

A COCKTAIL OF RECOMBINANT *ONCHOCERCA VOLVULUS* ANTIGENS FOR SEROLOGIC DIAGNOSIS WITH THE POTENTIAL TO PREDICT THE ENDEMICITY OF ONCHOCERCIASIS INFECTION

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Abstract. We report here the evaluation of the potential of a serologic test to determine the endemicity of onchocercal infection in hyper, meso, and hypoendemic communities by the detection of antibodies to a cocktail of recombinant antigens. Parasitologic parameters of infection prevalence and intensity were compared with serologic results. Infection prevalence by serology was consistently but not significantly higher than that defined by parasitology. Differences between the communities defined by microfilarial load (CMFL) and a measurement of *Onchocerca volvulus*-specific antibody levels (serologic index [SI]) were similar. When stratified by age, differences were more significant in the younger age groups. If a sentinel population of 5-15-year-old individuals was used to compare communities, all could be equally ranked by serologic and parasitologic parameters. The SI of the sentinel population gave a better distinction between each community than the SI of the whole and would be sufficiently sensitive to measure the changes in endemicity that would be required for onchocerciasis control programs.

An estimated 18 million people are infected by the parasitic filaria *Onchocerca volvulus*, the causative agent of human onchocerciasis or river blindness. Control of the disease is being attempted world-wide by vector control and/or large scale distribution of ivermectin, a drug effective against the pathogenic microfilarial stage of the parasite. The Onchocerciasis Control Program (OCP) in West Africa has been successful in lowering the risk of blindness due to infection with *O. volvulus*. Thirty million people in 11 West African countries have been estimated to be protected from damaging ocular lesions caused by this parasite.¹ A diagnostic test is urgently required by control programs such as the OCP to both monitor the success at reducing the prevalence of infection or to monitor recrudescence in areas where transmission has been successfully interrupted and vector control ceased.² The method of parasitologic diagnosis is the examination of skin snips for the presence of microfilariae (mf) collected with punches and, although exquisitely specific, it is not very sensitive, particularly in areas of low transmission. Skin snips are also unable to detect prepatent infection, which would result in a critical delay in the detection of recrudescence of transmission. Consequently, control operations would be instigated later and may require implementation on a much wider scale. Finally, with the increase in the prevalence of blood-borne viruses, the substitution of the skin snip is desirable due to problems of sterilization of the expensive punches.

Serologic assays based on antibody detection are one possible alternative method of determining infection by this parasite. There have been a number of reports of tests capable of this (for a review see Bradley and Unnasch³). We have previously reported the development of a serologic test able to detect specific *O. volvulus* antibodies to a cocktail of three recombinant antigens.^{4,5} This test compares favorably with any so far described, and the use of a cocktail of all but one of the same antigens has been assessed by the World Health Organization.² Since this test is highly specific,^{6,7} infections with other nematodes, frequently coendemic with *O. volvulus*, especially other human filarial parasites, do not affect

the results. We have also shown that responses to this assay decrease in a community after vector control⁶ and are beginning to decrease two years post-ivermectin treatment.⁸ If antibody detection assays are sensitive to the endemicity/intensity of infection in a community, it may be possible to calibrate the assay and provide a serologic index (SI). This measurement could then be monitored in at risk communities and if it increases above a defined level, appropriate control measures can be reinstated. Here we report the evaluation of a diagnostic cocktail of antigens to detect infections in hyper, meso, and hypoendemic areas of onchocerciasis in the Sanaga Valley of Cameroon.

PATIENTS AND METHODS

Informed consent was obtained from the patients or their parents or guardians, and guidelines for human experimentation from the Ministry of Health Cameroon were followed in the conduct of clinical research. This study received ethical clearance from the Cameroonian Ministry of Public Health.

Individuals living in areas endemic for onchocerciasis. Three villages in the Sanaga valley of Cameroon were selected from previous parasitologic studies as being representative of hypo, meso, and hyperendemic areas for onchocerciasis as defined by Prost and Hervouet.⁹ All areas were also endemic for two other human filariases: loasis and *Mansonella perstans* filariasis. The names of these villages were Nkolfepe, Nkolntsa, and Ntsan Mendouga, respectively. All individuals were informed of the purpose of the study and were subjected to a full questionnaire to ensure that they had lived continuously in the same area. Any individuals with a history of anti-filarial drug treatment were excluded from the study. Consenting individuals greater than five years of age underwent a parasitologic examination that involved the taking of two skin snips, one at each iliac crest, using a 1.5-mm Holth sclerocorneal punch.¹⁰ Snips were incubated for 24 hr in saline prior to counting. Individual microfilarial loads were calculated as the average of the counts recorded for each skin snip. Ten milliliters of venous blood was drawn



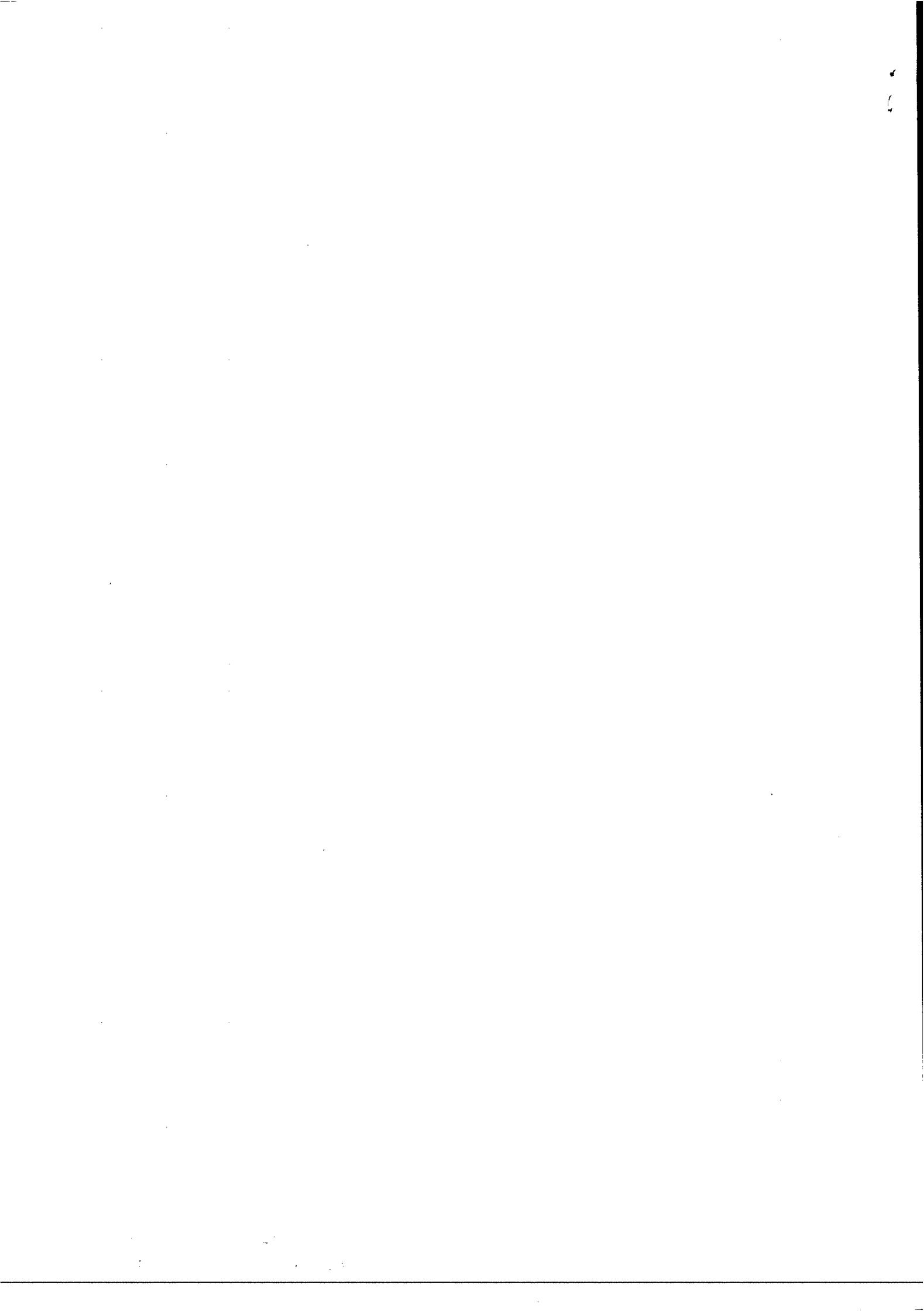


TABLE 1

Comparison of the population characteristics and the parasitologic and serologic indices of infection in three endemic areas*

	Location		
	Ntsan Mendouga	Nkolntsa	Nkolpép
N (Male/Female)	199 (90/109)	128 (57/71)	149 (80/69)
Mean age (SD)	24.53 (18.76)	26.06 (19.19)	25.81 (19.91)
Geometric mean Mf			
Males/Females	5.142/4.221	1.352/0.839	0.206/0.132
Mf+ (%)	134 (67)	54 (42)	17 (11)
Seropositive (%)	141 (71)	70 (55)	24 (16)
CMFL	8.117	1.562	0.279
SI	0.597	0.423	0.103

* Mf = microfilariae; CMFL = community microfilarial load; SI = serologic index.

from each individual using a vacutainer, allowed to clot, and the serum was separated and stored at -70°C until use. All individuals who had positive skin snips were subsequently treated with ivermectin.

Controls. A village was selected to provide a panel of control sera from an area that was located more than 50 km from the nearest vector breeding site where there was no onchocerciasis transmission. Individuals were carefully selected as never having visited or lived in an endemic area. This area was also known to be endemic for loiasis and *M. perstans* filariasis. Individuals also gave blood and serum that was separated as described in this report.

Antigens. Three recombinant antigens termed Ov MBP/10, Ov MBP/11, and Ov MBP/29 were produced and purified as previously described.⁵ They were used as fusion proteins with the maltose binding protein (MBP) of *Escherichia coli*. The MBP was obtained commercially (New England Biolabs, Beverly, MA).

Enzyme-linked immunosorbent assay. The ELISA to detect parasite specific IgG was performed as described previously with the antigens at a coating concentration of $1\ \mu\text{g}/\text{ml}$ for MBP alone, and a final concentration of $2\ \mu\text{g}/\text{ml}$ for OvMBP/10, OvMBP/11, and OvMBP/29 in the proportion 50% Ov MBP/11, 25% Ov MBP/10, and 25% Ov MBP/29.⁵ The ELISA was performed using the recombinant antigens still fused to MBP. Since anti-MBP responses are occasionally found in serum, an anti-MBP assay was performed in parallel and anti-MBP responses were subtracted. A cut-off value was established by taking the arithmetic mean of the results obtained from the control village plus three standard deviations.

Sera were used at a dilution of 1:200 throughout the study. This dilution was determined by titrating a representative sample of the sera to ensure that values were on the logarithmic part of the titration curve. All values were corrected to the mean of four replicates of a standard positive reference pool.

Indices and statistical analyses. Two parasitologic indices were calculated to evaluate the endemicity levels in the three villages. Parasitologic prevalence was the percentage of patients with mf found in the skin snips. Intensity of infection in the villages was evaluated using the community microfilarial load (CMFL), which is currently the reference index used in the OCP.¹¹ The CMFL is the geometric mean of the individual mf loads including negative counts in those patients greater than 20 years of age. The calculation was

performed using the $\log_e(x + 1)$ transformation, where x is the individual mf load. When intensity of infection was evaluated by age, the geometric mean microfilarial loads were calculated similarly to CMFL, but for individuals of the restricted age group.

Two serologic indices were used. First, the seroprevalence, defined as the percentage of patients who had optical density (OD) values at 492 nm values above the cut-off value obtained from the control patients. Second, the SI, which was defined as the geometric mean OD at 492 nm using the $\log_e(\text{OD} + 1)$ transformation of the population.

Since the distribution of responses was not normal, but positively skewed, nonparametric analyses had to be performed throughout the study. The chi-square test was used to compare parasitologic or serologic prevalence and the Mann Whitney-U and Kruskal-Wallis tests were used to compare mean microfilarial loads and SI. The relationship between parasitologic and serologic results at the individual level was evaluated using the chi-square test and Spearman's rank tests. All statistical tests were performed using the Statview program (Abacus Concepts, Berkeley, CA) on a Macintosh (Apple Computer, Inc., Cupertino, CA) computer.

RESULTS

Patient characteristics. The numbers of individuals evaluated in each village, sex distribution, and mean age are summarized in Table 1. The mean ages and the sex distribution in each village were not significantly different.

Parasitologic indices. Table 1 shows the parasitologic indices measured in the three communities. The parasitologic prevalences recorded in Ntsan Mendouga, Nkolntsa, and Nkolpép were significantly different ($P < 0.0001$). Similarly, the intensity of infection assessed by the mean mf densities in the total number of patients differed significantly between the three villages ($P < 0.0001$). The values of the parasitologic prevalence confirm that these villages are hyper, meso, and hypoendemic for onchocerciasis, respectively according to the criteria defined by Prost and Hervouet.⁹ These different levels of endemicity are due to the geographic locations of the three communities: with the parasitologic indices decreasing as the distance from the Sanaga river increases. In each village, the parasitologic prevalence and the mean microfilarial densities were not significantly different between males and females, although there was a trend for the indices

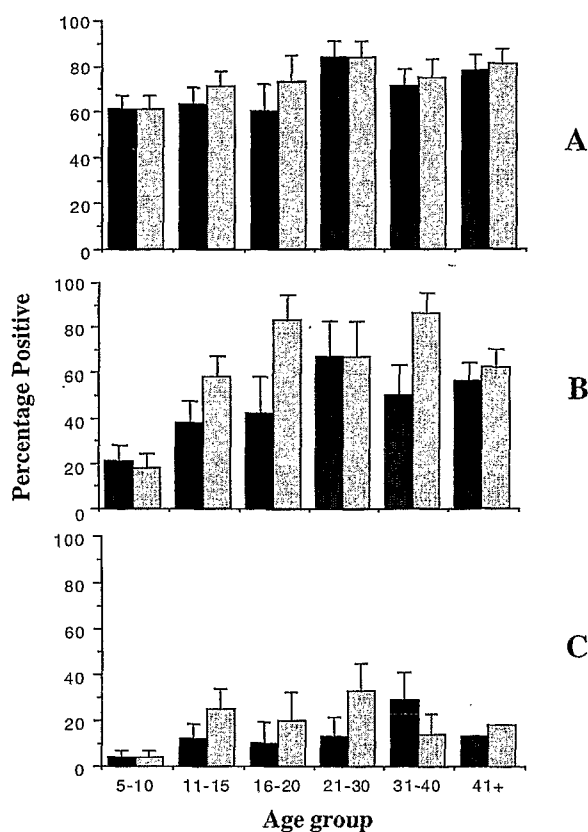


FIGURE 1. Comparison of parasitologic (closed bars) and serologic prevalence (shaded bars) of infection presented by age in three communities: A, Ntsan Mendouga (hyperendemic); B, Nkolntsa (mesoendemic); and C, Nkolfep (hypoendemic). Data are presented as the percentage positive in each age group. Bars represent the standard error for each age group.

to be higher in males than females. For further analyses, the sexes were therefore considered together.

Evolution of parasitologic prevalence with age is shown in Figure 1. In all three villages, the trends appear similar. The prevalence increased to the age of 20 and then plateaued or decreased. The prevalence of individuals ≤ 20 years of age was combined and compared with the similarly combined results of those more than 20 years of age and was significantly higher above the age of 20 in all study areas ($P < 0.002$).

The intensity of parasite load with age is shown in Figure 2. The mean mf densities increased continuously in Ntsan Mendouga apart from an apparent slight decrease at the age of 16–20, which was not statistically significant from the age groups on either side. In Nkolntsa, there was a progressive increase in the prevalence up to the age of 21–30 and the load decreased but not significantly above the age of 30 ($P = 0.26$). In Nkolfep, parasitologic intensity was low until the age of 20 when there was a significant increase ($P = 0.03$) when the ≤ 20 -year-old age group was compared with those greater than 20 years of age.

Serologic indices. The seroprevalences were calculated using the cut-off value obtained from the control patients (OD at 492 nm = 0.18). This and the SIs recorded for the whole communities are shown in Table 1. Both indices were significantly different between the three communities ($P <$

0.0001 and $P < 0.0001$). In each village, the general trend for both indices is an increase with age up to the age of 16–20 or 21–30 followed by a plateau. When the SIs were compared between those ≤ 20 and those > 20 years old, the results were significant for all communities (Ntsan Mandouga, $P = 0.002$; Nkolntsa, $P = 0.0007$; and Nkolfep, $P = 0.043$) (Figures 1 and 2).

Comparison of parasitologic and SIs. Using both the seroprevalence and SI recorded for the whole community, the villages could be ranked in an order similar to the endemicity levels recorded by parasitologic methods. However, in each village, the seroprevalence appeared slightly higher than parasitologic prevalence. With regard to the quantitative indices, the differences between the three communities were the same by CMFL and SI.

Analysis by age group showed that seroprevalence appeared higher than parasitologic prevalence in most age groups (Figure 1). Although this result was more marked in the meso and hypoendemic communities, in no case was the difference found to be significant.

Using the SI recorded for each age group, the communities could be ranked in an order similar to the endemicity levels determined by parasitologic methods. Comparisons between the SIs for each community for each age group were significant in all cases, but the degree of significance between the communities was greater ($P < 0.0001$) in the 5–15- and ≥ 41 -year-old age groups. Although, the mean microfilarial densities recorded in the 16–20-, 21–30-, and 31–40-year-old age groups were significantly different when compared between all the communities, the differences were not significant when just the hyper and mesoendemic communities were compared.

Parasitologic and serologic prevalence in the communities were therefore compared only in the 5–15-year-old age group (Table 2). The communities were clearly ranked by both parameters, with the serologic prevalence giving slightly higher results. The SI of each was also calculated as for the total population that could be compared with the CMFL and SI of the whole community (Tables 1 and 2). Since the communities can be clearly distinguished by this parameter and differences between the SIs for the 5–15-year-old age group were more pronounced than the same values for the total population, this population can be considered as an appropriate sentinel population for monitoring any increase or decrease in infection levels in a community.

Although we were looking only for a community based diagnostic test and have compared the level of endemicity ascertained by parasitologic and serologic means, it was also of interest to evaluate the correlation between the microfilarial load and serologic levels of individuals. We therefore analyzed the relationship between positivity and negativity of infection and the level of infection determined by mf burden or OD of individuals in age-stratified populations in all communities. The two variables correlated significantly for the communities when considered as a whole ($P < 0.01$), but when the results were analyzed by age group they were significant only in the 5–15-year-old age groups in the meso and hypoendemic communities.

DISCUSSION

The aim of the present study was to evaluate the ability of an SI for measuring the infection intensity/prevalence of

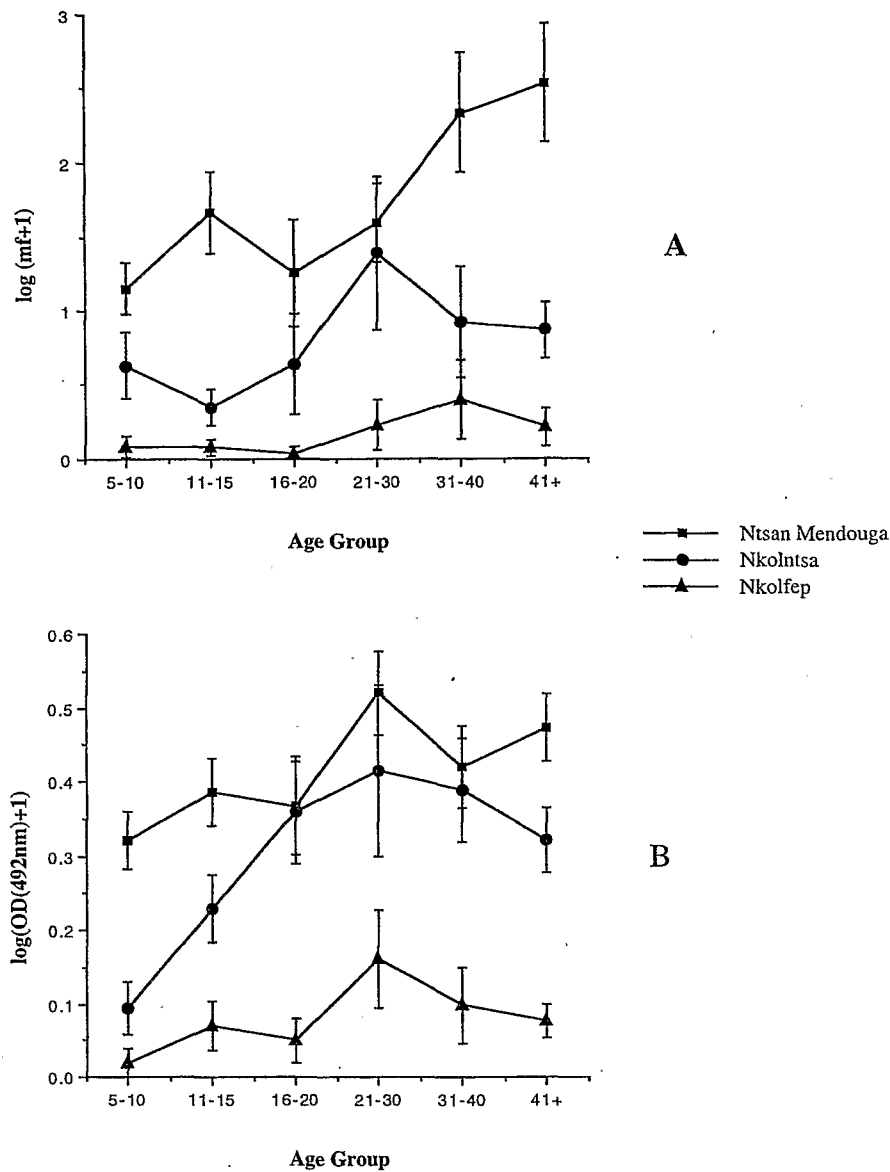


FIGURE 2. Intensities of infection by age: individuals in three endemic areas grouped by age. Values are the mean $\log_e(\text{microfilariae [mf]} + 1)$ (A) or as the mean $\log_e(\text{optical density [OD]}(492) + 1)$ (B). Bars represent the standard error for each age group.

TABLE 2

Comparison of seroprevalence and parasitologic prevalence obtained for a sentinel population 5–15 years of age and a measure of the population-specific antibody level for this group (SI) in three endemic areas*

Location	Age 5–15 Mf+ (%)	Age 5–15 years seropositive (%)	Age 5–15 years SI
Ntsan Mendouga	54/95 (56%)	59/95 (62%)	0.414
Nkolntsa	19/58 (33%)	20/58 (34%)	0.167
Nkolfep	5/70 (7%)	8/70 (11%)	0.037

* SI = serologic index; Mf = microfilariae.

an area and to compare it with the standard parasitologic parameters for determining endemicity. Three communities were selected on the basis of previous parasitologic surveys in the area (Boussinesq M, unpublished data) and for being at increasing distances from the blackfly breeding sites (the Sanaga River). The purpose was to compare typical hyper, meso, and hypoendemic areas. The parasitologic data showed an infection prevalence of 66%, 43%, and 11% for Ntsan Mendouga, Nkolntsa, and Nkolfep, respectively. Prost and Hervouet⁹ defined a hyperendemic area as one having a prevalence of greater than 60%; a mesoendemic one as having a prevalence of between 35% and 60%, and a hypoendemic one with a prevalence of less than 30%. The areas selected for this study therefore fitted well into the three different endemicity ranges.

The prevalence of infection defined by either serologic or parasitologic means of the whole community and as age-

stratified subpopulations were compared. Although the prevalence of infection in the community was consistently but not significantly higher when estimated by serology, the three communities were clearly distinguishable by both methods. When these results were age stratified, the gap between serologic and parasitologic positivity was more apparent in the communities of lower endemicity. It is impossible to evaluate the true prevalence of infection in any community because many infections remain cryptic. Skin snip data are likely to provide an underestimation because they cannot detect prepatent or low level infections, so it is not unexpected that serologic data would give higher values. Antibody detection assays, such as the one used here, cannot distinguish between exposure to infection, infection, or a history of infection. The fact that serology identified more individuals in the meso and hypoendemic areas than parasitology, whereas, this difference was not as great in the hyperendemic area, suggests that it is detecting low level infections and thus may be more sensitive as an indicator of prevalence of infection.

It is also useful to be able to describe the intensity of infection in a community. The CMFL has been used as such a parameter,¹¹ but if the skin snip is to be superseded, it is also useful to determine if serology could provide a sensitive method. The results presented here suggest that although the communities were statistically different from each other, if adults were evaluated, serology would not be as useful as CMFL. The statistical analyses of the communities, when age stratified, showed that the differences between the communities were more pronounced in 5–15-year-old children. This would suggest that a sentinel population of this age group would be the most useful indicator group for determining the intensity of infection in a community.

Examination of a sentinel population of this age group offers the advantage that sampling the population is relatively easy since it is possible to visit the schools. When parameters of infection were compared in this age group, parasitologic and serologic prevalences were very similar. The SI for this age group also showed pronounced differences between the communities.

The major aim of developing this diagnostic assay was to provide a method capable of monitoring recrudescence of infection after control measures have ceased in the OCP.² Our previous work has indicated that community antibody levels decreased after both vector control and treatment with ivermectin.^{6,12} This, when combined with the results shown here, indicates that this test is sensitive to levels of infection within a community and that it will be possible to monitor the increase (or decrease) of these parameters effectively. Perhaps the best method for defining reinfection within a community is to measure incidence of infection, i.e., new infections. A test such as this could be important in assessing the impact of the African Program for Onchocerciasis Control on the transmission of *O. volvulus* following prolonged annual treatment with ivermectin. Studies have shown that prolonged ivermectin treatment can dramatically reduce transmission levels in flies.¹³ Since children less than five years of age are not given ivermectin, a serologic assay could be used to assess the prevalence and intensity (thus incidence) of infection in each new entry of children into the ivermectin control program. This test has been developed

as an ELISA using fingerstick blood spots (Bradley JE, unpublished data), which may be more acceptable to obtain in this age group than skin snips.

The test described here has been developed for community based diagnosis as an epidemiologic tool. Unfortunately, results indicate that such an assay is not useful for individual diagnosis because microfilarial levels and *O. volvulus*-specific antibody levels did not show any significant association. This is probably due to the fact that the ODs, as a measurement of antibody load, reached a plateau in the older age groups. To evaluate this further, it would be necessary to quantify the actual immunoglobulin level in each individual to see if this is related to worm burden.

The ability to measure the level of infection in a community is not only useful for monitoring the success (or failure) of a control program but could also predict the likelihood of prevalence of disease. Prost and Hervouet⁹ found that there was no disease apparent in hypoendemic areas, but it could become intolerable in hyperendemic villages. Prevalence of infection, however, is not a good indicator for the severity or likelihood of the presence of disease in a community, but CMFL as a measure of infection intensity has been shown to be a better parameter.^{11,14} The relationship of CMFL as an indicator of infection intensity with eye disease was investigated in both forest and savannah regions of West Africa.^{15–18} A clear linear relationship between most indices of ocular onchocerciasis and CMFL were found in savannah but not forest regions.¹⁸ Serologic prevalence and/or the SI index could correlate with disease manifestation as does CMFL and provide an evaluation of the risk of developing complications of infection for individuals living in areas with a high serologic value. The area studied here was not ideal for evaluating such a possibility because the prevalence of severe eye pathology in the community was low. In addition, the CMFL value of the hyperendemic area was < 10, the threshold at which the risk of blindness becomes high.¹⁸ It would be necessary to examine a large number of communities, particularly in savannah areas, to evaluate this possibility.

In conclusion, serology can provide a measurement of infection, particularly if the prevalence of infection in a community is required. The use of the 5–15-year-old sentinel population is better for distinguishing between communities and detecting recrudescence of transmission. The results presented here suggest that it is possible to impose an SI index on a community that can be monitored over time to evaluate the success (or failure) of any onchocerciasis control program.

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