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Biomarker Discovery and Assay Development to Detect Antibodies to *Onchocerca volvulus* based on OvCol-1 and Ov7

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BACKGROUND

Onchocerciasis affects ~21 million people, with 99% of cases reported in 31 sub-Saharan countries. It is a high burden disease and is therefore targeted for elimination. The control and elimination programs of the disease is primarily based on mass-drug administration (MDA) using the anti-helminthic drug ivermectin. The elimination program strategy is a multi-phase process. Due to the differences in control or elimination stages, tools needed to support the program are needed. The tools needed for monitoring and evaluation are mostly based on detecting antibodies in the population. The World Health Organization (WHO) neglected tropical diseases road map for 2021-2030 highlighted the need for better diagnostic tests to support control and elimination of onchocerciasis. WHO's target product profiles (TPP) for new onchocerciasis tests set minimum criteria for mapping as a laboratory-based assay to detect exposure to *Onchocerca volvulus (O.volvulus)* with ≥ 60% sensitivity and ≥ 99.8% specificity. The minimum criteria for tests to make MDA stopping decisions have the same specificity but higher sensitivity (≥ 89%). The requirement for very high specificity for both program use cases may necessitate the use of multiple biomarkers in combination, as studies has shown the necessary tool with a high specificity could not be achieved using a single antigen, such as the *Ov16* based antibody-based detection. Therefore, with that understanding, we sought to identify more potential antigens that could be utilized to this end. We conducted a serum epitope repertoire analysis (SERA) to identify biomarkers to detect anti-IgG, anti-IgG1, and anti-IgG4 epitopes using sera from 60 *O. volvulus* confirmed positive individuals and 60 *O. volvulus* negative individuals from Guatemala and 60 *Wuchereria bancrofti* positive sera from Haiti to control for cross-reactivity. SERA identified 22 target proteins for IgG, 18 for IgG1, and 11 for IgG4. Two identified proteins were previously described antigens, ***OvCol-1*** and ***Ov7***. We expressed these two proteins as GST-fused recombinant antigens and developed a multiplex bead assay (MBA).

OBJECTIVES

- To develop more effective tests with high sensitivity and specificity for monitoring residual infection levels in endemic areas.
- To Identify additional antigens that could be used alone or in combination with *Ov16* for Onchocerciasis MDA screening and stopping.

METHODS

SERA

SERA incorporates a large random peptide library, Next Generation Sequencing (NGS), and custom bioinformatics tools to map immunogenic antigen epitopes targeted for a given infection.
An antibody-containing patient sample (typically serum or plasma) is incubated with a bacterial library displaying random peptides at the cell surface. Antibodies bind to peptide library members that mimic the binding site on their natural protein target. Antibody (IgG, IgG1, and IgG4 subclasses) bound bacteria are magnetically separated to isolate a unique set of antibody-binding peptides for each patient. NGS is used to sequence each patient's set of millions of antibody-binding peptides (i.e. each patient's antibody epitope repertoires). From these peptide datasets, custom computer algorithms identify amino acid motifs that occur in the diseased patient samples and are absent from controls. Motifs are aligned to proteome sequences of interest to identify likely antigen candidates eliciting the patient's antibody response. The most sensitive and specific motifs and antigens are identified for the disease cohort compared to controls and additional databased samples. A validation cohort of samples is then used to evaluate the performance of the selected motifs and antigens. The best-performing antigens are candidates for testing using a simple diagnostic platform such as ELISA or lateral flow (Kamath, K, et al. Sci Rep. 10, 5294 (2020); Reifert, J, et al. J Clin Microbiol. 59, e01836-20 (2021); Pantazes, RJ, et al. Sci Rep. 6, 30312 (2016); Haynes, WA, Kamath, K, Wait,z R, Daugherty, PS, Shon, JC. Front Immunol. 12, 625311 (2021)).

Recombinant Protein Expression and Purification

The proteins were expressed using *E. coli* BL21 DE3 bacterial expression system and purified using the glutathione *S-transferase* (GST) column. Western blot was done on the purified proteins to identify the targets based on size and specificity (Tsang et.al.,1989).

SCAN HERE FOR MORE INFORMATION

RESULTS

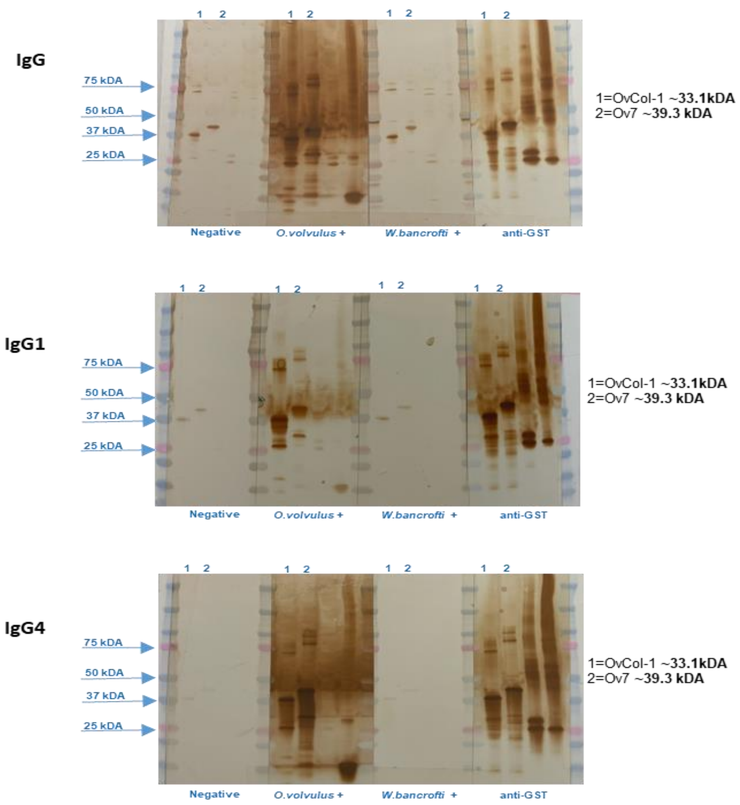

Figure 1: Western blot of the OvCol-1 and Ov7 proteins run against negative sera, *O.volvulus* positive and *W. bancrofti* positive sera using IgG, IgG1- and IgG4- specific antibodies.

Multiplex Bead Assay

The proteins were coupled to magpix beads (microspheres) in an MBA format. Both antigens showed high signal to noise (S/N) ratios against the antibodies tested(~1200 - 34000) obtained using the median fluorescent intensity (MFI) from the assay run on Magpix assay analyzer (**Table 1**). To identify the best coupling buffer, the average MFI was used to calculate the S/N) ratio. Briefly, the result from the "signal" serum (the MFI from the ***O. volvulus*** positive serum) was divided by MFI of the "noise" serum (the MFI of the negative ("normal" serum) or *W. bancrofti*). A S/N ratio greater than 1 shows that more reactivity was seen with the *O. volvulus* serum than the comparator serum sample. The higher the S/N ratio, the greater the difference is between the reactivities of the 2 sera.

Table 1: Optimized coupling of the proteins to Magpix beads for MBA showing the S/N ratio of the positive control (*O. volvulus*) against negative control and *W. bancrofti* (cross reactor)

	OvCol_1			Ov7		
	IgG	*IgG1*	*IgG4*	*IgG*	*IgG1*	*IgG4*
Negative	25	1	1	27	19	3
O.volvulus (Ov)+	32189	34206	10992	33388	36043	45063
W.bancrofti (Wb)+	29	1	1	48	2	5
S/N Negative	1305	34206	10992	1252	1931	15021
S/N Wb	1123	34206	10992	700	18021	9656
Buffer	Borate, pH 8.0	PBS, pH 7.2	PBS, pH 7.2	MES, pH 5.0	PBS, pH 7.2	MES, pH 5.0
Protein amount (ug/ 1X coupling scale)	1.0	1.0	3.0	3.0	6.3	3.0

All values in median fluorescent intensity (MFI)

After protein amount and buffer was optimized, assay linearity was determined using a standard curve. Inter-plate **(Table 2)** and Intra-plate (data not shown) variation were determined from testing the linearized positive and negative sample 24 times. Data were tabulated and inter-plate coefficient variation was calculated using Microsoft Excel.

RESULTS (CONT'D)

Table 2: Inter-plate variation of the proteins showing the coefficient of variation from running the linearized positive and negative sample 24 times on different days in consideration of assay variability and reproducibility.

IgG	Ovcol-1		Ov7	
	Neg	Pos	Neg	Pos
Mean	11.2	7463.4	15.4	8556.4
SD	2.8	607.7	3.3	1220.5
CV	24.9	8.1	21.5	14.3
Mean - 2SD	5.7	6248.1	8.8	6115.3
Mean + 2 SD	16.8	8678.7	22.0	10997.4
IgG1	**Ovcol-1**		**Ov7**	
	Neg	Pos	Neg	Pos
Mean	8	8102	8	7099
SD	6	1156	2	1243
CV	76	14	30	18
Mean - 2SD	-4	5791	3	4614
Mean + 2 SD	21	10413	13	9584
IgG4	**Ovcol-1**		**Ov7**	
	Neg	Pos	Neg	Pos
Mean	2	1738	1	19294
SD	2	316	1	1563
CV	84	18	40	8
Mean - 2SD	-1	1107	0	16167
Mean + 2 SD	6	2370	2	22420

ROC curve graphical representation showing the sensitivities and specificities of the both proteins for IgG. IgG1 and IgG4 reactivity. Determination of the cut-off value and assay performance (sensitivity and specificity) was obtained using R statistical software and pROC package. To combine the results of diagnostic tests, we used a method which finds an optimal linear combination of MFI values of multiple antigens. In this study, a total of 494 *O. volvulus* positive, 64 *W. bancrofti* positive, 197 normal human serum (negative sera) were tested.

Table 3: Performance of *O. volvulus* in MBA

	OvCol-1			Ov7			Combo
	IgG	IgG1	IgG4	IgG	IgG1	IgG4	IgG4*
Threshold	58.6	116.5	5.5	62.5	7.5	2.5	0.241
Sensitivity	90.4	85.6	70.7	83	90.4	93.4	93.9
Specificity	90.5	95.4	97.9	91.2	78.9	95.4	95.4

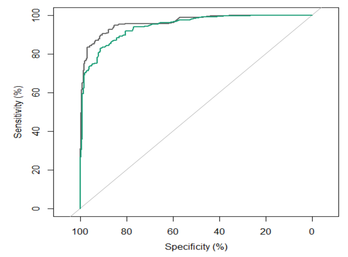

Figure 3A: ROC curve of both proteins against IgG

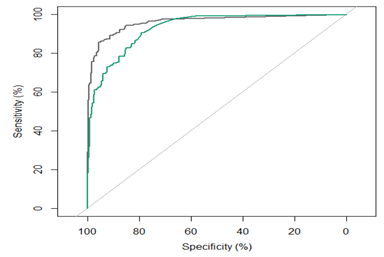

Figure 3B: ROC curve of both proteins against IgG1

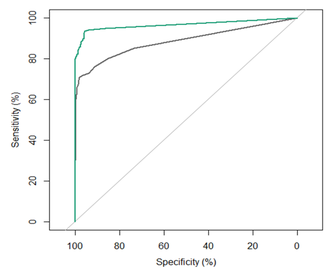

Figure 3C: ROC curve of both proteins against IgG4

DISCUSSION and CONCLUSION

Antigens that can be used for immunodiagnosis of onchocerciasis could benefit both control programs (at elimination mapping and MDA stopping stages) as well as individual patient (travelers and immigrants) diagnosis. In this study, using SERA, potential candidates were identified and evaluated by both immunoblot and MBA. The proteins in this study (***OvCol-1 and Ov7***) were highly specific for *O. volvulus* and showed the littlest cross reactivity with the negative control or *W. bancrofti* positive sera in either IgG4-specific Western Blot or MBA. These proteins demonstrated very strong (~1200 - 34000) signal to noise ratio (S/N) compared to potentially cross-reactive sera from persons with *W. bancrofti* infection in multiplex assays. Two defined sets of *O. volvulus*-positive sera, from different geographical regions were used to optimize and validate the antigens. As individual antigens, IgG4 reactivity of sera from *O.volvulus* infected persons was higher with ***OvCol-1*** having a specificity of 97.9% compared to the ***Ov7*** of 95.4% specificity. ***Ov7*** produced a more sensitive test than ***OvCol-1*** across the all the 3 antibodies tested. By themselves, none of these proteins met the WHO criteria of target product profile (TPP) for onchocerciasis stopping determination (specificity ≥98.9%, and sensitivity ≥89%). However, the combination of both proteins only increased the sensitivity to 93.9% but not specificity when detecting IgG4. It may be possible to use these two antigen to develop an assay for onchocerciasis MDA stopping decisions in combination with Ov16 or other *Onchocerca* antigens, more tests is being done to this effect.

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