of 98.5% and a specificity of 97.7% and OvH3 showed a sensitivity of 98.5% and a specificity of 95.35%. All non-responders were from Ecuador. These results surpass those of existing single recombinant antigens, suggesting that our hybrid proteins combined the sensitivity of the two parent proteins. Tests based on OvH2 should prove suitable for monitoring onchocerciasis control programs and individual diagnosis.

INTRODUCTION

Onchocerciasis, which is caused by the filarial nematode *Onchocerca volvulus*, affects approximately 18 million individuals worldwide. The disease is usually diagnosed by the detection of microfilariae in bloodless skin biopsies, an approach that is considered to be insensitive for the detection of prepatent and low level infections. Comparable results can also be obtained by a skin reaction evoked by topical application of diethylcarbamazine, a drug that eliminates microfilariae. Major efforts have been undertaken by the Onchocerciasis Control Program of the World Health Organization (WHO) to interrupt the transmission of *O. volvulus* through control of the blackfly intermediate hosts and by transient elimination of the microfilariae with ivermectin. These measures have led to the elimination of the parasite or reduction of its prevalence in wide areas of West Africa and Latin America. The remaining low-level infections or newly upcoming infections in controlled areas are difficult to diagnose, yet such a diagnosis is important as a basis for decisions on further interventions. In such situations, tests have to be very specific to keep the risk of detecting false-positive cases in large populations at a minimum, while the sensitivity is also important.

Although sensitive polymerase chain reaction (PCR) assays have been developed, they also depend strictly on the presence of parasite DNA in the skin and as such have disadvantages similar to parasitologic diagnosis. Therefore, antibody detection assays have been developed using recombinant *O. volvulus* antigens. Some of these antigens were tested in a multicenter trial organized by the Tropical Diseases Research Program of the WHO that identified highly specific proteins, and high sensitivity of these tests was achieved by combining three proteins in a diagnostic cocktail. However, these antigens were difficult to produce and the fact that they were expressed as fusions with a carrier protein made it necessary to run parallel tests to rule out antibody reactions with the carrier. These limitations have prevented the *O. volvulus* antigen cocktail from becoming a widely used diagnostic tool. Meanwhile, formats of antibody tests for onchocerciasis have been produced that allow rapid serodiagnosis directly on the spot of sampling. Such developments make it attractive to refine recombinant antigens to create a new generation of tests, which combine high specificity and sensitivity with easy handling.

As a prerequisite for such tests, the present investigation re-evaluated two *O. volvulus* antigens, Ov33 and Ov20, which were found to be recognized by greater than 90% of the onchocerciasis sera and had a very high specificity when produced as fusion proteins. Previous epitope mapping studies showed that frequently recognized continuous B cell epitopes were present in several parts of Ov33, whereas the N-terminus of Ov20 did not appear to contribute much to the overall B cell reactivity (Bradley JE, unpublished data). Therefore, slightly different elements of Ov20 in combination with the same region of Ov33 were used in the production of two hybrid proteins, OvH2 and OvH3, and their serodiagnostic potentials were evaluated with onchocerciasis sera from different geographic regions. Our aim was to create a reagent that would emulate the properties of the diagnostic cocktail, while eliminating its shortcomings.

MATERIALS AND METHODS

Sera. A total of 132 sera from onchocerciasis patients with proven skin microfilariae were obtained from five countries in different geographic regions of Africa and Latin America (Table 1). In addition, we tested 28 sera from persons in Ecuador who lived in the same areas as the onchocerciasis patients and were exposed to *O. volvulus* transmission, but did not harbor skin microfilariae. These persons have been described as putatively immune individuals. Furthermore, we studied 33 sera from persons living in endemic areas in Togo, who were exposed to *O. volvulus* transmission, but did not harbor microfilariae in their skin (Soboslay P, unpublished data). Sera from Cameroon were obtained from characterized patients living near the Sanaga River in the Central Province of Cameroon, a forest region endemic for onchocerciasis. For the field study in Cameroon, informed consent was obtained from all human adult participants and from parents or legal guardians of minors. The study was approved by the University of Yaounde I Ethical Committee. Onchocerciasis patient sera from Ivory Coast, Togo, Ecuador, and Guatemala were obtained from the WHO Filariasis Serum Bank and the Edna McConnell Clark Foundation Serum Bank, respectively. Sera from individuals infected with *Wuchereria*
designed and constructed from amino acids 17–22 (GenBank Accession No. X13313). Computer-based analysis of the nucleotide sequence encoding amino acids 22–178 of Ov20, the 5’ portion of this primer also codes for the amino acid sequence in the spacer region linking the proteins. The 5’ primer used for the amplification of the nucleotide sequence encoding amino acids 74–178 of Ov20 had the sequence 5’-GGATCCAAAATCGGATAAACTC-GAG-3’. The anti-sense primer was the same as that used for the amplification of the nucleotide sequence encoding amino acids 17–178 of Ov20.

For the amplification reactions, 50 pM of each forward and reverse primers, 200 μM dNTP, 50 ng of plasmid DNA, one unit of Pwo polymerase enzyme (Angewandte Gentechnologie Systeme [AGS], Heidelberg, Germany), and 1× complete buffer in a total reaction volume of 50 μl were used. The amplification reactions were carried as follows. For amplification of the Ov33 nucleotide sequence, after an initial denaturation step of 95°C for 3 minutes, the primers were annealed at 60°C for 1 minute and nascent DNA was synthesized at 72°C for 1 minute for 30 cycles. Afterwards, there was a final synthesis step at 72°C for 10 minutes. For the amplification of the Ov20 nucleotide sequences, the cycling conditions were similar to those of Ov33 with the exception that the primers were annealed at 56°C for 1 minute.

Plasmid constructions. Standard procedures used for the cloning of amplified DNA were as previously described. All DNA-modifying enzymes used were obtained from AGS unless otherwise stated. The PCR products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany).

bancrofti from India and Sri Lanka were obtained from the WHO Filariasis Serum Bank (Table 2). Sera from individuals infected with Brugia malayi were kindly provided by Dr. M. Yazdanbakhsh and Dr. E. Sartono (Department of Parasitology, University of Leiden, Leiden, The Netherlands). The brugian sera were collected from individuals who were microfilaremic or microfilaremic, and also from patients with elephantiasis. European control sera were obtained from adult German students who had never travelled to the tropics.

Structure of hybrid proteins OvH2 and OvH3. The two hybrid proteins, designated OvH2 and OvH3, were composed of elements of the immunodominant O. volvulus proteins Ov20 (GenBank Accession No. L27686) and Ov33 (GenBank Accession No. X13313). Computer-based analysis of the amino acid sequences of each designed hybrid protein was carried out to evaluate its stability and solubility for efficient expression in Escherichia coli and purification. OvH2 was designed and constructed from amino acids 17–178 of Ov20 linked by a spacer (GPGKK) to amino acids 22–239 of Ov33. OvH3 was composed of amino acids 74–178 of Ov20 joined to amino acids 22–239 of Ov33 by a similar spacer. The spacer was introduced through the 3’ primer used for amplification of Ov20.

Polymerase chain reaction. The nucleotide sequences of Ov33 in plasmid pGEX 2a and Ov20 in plasmid pUC 18 were amplified with primers containing specific DNA restriction endonuclease sequences at their 5’ and 3’ ends to facilitate subsequent ligation and cloning reactions. Primers for the amplification of the nucleotide sequences of interest were designed using the Oligo Program. For amplification of the nucleotide sequence encoding amino acids 22–239 of Ov33, the 5’ primer had the sequence 5’-GAGCTCGGTGGTAG-TAAAAGGTACAATAACGT-3’ and the 3’ primer had the sequence 5’-GGATCCATAAGATTGCGACGCAG-3’.

- **TABLE 1**
  Characteristics of onchocerciasis serum donors

<table>
<thead>
<tr>
<th>Country</th>
<th>Microfilariae positive</th>
<th>Mean ± SD microfilariae/mg of skin (range)</th>
<th>Mean ± SD age, years (range)</th>
<th>Microfilariae negative</th>
<th>Mean ± SD age, years (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cameroon</td>
<td>35</td>
<td>46.2 ± 78.5 (0.78–410.1)</td>
<td>43.6 ± 15.5 (13–78)</td>
<td>33</td>
<td>32.7 ± 14.4 (12–75)</td>
</tr>
<tr>
<td>Ivory Coast*</td>
<td>27</td>
<td>65.1 ± 57.7 (2.0–242)</td>
<td>25.2 ± 11.6 (5–50)</td>
<td>28</td>
<td>33.1 ± 9.6 (5–20)</td>
</tr>
<tr>
<td>Togo†</td>
<td>19</td>
<td>51 ± 62.1 (0.4–259.4)</td>
<td>38.6 ± 10.4 (14–60)</td>
<td>33</td>
<td>32.7 ± 14.4 (12–75)</td>
</tr>
<tr>
<td>Ecuador†</td>
<td>21</td>
<td>38.4 ± 31.9 (5.5–95.5)</td>
<td>41.8 ± 17.2 (16–72)</td>
<td>28</td>
<td>33.1 ± 9.6 (5–20)</td>
</tr>
<tr>
<td>Guatemala*</td>
<td>30</td>
<td>41.4 ± 36.0 (08.5–130)</td>
<td>32.6 ± 10.3 (20–58)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>132</td>
<td>61</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Sera obtained from the World Health Organization Filariasis Serum Bank.
† Sera obtained from Edna McConnell Clark Foundation Filariasis Serum Bank.

- **TABLE 2**
  Characteristics of control serum donors*

<table>
<thead>
<tr>
<th>Type of serum</th>
<th>No. of sera</th>
<th>Mean ± SD age, years (range)</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brugia malayi infection</td>
<td>21</td>
<td>37.5 ± 12.6 (17–70)</td>
<td>Indonesia</td>
</tr>
<tr>
<td>Wuchereria bancrofti infection</td>
<td>22</td>
<td>27.36 ± 11.8 (10–55)</td>
<td>India and Sri Lanka</td>
</tr>
<tr>
<td>European sera</td>
<td>12</td>
<td>ND</td>
<td>Germany</td>
</tr>
<tr>
<td>Total</td>
<td>55</td>
<td>27.36 ± 11.8 (10–55)</td>
<td></td>
</tr>
</tbody>
</table>

* ND = not determined.
as described by the manufacturer. The purified Ov33 ampi-
con was digested overnight with Sac I and Sal I and subcloned into pBluescript SK+ (Stratagene Europe, Amsterdam, The Netherlands) previously digested with the same enzymes. The purified Ov20 amplicons were digested overnight with Bam HI and Sac I and similarly subcloned. To construct the nucle-
otide sequence of OvH2, the expression plasmid pQE 30 was
digested with Bam HI and Sal I. The previously digested
Ov20 and Ov33 amplicons were ligated into the plasmid using
a rapid DNA ligation kit (Boehringer Mannheim, Mannheim,
Germany) as described by the manufacturer. The ligation of
the Ov33 sequence and the sequence encoding amino acids
74–178 of Ov20 into a similarly digested pQE30 plasmid gave
rise to the complete nucleotide sequence of OvH3. The plas-
mids were used to transform competent E. coli M15 already
containing the repressor plasmid pREP4. Positive clones were
selected against an ampicillin/kanamycin background.

Expression and purification of hybrid proteins. The se-
lected positive E. coli clones were induced with 1.0 mM iso-
propyl-β-D-thiogalactopyranoside (IPTG) for 3 hr at 25°C and
the OvH2 and OvH3 proteins were affinity purified under
denaturing conditions using the Ni-NTA matrix (Qiagen) as
described by the manufacturer with some modifications, as
follows. For the purification of OvH2, the pellet of the in-
duced bacteria was lysed in a buffer containing 6 M GuHCl,
0.1 M NaH₂PO₄, 0.01 M Tris-Cl, pH 8.0, and 20 mM β-mer-
captoethanol on ice. The bacterial lysate was centrifuged at
27,000 × g for 20 minutes at 4°C and the supernatant was
mixed with Ni-NTA agarose previously equilibrated with lysis
buffer without β-mercaptoethanol. For sufficient binding of
the expressed 6-histidine tagged protein to the matrix, the
mixture was kept shaking for 3 hr at 4°C. The slurry was
loaded onto a column and washed with the lysis buffer. Sub-
sequent washes were done with a buffer containing 8 M urea,
0.1 M NaH₂PO₄, 500 mM NaCl, 0.1% Triton X-100, 0.01 M Tris-Cl, pH 8.0, followed by a buffer containing 8 M urea, 0.1 M NaH₂PO₄, 500 mM NaCl, 0.01 M Tris-Cl, pH 8.0 and then
with the same buffer without 500 mM NaCl, pH 6.3. The
bound proteins were eluted using the last washing buffer at
pH 4.0. The purification of OvH3 was similar, except that the
third washing step was with a buffer containing 8 M urea, 1 M
NaCl, 20% glycerol, 0.1 M NaH₂PO₄, 0.01 M Tris-Cl, pH 8.0.
The purified proteins were analyzed by electrophoresis on a
13% polyacrylamide gel. The pure fractions of each hybrid
protein were pooled and dialyzed against a buffer containing
1 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-Cl, pH 7.4 and then
exhaustively dialyzed against phosphate-buffered saline
(PBS), pH 7.4.

Enzyme-linked immunosorbent assay (ELISA). A stan-
dard ELISA technique was used to investigate the reactivity
of sera with the purified hybrid proteins. The optimal antigen
concentration required for the coating of the Maxisorp
ELISA plates (Nunc, Wiesbaden, Germany) was determined
by a checkerboard titration using several well-characterized
onchocerciasis sera. The ELISA plates were coated with 50
µl/well of antigen (OvH2 or OvH3, 3 µg/ml or OvAg, 2.5
µg/ml) in 0.2 M carbonate buffer, pH 9.6, and the plates were
incubated overnight at 4°C. After the plates were washed
with PBS, pH 7.4, they were blocked with 3% bovine serum
albumin in PBS (200 µl/well) for 2 hr at room temperature.
Serum samples diluted in blocking buffer at 1:1,500 for the
determination of IgG antibody responses were added in du-
licate to the plates (50 µl/well) and incubated overnight at
4°C. Thereafter, the plates were washed three times with PBS
containing 0.025% Tween-20. Detection of bound human IgG
was performed with a monoclonal antibody against human
IgG (Immunotech, Hamburg, Germany) diluted 1:2,000 in
blocking buffer, which was added to the plates and incubated
at 37°C for 3 hr. The plates were washed and alkaline phos-
phatase-conjugated anti-mouse antibody (Dianova-Jackson,
Hamburg, Germany) diluted 1:3,000 in blocking buffer was
added. The plates were incubated for 1 hr at 37°C and un-
bound antibodies were washed away. Bound antibodies were
revealed by adding the substrate (5 mg of p-nitrophenyl phos-
phate [Sigma, Deisenhofen, Germany] dissolved in 10 ml of
carbonate coating buffer containing 1 mM MgCl₂). The en-
zyme reaction continued in the dark until the positive control
sera attained an approximate absorbance of 1.1 at 405 nm.
The quotient of the observed mean absorbance of the positive
control sera of each plate relative to the expected absorbance
was used to multiply all observed optical density values of
that plate to give the absorbance units. All serum samples
were analyzed in duplicate.

Serologic parameters. The cut-off ELISA value for the de-
termination of the sensitivity was calculated from the arith-
etic mean plus three standard deviations of the lymphatic
filariasis sera (cumulative W. bancrofti and B. malayi sera).
All values below that line were considered negative. Accord-
ingly, the sensitivity was the percentage of sera above the
cut-off value. The specificity was 100% minus the percentage
of lymphatic filariasis sera with absorbances above the cut-off
value. The rating index (J index), the positive predictive value
(PPV), and the negative predictive value (NPV) were calcu-
lated as described.25

RESULTS

Expression and purification of the hybrid proteins. The two
hybrid proteins OvH2 and OvH3 were expressed in E. coli
after induction with IPTG. Induced bacteria produced a 6-his-
tidine-tagged protein with a molecular mass of 44 kD (OvH2),
which represented approximately 30% of the bacterial bio-
mass, and a second protein with a molecular mass of 38 kD
(OvH3), which represented approximately 30% of the total
bacterial proteins. The affinity-purified hybrid proteins were
soluble and stable after exhaustive stepwise dialysis against
PBS. From one liter of E. coli culture, approximately 10 mg of
OvH2 protein and 12 mg of OvH3 protein could be purified.

Immunodiagnostic properties of OvH2 and OvH3. To es-
establish the immunodiagnostic properties of OvH2 and OvH3,
the IgG reactions against the hybrid proteins were evaluated
using characterized sera as summarized in Tables 1 and 2. As
a positive control, we evaluated the reaction of the sera with
O. volvulus total extracts (OvAg). In four of the five serum
samples groups tested, 100% of the onchocerciasis sera recog-
nized the two hybrid proteins. Onchocerciasis sera from Ecuador were
slightly less reactive. Less than 5% of the lymphatic filariasis
sera and none of the European control sera reacted with
OvH2 or OvH3. OvH2 was relatively more specific than
OvH3. The ELISA values are shown in Figures 1 and 2 and
the sensitivities, J-rating indices, and predictive values are
summarized in Tables 3 and 4.

OvH2. Of the 132 onchocerciasis sera from microfilariae-
positive patients, 130 reacted positively with OvH2 (sensitiv-
ity = 98.5%). All sera from four geographic regions (Cameroon, Ivory Coast, Togo, and Guatemala) recognized OvH2 in the IgG ELISA (sensitivity = 100%, Table 3), while 19 of 21 sera from Ecuador were positive (sensitivity = 90.5%). The two individuals from Ecuador who had no specific IgG antibody to OvH2 had very low microfilarial densities of 7 and 8 microfilariae/mg of skin biopsy, respectively, and showed a very weak IgG response against OvAg with absorbance units of 0.06 and 0.12, respectively. One of the 43 lymphatic filariasis sera (a W. bancrofti-infected serum) showed weak IgG reactions, with absorbance units of 0.21 and 0.14, respectively, compared with the cut-off value of 0.139 (Figure 2). Thus, the specificity of the IgG antibody responses against OvH3 was 95.3%. Consequently, the J rating indices were 95.3% for all countries tested, except Ecuador (85.8%). Similarly, the PPV was 100% for all countries tested, except Ecuador (90.5%) and the NPV was 95.3%.

Testing of the sera from individuals who were free of skin microfilariae despite living in the same areas as the onchocerciasis patients showed that 57.1% and 66.7% of the sera from

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**Table 3**

<table>
<thead>
<tr>
<th>Country</th>
<th>Sensitivity (%)</th>
<th>J Index (%)</th>
<th>PPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cameroon</td>
<td>100 (0/33)</td>
<td>97.7</td>
<td>100</td>
</tr>
<tr>
<td>Ivory Coast</td>
<td>100 (0/27)</td>
<td>97.7</td>
<td>100</td>
</tr>
<tr>
<td>Togo</td>
<td>100 (0/19)</td>
<td>97.7</td>
<td>100</td>
</tr>
<tr>
<td>Ecuador</td>
<td>90.5 (2/19)</td>
<td>88.2</td>
<td>90.5</td>
</tr>
<tr>
<td>Guatemala</td>
<td>100 (0/30)</td>
<td>97.7</td>
<td>100</td>
</tr>
</tbody>
</table>

* The sensitivity cut-off value is the mean ± 3 standard deviations of the cumulative absorbance units of sera infected with Wuchereria bancrofti and Brugia malayi sera. PPV = positive predictive value; neg = negative; pos = positive.
of these antigens has a sensitivity and specificity of 100%, and sensitivity, J rating indices, and positive predictive values of the IgG results,9,11 geographic regions tested is an improvement of previous properties. The results presented in Figures 1 and 2 confirm this.

A theoretic combination of three recombinant Ov volvulus antigens was previously reported to have a specificity of 100%.13 However, another study reported that Ov20, the major component of the cocktail, displayed a low degree of cross-reactivity with sera from individuals infected with W. bancrofti.25 Thus, we assume that the specificity of OvH2 is comparable to this cocktail. Our attempts to increase the specificity of the test by detection of IgG4 antibodies against OvH2 and OvH3 did not yield better results, since the sensitivity and specificity were slightly lower than for IgG detection. This is surprising since IgG4 antibodies in filarial infections were repeatedly shown to be more specific than the total IgG response.9,19,30 The presence of a 6-histidine tag on the hybrid protein did not interfere significantly with the specificity of the test, confirming our results.

We also evaluated the antibody responses of microfilariae-negative individuals who were exposed to the transmission of Ov volvulus to the hybrid proteins. The serum donors lived in endemic areas together with infected individuals. The sera from Ecuador were collected from putatively immunes, i.e., well-characterized persons who were probably immune to Ov volvulus infection, while the sera of microfilariae-negative exposed individuals from Togo might include sera of persons with low level and prepatent infections, as well as putatively immunes. The positive reactions of many sera from these exposed persons suggest that the early stages of the parasite induce diagnostic antibody responses against Ov33 and Ov20. This is supported by previous data that demonstrated that Ov33 is recognized by sera from patients with onchocerciasis before the onset of patency.26 Early seroconversion is conceivable, since both Ov20 and Ov33 are expressed by infective larvae of Ov volvulus,8,14 which makes the hybrid protein a sensitive tool to monitor exposure to Ov volvulus. The potential of OvH2 for diagnosing exposure to Ov volvulus transmission as well as early and light infections, might be particularly valuable, i.e., in situations where it is difficult to diagnose onchocerciasis by detection of microfilariae or microfilarial DNA, e.g., in situations after interruption of transmission and/or following prolonged treatment with ivermectin. Further studies are needed to address this question.

Consequently, our approach in constructing hybrid proteins for the diagnosis of onchocerciasis has led not only to an improvement of diagnosis but has also yielded a reagent that is easy to produce. Similar reagents might be useful in cases where diagnosis with a single protein or with cocktails of antigens is unsatisfactory.

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