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SEROEPIDEMIOLOGY OF SCHISTOSOME CERCARIAL PENETRATION ENZYME IN A COMMUNITY IN KENYA (AUGUST 1989)

FINAL REPORT

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This report is based on the research project carried out with the support of the U.S. Agency for International Development through a grant to the Charles R. Drew University of Medicine and Science and the cooperation of Drew University, The Johns Hopkins University , University of California, San Francisco and University of Nairobi. The main issue which is addressed in this work relates to the detection of an immune response to cercarial elastase in people who habitually contact infected water in a particular locality in Kenya.

Cercarial elastase was purified at Drew University and sufficient anti-elastase antigen and ELISA plates were prepared and sensitized at University of California San Francisco. Supplies of additional cercariae and cercarial secretions were prepared by Dr. Shiff at Johns Hopkins University and were dispatched to Dr. McKerrow and myself.

My collaborators in the field work in Kenya were Prof. George Kinoti, Department of Zoology, University of Nairobi, assisted by two university technicians and Dr. Clive Shiff, Johns Hopkins University. A medical student, Ernest Rector from University of California San Francisco, was also present for part of the time. His travel and arrangements were made by Dr. Jim McKerrow and he came along for the experience.

Field survey according to the agreed protocol was to be carried out in the Kirinyaga and Morangu Districts of the Central Province Division of Mwea and Kandara. The Mwea irrigation scheme is over 6,000 hectares in extent and grows most of the rice produced in Kenya. It has a population of over 30,000 people who live in villages located around the scheme. It lies at the foothills of Mount Kenya, at an altitude of 1,200 meters above sea level and about 100 Km north east of Nairobi.

Prof.Kinoti had visited the areas and made local arrangements with headmaster, teachers, health officials and administrators. A special trip was made to the office of the President to obtain official approval and a permit for the field work.

This study was carried out as a single cross sectional investigation among school children attending four schools in the Central Province. Children were examined parasitologically to determine the overall prevalence of <u>schistosomiasis</u> and other

helminth infections and serologically to see if there was any response to cercarial elastase and to crude schistosome antigens.

SELECTION OF THE STUDY SIZE

In order to survey elastase antibody levels in communities exposed to high and low transmission of schistosomiasis, samples of school children from communities living under these conditions were examined. School children from standards 2-5 (8-13 yrs old) form the main study group. Three of the schools, Muthangauta, Mutithi and Mukou serve communities living in a rice growing irrigation area (Mwea District) where previous information has revealed high transmission rates of Schistosoma mansoni. Two of these schools had recently participated in a mass chemotherapy campaign using Praziquentel. The Mukou school serves a community in the same district where no such chemotherapy was under way.

The fourth school, Kiguoyo, serves a dryland farming area in the Kandara District where there is no schistosomiasis transmission. However, since a major objective was to determine whether other common helminths would interfere with the detection of anti-elastase antibodies, the population at this school was selected to represent proportionately more individuals positive for Ascaris or hookworm than the other schools. This was based on the results of a survey recently carried out by the Division of Vector Borne Diseases.

Urine and stool specimens were collected from approx 600 school children from four schools, three in the Mwea and one at Kandara. All the specimens were brought to Nairobi and stool samples examined fresh using Kato thick smear, then preserved in 10% formal saline, sedimented, centrifuges and examined microscopically (to ensure that all intestinal parasites could be detected). A number of children were re-examined when there was some doubt in the diagnosis. Schistosome eggs were counted and expressed as eggs per gram of stool. However, for the analysis, results were grouped in four catagories, negative; 1-50 egg/gm (low infection); 51-300 eggs/gm (moderate infection) and greater than 300 eggs/gm as high infection. Hookworms, Ascaris and Trichuris infections were recorded, but egg counts were not made.

Following these examinations, small quantities of blood were drawn from selected children by finger prick. These specimens were brought back to Nairobi and subsequently examined by ELISA for antibodies to schistosome parasites, and to the cercarial elastase.

Unfortunately, a number of serum specimen from Muthangauta school were lost, the remainder, were included with those from Muthiti school as both schools serve the same general community. Thus the study was carried out on three discrete groups, a high prevalence area with organized chemotherapy, a high prevalence area with some recent chemotherapy targeted on children, and a low prevalence area without recent chemotherapy.

ELISA was carried out using normal methods with elastase antigen coated plates. Sera were diluted 1:20, 1:100 and 1:200 and goat anti-human IgM/ HRPO conjugate at 1:1000 was used as detector antibody. The substrate used was ABTS and plates were read at 405 nm after 30 min incubation at room temperature.

The test system with cercarial elastase had been standardised previously and shown to discreminate between controls and patients. In this work the cercarial elastase was used in an attempt to determine patterns of exposure, not current infections. Therefore there has been no attempt to identify a "cut-off" optical density in the ELISA. This is not appropriate to the study. What is important is to see which group of people show evidence of exposure to cercariae. This is best done by classifying people into age or sex groups, community groups etc., with defferent risk factors for exposure and then determining the mean response for those groups.

Optical density measurments carried out in duplicate for each participant were averaged if within 30%, and the average value used in the analysis. The distribution of the study population was compared for sex and parasitological diagnosis by using the chi2 statistic. To achive a normal distribution of the optical densities, square root transformation was performed prior to statistical analysis. The two sample "t" test was used to compare the mean otical densities of two groups and a one-way analysis of variance (ANOVA) was used if more than two groups were being compared.

Equipment supplied to the university of Nairobi by the project as well as equipment in the Department of Zoology, University of Nairobi was used in the work.

RESULTS

Overall the study population showed a mean age of 10.96 years; range 8-16 yrs, with 48.6% male and 51.4% female. There is no bias in the sex distribution in the sample, and apart from Kiguoyo school, the age distribution in the sample is similar.

The two schools Muthangauta and Muthiti serve communities where some of the population had participated in mass chemotherapy show low levels of schistosome infection detected by stool examination. However, it is unlikely that this treatment program had interfered with the patterns of exposure to cercariae as only a small proportion of the overall community was involved in the program.

There is no evidence of an association between O/D measured for the schistosome antigen and hookworm or Ascaris infections. However it is noteworthy that a significantly higher value in mean O/D is found for those infected with S.mansoni than those found negative by stool examination.

There is also a difference in the elastase O/D between girls and boys (P= 0.0379), with the former showing the higher response. This is likely to be associated with domestic activities allocated to the young female population bringing them into contact with infective water more frequently than boys and is often a feature of epidemiological surveys carried out in areas endemic for schistosomiasis

There are considerable differences between the mean O/D's measured at the three schools. This is realized clearly when the data from the high transmission areas are combined and compared with those from the low transmission area. The second group of children with a different response come from Kiguoyo school. This school serves a community living in a dryland farming area where there is no transmission of schistosomiasis. These children have low mean O/D values for elastase whereas the children living in communities where there is greater risk of exposure to cercariae have significantly higher mean values for this parameter. The mean O/D at Kiguoyo is significantly less than in the combined other schools (P=0.0004). No evidence fo S. haematobium was found in the entire survey.

It is difficult to account for the variation in mean O/D between the schools in the high transmission area, particularly when it is the school with the highest prevalence that displays a low mean O/D. This may be a factor of the exposure pattern in the community, with the test being sufficiently sensitive to discriminate between different levels of cercarial bombardment to which people are exposed. Unfortunately it was not possible to study the behavior patterns and risk of infection of the children who participated in this study and follow up information is necessary for further interpretation of these results.

ELISA tests were also performed on the sera collected using cercarial antigen and whole worm antigen. This is to be expected in an area where schistosomiasis is endemic. The antibodies detected by such antigens are of long duration and do not give information on the current state of infection or exposure. What is interesting is the significant difference noted in the mean O/D figures for persons with or without the schistosome infection. This is probably a reflection on the low titre recorded at Kiguoyo, and the absence of infection at that school. It remains to be seen whether the elastase test will be of value in individual diagnosis.

Clearly. the use of cercarial elastase as a stage specific antigen will have an important role to play in future epidemiological studies. Further work needs to be done to optimize the ELISA procedure to make it more sensitive for testing individuals, particularly if it is to be used as an indication of personal exposure. However to be useful as an epidemiological tool, there must be additional information on the duration of the antibody response, as well as its correlation with natural fluctuation of cercarial prevalence in the water. Such studies are planned and will be carried out in the near future.

ABSTRACT

Antibody levels to *Schistosoma mansoni* cercarial elastase were measured by ELISA in a cross sectional study among children from four schools in Central Kenya. Three of the schools serve communities living close to a rice irrigation scheme where there is a high prevalence of *S. mansoni*, the fourth school serves a community where the prevalence is low. Optical densities to elastase antigen measured in the high prevalence, high transmission area, were higher than those detected in the low transmission area (P=0.0001). Girls also showed higher O/D than boys, which may be associated with domestic chores and more water contact than seen among the boys. Measurement of elastase antibodies has potential as a serological tool.

INTRODUCTION.

The process of analyzing transmission patterns of schistosomiasis requires information on the nature and extent of exposure to cercariae by target groups of people under study. This is important if one wants to determine whether people, having been treated, are being re-exposed to infection, to help define high risk behaviour or to help identify the locality or extent of transmission sites. Additionally information on exposure to cercariae would be crucial if the protective effects of a test vaccine were to be assessed. This would be forthcoming if it is possible to determine whether the study population or components of

the population have recently been exposed to schistosome cercariae.

Presently studies to determine exposure patterns (Husting,1970; Dalton and Pole, 1978; Wilkins et al, 1987;) involve complex observation components to detect and describe the normal activity patterns of the study group. There is need for a serological test which can be used to measure the extent of exposure to schistosome cercariae both from the individual and group standpoint. McKerrow et al 1985, described the separation and purification of an elastinolytic protease from cercariae of S. mansoni, and in a subsequent paper, Toy et al (1987) showed that the enzyme was immunogenic and there was a strong but time limited immunological response which could be detected from sera of monkeys exposed to single or multiple challenges of cercariae. In these animals as well as in mice the antibody response was predominantly IgM and the titre peaked approximately nine weeks after exposure. No enhancement of this titre was seen following multiple exposure to S. mansoni cercariae (Toy et al,1987). In contrast IgG as well as IgM antibodies to cercarial elastase were detected in sera from humans infected with schistosomiasis. Control sera taken from naive humans show low optical densities (O/D) in the IgM, but with a wider range of response in the IgG (Toy et al, loc.cit.). This was confirmed with 11 sera taken from U.S. volunteers and analysed in Baltimore.

This would suggest that antibodies to the cercarial elastase may be of value in detecting whether people have been exposed recently to

cercariae, therefore the following work was undertaken to determine the potential epidemiological significance of the cercarial elastase in an ELISA test system. This was be done by examining antibody levels to cercarial elastase in children living in several localities with different degrees of schistosome endemicity in Kenya.

MATERIALS AND METHODS

1. The Study Population:

This study was carried out as a single cross sectional investigation among school children attending four schools in Central Kenya. Children were examined parasitologically to determine the overall prevalence of bilharzia and other helminth infections and serologically to see if there was any response to cercarial elastase and to crude schistosome antigens.

2. Selection of Study Sites:

In order to survey elastase antibody levels in communities exposed to high and low transmission of schistosomiasis, samples of schoolchildren from communities living under these conditions were examined. School children from Standards 2-5 (8-13 yrs old) form the main study group. Three of the schools, Muthangauta, Mutithi and Mukou serve communities living in a rice growing irrigation area (Mwea District) where previous information has revealed high transmission rates of Schistosoma mansoni. Two of these schools had recently

participated in a mass chemotherapy campaign using Praziquantel. The Mukou school serves a community in the same district where no such chemotherapy was under way. The fourth school, Kiguoyo, serves a dryland farming area in the Kandara District where there is little or no schistosomiasis transmission. However, since a major objective was to determine whether other common helminths would interfere with the detection of anti-elastase antibodies, the population at this school was selected to represent proportionately more individuals positive for Ascaris or hookworm than the other schools. This was based on the results of a survey recently carried out by the Division of Vector Borne Diseases.

Unfortunately, a number of serum specimens from Muthangauta school were lost, the remainder, a total of seven were included with those from Mutithi school as both schools serve the same general community.

Thus the study was carried out on three discrete groups, a high prevalence area with no organised chemotherapy, a high prevalence area with some recent chemotherapy targeted on children, and a low prevalence area without recent chemotherapy.

3. Parasitology:

All children examined in this work provided duplicate stool and single urine specimens. Sera were obtained from finger prick and were collected in 100 ul haematocrit tubes which were later centrifuged and separated from red cell debris. All stool specimens were examined

fresh by Kato thick smear, then preserved in 10% formal saline, sedimented, centrifuged and examined microscopically. Schistosome eggs were counted and expressed as eggs per gram of stool. However, for the analysis, results were grouped in four categories, negative; 1-50 e/gm; 51-300 e/gm and > 300 e/gm. Hookworm, Ascaris and Trichuris infections were recorded, but egg counts were not made.

4. The Test Systems:

a) Cercarial Elastase Antigen (ELAS): Cercarial secretions were collected by stimulation of cercariae with skin lipids in water. Following filtration the solution was lyophilized and subsequently fractionated. The elastase was purified from cercarial secretions using molecular sieve chromatography followed by HPLC chromatofocussing (McKerrow et al, 1985). Approx 68 mg of crude secretory protein yield 0.5 mg of elastase. This is the product of approx. 2 x 10^6 cercariae. Plates were coated with 500 ng/well in 50 ul TBS (10 mM Tris pH 7.4/ 150 mM NaCl) overnight at 4 C. Following they were rinsed in TBS, dried and packed dry for shipment.

b) Cercarial Antigen (CA): Approximately 200,000 S. mansoni cercariae were collected from Biomphalaria glabrata, filtered to remove snail debris, centrifuged and washed in distilled water, then homogenized in 1.0 ml PBS. The homogenate was triturated at 4 C for four hours, and solid debris removed by centrifugation. Following

determination of protein content in the clear supernatant, the antigen was adjusted to 2.0 ug/ml in KCl/EDTA coating buffer (Tsang et al 1983) and the plates coated with 50 ul/well overnight at 4 C.

c) Whole Worm Antigen (WWA): Approximately 200 male and female S. mansoni harvested from previously infected mice were washed in several rinses in PBS to remove host tissue. The worms were homogenized in 1.0 ml PBS at 4 C, then triturated for four hours at the same temperature. Following centrifugation, the supernatant was analyzed for protein content which was then adjusted to 2.0ug/ml with KCl/EDTA coating buffer. Plates were coated and prepared as above.

ELISA was carried out using normal methods with antigen coated plates. Sera were diluted 1:20 and 1:100 for the elastase plates, goat anti-human IgM/ HRPO conjugate at 1:1,000 was used as detector antibody. For the other antigens sera were diluted 1:100 only, goat anti-human IgG/ HRPO conjugate at 1:1,000 was the detector reagent. The substrate used was ABTS (Kirkegaard & Perry) and plates were read at 405 nm after 30 min incubation at room temperature. The test system with cercarial elastase had been standardised previously (Toy et al, 1987), and shown to discriminate between controls and patients. In this work the cercarial elastase was used in an attempt to determine patterns of exposure, not current infections. Therefore there has been no attempt to identify a "cut-off" optical density in the ELISA. This is not appropriate to the study. What is important is to see which

groups of people show evidence of exposure to cercariae. This is best done by classifying people into age or sex groups, community groups etc., with different risk factors for exposure and then determining the mean response for those groups.

5. Statistical Analysis:

Optical density measurements carried out in duplicate for each participant were averaged if within 30%, and the average value used in the analysis. Three readings were excluded from further analysis because the difference between the duplicate values was greater than 30%. The distribution of the study population was compared for sex and parasitological diagnosis by using the Chi^2 statistic. To achieve a normal distribution of the optical densities, square root transformation was performed prior to statistical analysis. The two sample "t" test was used to compare the mean optical densities of two groups and a one-way analysis of variance (ANOVA) was used if more than two groups were being compared.

RESULTS

Overall the study population had a mean age of 10.96 years; range 8-18 yrs, with 48.6% male and 51.4% female. Table 1 shows age and sex distribution of the study population with respect to schools and parasitological diagnosis. There is no bias in the sex distribution in the sample, and apart from Kiguoyo school, the age distribution in

the samples is similar. The striking differences between the parameters recorded from children at Kiguoyo and the other schools as regards parasitological diagnoses are due to selection of participants at that school. The two schools Muthangauta and Mutithi serve communities where some of the population had participated in mass chemotherapy using Praziquantel. This is reflected in low levels of schistosome infection detected by urine and stool examination (Table 1). However, it is unlikely that this treatment program had interfered with the patterns of exposure to cercariae, as only a small proportion of the overall community was involved in the program.

Table 2 summarizes mean optical densities obtained from children at the various schools. Although there are considerable differences between the mean O/D's measured at the three schools, the results are seen most clearly when the data from the high transmission areas are combined and compared with those from the low transmission area (Tables 2 and 3). The mean O/D at Kiguoyo is significantly less than in the combined other schools ($P = 0.0004$).

Mean optical densities in groups which were positive for hookworm and Ascaris infection were compared with those showing no infection by "t"-test (Table 3). There is no evidence of an association between O/D measured for the schistosome antigen and hookworm or Ascaris infections. However it is noteworthy that a significantly higher value in mean O/D is found for those infected with S. mansoni than those found negative by stool examination. There is also a difference in the

elastase O/D between girls and boys (P = 0.0379), with the former showing the higher response. The different distribution of O/D measurements for the sexes is clearly seen in Fig.1.

No evidence of S. haematobium was found in the entire survey.

DISCUSSION

Transmission of schistosomiasis is focal in space and time (Woolhouse, 1989) and the majority of infections take place at well defined contact points. New methods for defining these important epidemiological regions are needed. Thus the ability to detect exposure to schistosome cercariae represents a novel approach in sero-epidemiology. It would allow public health workers to identify people in communities who are habitually attacked by cercariae and help understand better the source, locality and perhaps time of infection.

McKerrow et al (XXXX) have indicated that the schistosome elastase is different from that of hookworm larvae, and Toy et al, (1987) have shown that the immune response to this elastase differs from other trematodes such as Schistosomatium douthitti, the common cause of swimmers itch in North America. From evidence presented in this work, there is no reason to suspect cross reaction with either hookworm or Ascaris infections. Therefore, it is reasonable to expect that antibodies to the cercarial elastase will be specific indicators of recent exposure to cercarial bombardment. High O/D's recorded for a specific group would then indicate a high risk of transmission.

In these studies two groups of people clearly show disparate O/D mean values. Overall, the girls have significantly higher O/D values than boys. This is likely to be associated with domestic activities allocated to the young female population bringing them into contact with infective water more frequently than boys and is often a feature of epidemiological surveys carried out in areas endemic for schistosomiasis (Clarke, 1966; Jordan, 1985). The second group of children with a different response come from Kiguoyo School. This school serves a community living in a dryland farming area where there is little or no transmission of schistosomiasis (Table 1). These children have low mean O/D values for elastase whereas the children living in communities where there is greater risk of exposure to cercariae have significantly higher mean values for this parameter. This indicates that one can expect to find higher titres of anti elastase antibodies in communities or population groups with high risk of transmission in endemic areas.

It is difficult to account for the variation in mean O/D between the schools in the high transmission area, particularly when it is the school with the highest prevalence that displays a low mean O/D. This may be a factor of the exposure pattern in the community, with the test being sufficiently sensitive to descriminate between different levels of cercarial bombardment to which people are exposed. Unfortunately it was not possible to study the behaviour patterns and risk of infection of the children who participated in this study and

follow up information is necessary for further interpretation of these results.

Elisa tests were performed on the sera collected using cercarial antigen and whole worm antigen. However, there was little difference in the mean O/D recorded for the various groups studied and the two antigens. This is to be expected in an area where schistosomiasis is endemic. The antibodies detected by such antigens are of long duration and do not give information on the current state of infection or exposure. What is interesting is the significant difference noted in the mean O/D figures for persons with or without the schistosome infection. This is probably a reflection on the low titres recorded at Kiguoyo, and the absence of infection at that school. It remains to be seen whether the elastase test will be of value in individual diagnosis.

Clearly, the use of cercarial elastase as a stage specific antigen will have an important role to play in future epidemiological studies. Further work needs to be done to optimize the ELISA procedure to make it more sensitive for testing individuals, particularly if it is to be used as an indication of personal exposure. This would be useful for determining the protective value of a schistosome vaccine, particularly in phase II trials. However to be useful as an epidemiological tool, there must be additional information on the duration of the antibody response, as well as its correlation with natural fluctuation of cercarial prevalence in the water. Such studies

are planned and will be carried out in the near future.

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Table 1.

Comparison of Demographic and Parasitological Factors in the Study Sites.

	Mutangauta n = 67	Mutithi n = 67	Mikou n = 86	Kiguoyo n = 70
1. AGE: mean	10.99	10.93	10.02	12.13
(range)	(8-16)	(9-13)	(8-14)	(9-18)
2. SEX males (%)	29 (43.3)	37 (55.2)	44 (51.2)	31 (44.3)
females (%)	38 (56.7)	30 (44.8)	42 (48.8)	39 (55.7)
3. SCHISTOSOMIASIS:				
negative (%)	46 (68.66)	15 (22.39)	19 (22.09)	70 (100)
1-50 eggs/gm	12 (17.910	23 (34.33)	14 (16.28)	0
51-300	8 (11.94)	20 (29.85)	22 (25.58)	0
> 300	1 (1.49)	9 (13.43)	31 (36.05)	0
4. HOOKWORM:				
negative (%)	54 (93.1)	58 (83.33)	85 (77.91)	56 (80.00)
positive (%)	4 (6.9)	8 (12.12)	1 (1.16)	14 (20.00)
5. ASCARIS:				
negative (%)	47 (81.03)	55 (83.33)	67 (77.91)	33 (47.14)
positive (%)	11 (18.97)	11 (16.67)	19 (22.09)	37 (52.86)

Note: Children at Kiguoyo were selected for both hookworm and Ascaris infection from Ministry of Health information.

TABLE 2.

Optical Densities by ELISA using Schistosoma mansoni cercarial elastase antigen (ELAS)

School	n	mean	SD	Transformed mean	Transformed S/D	F	P
Mutangauta-Mutithi[a]	29	0.290	0.152	0.516	0.154		
Mukuo	34	0.127	0.094	0.328	0.142		
Kiguoyo	27	0.089	0.052	0.284	0.093	24.54	0.0001 [b]
Total Schools	90	0.168	0.138	0.376	0.165		
Combined Schools:							
High Transmission	63	0.202	0.148	0.415	0.174		0.0002 [c]
Low Transmission	27	0.089	0.052	0.284	0.093		0.0004 [c]

a = Data from Muthangauta and Mutithi combined
b = Based on one way ANOVA
c = Based on 2 sample t-test

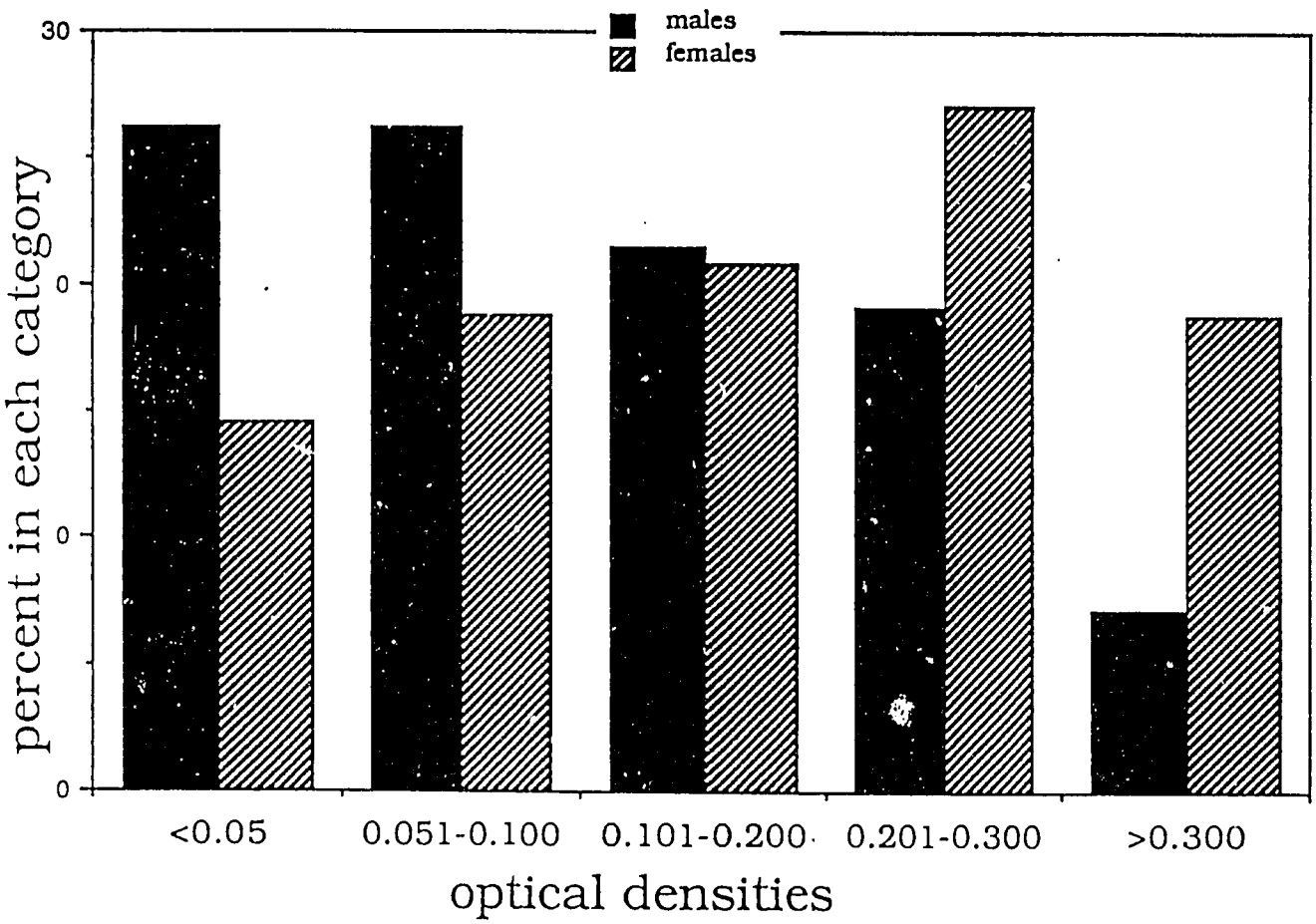
TABLE 3

Transformed Optical Densities (square root) measured against *Schistosoma mansoni* cercarial elastase antigen compared with other parasite infections, sex and transmission levels for schistosomiasis.

	n	mean	S/D	T	P
1. ASCARIS:					
negative	61	0.383	0.175		
positive	28	0.357	0.146	0.6855	0.4948
2. HOOKWORM:					
negative	78	0.372	0.161		
positive	11	0.398	0.208	-0.4847	0.6291
3. S. MANSONI INFECTION:					
negative	36	0.319	0.118		
positive	54	0.413	0.182	-2.7415	0.0074
4. TRANSMISSION LEVEL:*					
high	63	0.415	0.174		
low	27	0.284	0.093	3.6704	0.0004
5. SEX					
males	42	0.336	0.154		
females	48	0.411	0.168	-2.1833	0.0317

* = High area represented by Mutangauta, Mutithi and Mukou schools.

Low transmission area was representated by Kiguoyo school.


males
females
30
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percent in each category
<0.05
0.051-0.100
0.101-0.200
0.201-0.300
>0.300
optical densities