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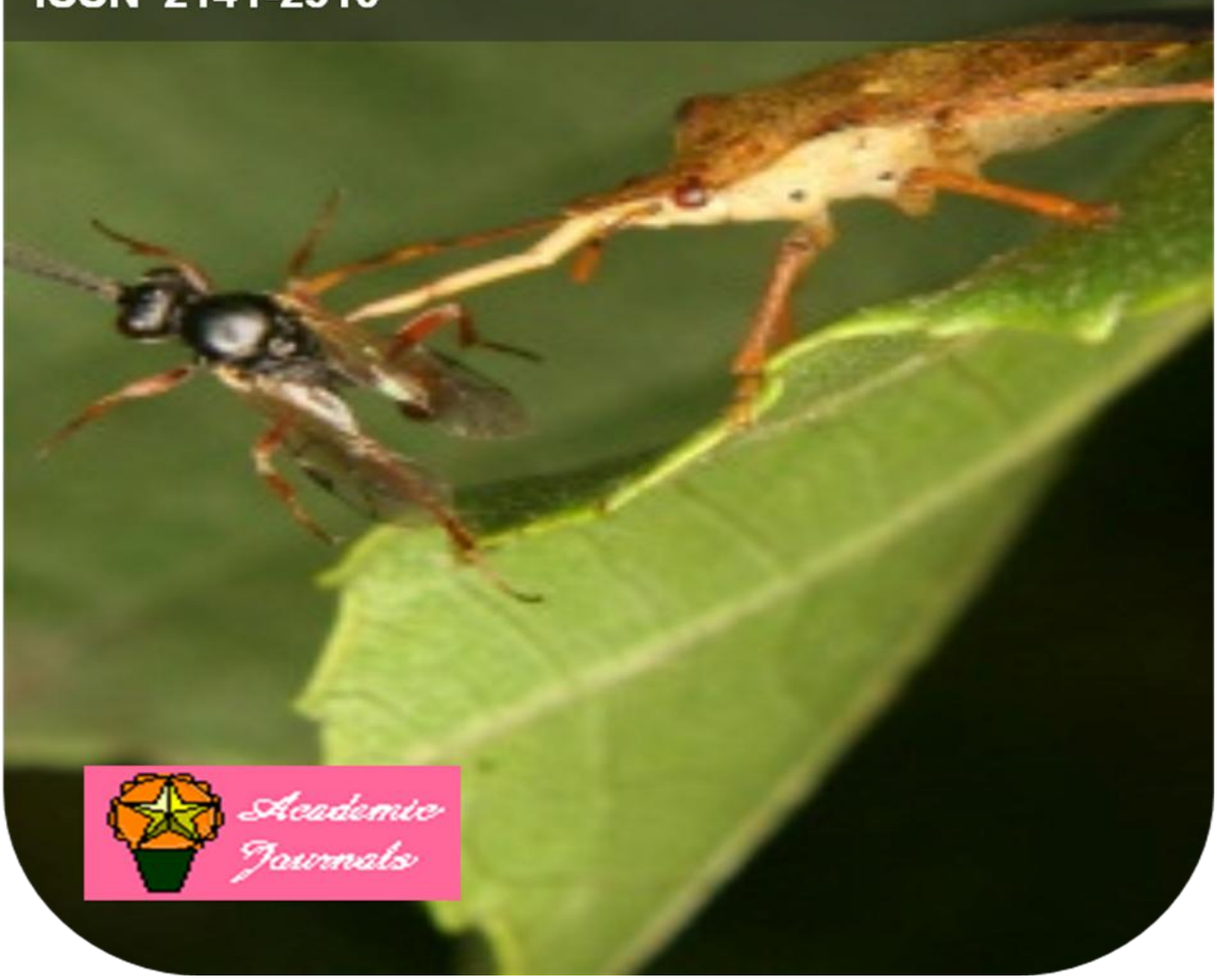

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Full Length Research Paper

Assessment of the evolution of malaria and intestinal helminthes infections from 1983 to 2014 in the Hevecam agroindustrial complex, south region of Cameroon

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Man-made development projects have usually been pointed to significantly influence the epidemiological features of communicable parasitic diseases in sub-Saharan countries as a result of anthropogenic environmental changes. This study aimed to assess the current health status of dwellers of the Hevecam agroindustrial complex established after rainforest deforestation in Southern Cameroon, and to monitor its evolution compared to data collected 31 years ago. A prospective cross-sectional study was carried out in 2014 in the same four villages which were previously investigated 31 years ago in the Hevecam area. Assessment focused on prevalence of malaria and soil-transmitted helminthiasis, and the risk factors of these diseases. Present data were then compared with those collected in 1983. Data from four villages investigated indicated that malaria and soil-transmitted helminthiasis were mesoendemic and hypoendemic, respectively in the area. The malaria endemicity level was similar in either study village according to both parasitological results and spleen index. *Plasmodium falciparum* and *Plasmodium malariae* caused 98.8 and 1.2% of malaria cases recorded. *P. falciparum* parasites loads ranged from light to heavy. Prevalence rates of *Plasmodium* carriage were significantly influenced by sex, age and occupation. Intestinal helminths parasites occurred in 14.1% subjects. The main infective agents were *Ascaris lumbricoides* and *Trichuris trichiura*, found either as monospecfic or mixed infections. *A. lumbricoides* infections were the most frequent. Helminths intensities of infections were predominantly light. Prevalence significantly varied with villages, age, occupation and regular deworming status. Mixed infections by malaria and helminths parasites were recorded in some subjects. Comparing current to previous data, malaria and intestinal helminthiasis have evolved significantly in divergent ways in 31 years within the Hevecam villages. Malaria infections increased from hypoendemic to mesoendemic, while intestinal helminth infections decreased from hyperendemic to hypoendemic. Evolution of these diseases indicated a need for health facilities located in Hevecam to improve specific control measures for malaria and sustained helminth control tools.

Key words: Malaria, intestinal helminthiasis, prevalence, evolution, Hevecam.

INTRODUCTION

In most rural and urban countries of sub-Saharan Africa, anthropogenic environmental changes for implementation of development projects have usually been demonstrated to influence the epidemiological profile of communicable

diseases through changing the transmission pattern of existing diseases and importation of new pathologies by immigrants (Hunter et al., 1993; Keiser et al., 2005; Ripert et al., 1979). In fact, the functioning of the manmade projects need a manpower which is usually made of non-indigenous workers and their families attracted by the new job opportunities. In this situation, people were sometimes deported from other regions and resettled in the project area leading to creation of cluster settlements made of villages whose inhabitants were mostly immigrants.

Creation of development projects therefore influences greatly the dynamics of parasitic and other communicable diseases by introduction of new diseases and/or new parasites strains in the development site, and also through changes in environmental conditions favourable to vectors development and changing in human being habits. These observations have been a rule concerning dams construction and hydroagricultural projects which usually lead in many countries of sub-Saharan Africa, and other continents to a sustainable increase of prevalence rates of waterborne and vector borne parasitic diseases namely malaria, schistosomiasis, lymphatic filariasis and other filariasis in Africa (Hunter et al., 1994; Keiser et al., 2005; Ripert et al. 1979). Like waterborne and vector diseases, epidemiology of other communicable diseases like soil-transmitted helminth infections (STHs), intestinal protozoan, sexually transmitted diseases is likely to be influenced by the project. Like water-related development projects, other manmade environmental changes such as deforestation and changes in land use can be potential causes of dynamic changes in communicable diseases risk in absence of an adequate safeguard for disease surveillance.

Beyond such health negative impact of development projects there was a lack in implementation of an adequate safeguard for disease surveillance (Parent et al., 1997). A major prerequisite to build development projects in diseases endemic areas is implementation of health impact assessments to be followed by creation of an adequate safeguard for disease tracking system or prevent transmission according to country directorates of national disease control program. However, when such studies are done, results are not often used to design adequate scheme to prevent health negative impact of the project.

Malaria and intestinal helminthiasis are controllable, and recommended control tools are largely implemented in Cameroon through specific national control programs of the country Public Health Ministry. Infection with soil-transmitted helminth is intimately connected with poverty, with the highest prevalence rates observed in low- and middle-income countries where hygiene is poor, access to safe and clean water is lacking, and sanitation is absent or inadequate (Brooker, 2010; WHO, 2002, 2012, 2006; Hotez et al., 2006). The morbidity caused by intestinal helminth is most commonly associated with infections of heavy intensity (Hotez et al., 2006; Hotez et al., 2006). Anaemia and other morbidities (example, reduced physical and cognitive development) are the main reasons for this large global burden (Brooker et al., 2006; Partnership for Child Development, 1998). People are infected after ingesting eggs from contaminated soil or food (*Ascaris lumbricoides* and *Trichuris trichiura*), or through active penetration of the skin by infective larval stages present in contaminated soil such as hookworm (Brooker et al., 2006; Bethony et al., 2006). More than half of the Cameroon population is thought to be affected by soil-transmitted helminthiasis (Brooker, 2010).

Improving access to sanitation, safe water and hygiene practices (WHO, 2002, 2006, 2012; Utzinger and Keiser, 2004; Horton, 2003) together with annual mass drug administration of anti-helmintic drugs (WHO, 2012; Bartram and Cairncross, 2010; Ziegelbauer et al., 2012; Strunz et al., 2014) are affordable and simple measures to be implemented for a sustainable control of intestinal parasitic diseases under public health importance. Strategic plans have been so far lunched in order to eliminate soil-transmitted helminthiasis (STH) related morbidity in children by 2020 through school-based deworming (WHO, 2012). The World Health Organization (WHO) guidelines for STH control generally focus on routine mass drug administration for school-aged children, aiming primarily to reduce the prevalence of high to moderate-intensity infections by achieving in endemic countries a minimum target of regular deworming of at least 75% and up to 100% of school-aged children and other groups at risk of morbidity by 2010 (WHO, 2002, 2006). Cameroon Ministry of Public Health adopted the annual nationwide school-based mass deworming strategic plan for the control of schistosomiasis and STH in 2004 with its completion in 2007 (Tchuem and N'goran, 2009; Tchuem et al., 2013). Implementation of school-based annual deworming from 2007 to 2010 showed a 73% decrease of STH infections in school age children in all health districts of the Centre region of Cameroon (Tchuem et al., 2012).

Since 2005, a target was set for the reduction of malaria cases and deaths by 75% by 2015 (WHO, 2014). Such achievements could be attained in endemic countries only after wide implementation of WHO recommended malaria control guidelines to endemic countries mainly based large scale use of insecticide-

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treated mosquito nets (ITNs) in households, indoor residual spraying, intermittent preventive treatment in pregnancy (IPTp), proper management of diagnosed cases complemented with a tracking system as recommended in the T3: Test. Treat. Track Initiative (WHO, 2012, 2013, 2014). It was estimated that the only ownership and proper use of ITNs reduce the incidence of malaria cases by 50% in a variety of settings, this also reduce malaria mortality rates by 55% in children under 5 years of age in sub-Saharan Africa, as well reducing maternal anaemia, placental infection and low birth weight (WHO, 2013, 2014).

The Hevecam agro-industrial society was established in 1975 at Niete neighbourhood in southern Cameroon following an important deforestation of the evergreen forest. A previous assessment of the health status of the employees and their families undergone in 1983 showed an hyperendemic level of intestinal helminthiasis whereas malaria was hypoendemic (Moyou et al., 1984). Considering the ecological and human habits changes in the area, the morbidity of these diseases could likely change in absence of adequate control measures. This study aimed to assess the present endemicity levels of malaria and intestinal helminthiasis in villages of the Hevecam company, investigate potential risk factors of their transmission, and appreciate the evolution of these diseases compared to data collected 31 years ago.

MATERIALS AND METHODS

Study type, period and place

This was a prospective cross-sectional study undergone in 2014. Data collected were then compared with previous data from a study done 31 years ago in the same area. The study took place in 4 villages of the Hevecam agroindusrial complex namely villages 2, 5, 6 and 8 for recruitment of participants. A first stool examination and staining blood smears took place in the Hevecam Central Hospital laboratory. A counter-examination of stool samples and stained blood films took place in the laboratory of the Faculty of Medicine and Pharmaceutical Sciences of the University of Douala and also in the Parasitology laboratory of the Yaoundé University Teaching Hospital in Cameroon. The Hevecam agroindustrial complex is specialized in cultivation of rubber trees and harvesting natural rubber. The Hevecam company was established in 1975 at Niete, a village situated deeply in the rainforest at 40 km from the nearest town Kribi. Its plantation covers nearly 40 000 ha area created after the destruction of the rainforest. The population of the Hevecam is estimated at 20 000 inhabitants of whom 6000 are employees of the society. Employees of the plantation and their families live in 16 encampments numbered from 1 to 16. Working as Hevecam employee, petty trading and peasant farming are the major occupation of the population. Sanitary conditions are poor in large parts of the community. The Hevecam company constructed a hospital with well trained personnel, and has also built health centers in each of the sixteen villages. Niete is located in an equatorial climate where mean atmospheric temperature is around 26.2°c in the rainy season and 28.7°c in the dry season. The hydrographic network is dominated by the Nyete river which traverses the Hevecam plantation. The Hevecam residents collect drinking and domestic water from pipes and taps constructed by the company.

Ethics

Permission to carry out this research work was obtained from the Ethical Committee of the University of Douala, the Regional Delegate of Public Health of the South Region in Cameroon and the administration of the Hevecam hospital. Then community leaders of the selected villages held a meeting during in which the study was explained by the research investigators. The date for participant recruitment was fixed together with the residents.

Study criteria

All inhabitants of each selected village were targeted as the study population. Visitors and those who (or whose parent) did not sign the study inform consent were not admitted in the study.

Data collection

In each village, residents were convened at the local health facility for recruitment. The aim and procedures of the study was carefully read and explained to the participants or the legal guardian (for children), then each participant was asked for consent before his (or his child) enrolment into the study. He (or the parent) was asked to sign the study informed consent sheet if he accepted to participate. Each person who agreed to participate in the study was questioned for the following data: sex, age, occupation, mosquito bites preventive tools used, sanitation types, provision of drinking water, history of any anti-malaria or regular anti-helminthic treatment. Children of two to 9 years old were examined for existence spleen enlargement to determine the spleen index. Participants were classified into five occupational groups namely preschool children, school-aged children, Hevecam rubber plantations workers, housewives and independent occupations. Independent occupations comprised trade, small-farming and jobless. The volunteer was then asked to give a stool sample in a container given by the investigator. Lastly, the participant underwent a thick and thin blood smears from finger pricking for microscopy identification and count of Plasmodium stages. Stool samples and blood smears were then transported to laboratory for analysis.

Thick and thin blood smears were stained with Giemsa, then dry at room temperature and examined under light microscope at high magnification. Plasmodium loads were expressed per microliter of blood with the assumption that each participant had a mean 7500 white blood cells/µl of blood. Stool samples were analysed in laboratory using the Kato-Katz technique by microscopic examination and count of all helminth eggs contained in a calibrated thick smear made from 41.66 mg moulded faeces (Katz et al., 1972; Martin and Beaver, 1968). Parasitic load of each helminth parasite was then brought to number of egg per gram of faeces for each participant.

For each of the diseases assessed, prevalence rates, intensity of infection and endemicity level was estimated. Prevalence of each diagnosed infection was estimated as the percentage of subjects who harboured the parasites in the biological specimen after laboratory examination. The malaria endemicity level was estimated according to spleen enlargement index according to WHO classification (Spencer, 1963; Hay et al., 2008). Spleen index classification defines hypoendemicity as spleen index $0 < SI \leq 10$ %; mesoendemicity as spleen index $10 < SI \leq 50$%; hyperendemicity

Table 1. Malaria inction prevalence according to Plasmodium species, sex, age groups and spleen index.

Villages	N	Plasmodium infection prevalence																	
		Specific infections			Sex				Age groups (years)								Spleen index	
					F		M		0-4		5-9		10-14		≥15			
		Overall	*P. f*	*P. m*	N	%	N	%	N	%	N	%	N	%	N	%	N	Index
Village 2	148	32.4	32.4	0.0	87	27.7	61	39.3	45	17.8	29	62.1	25	40.0	49	24.5	62	21.0
Village 5	110	44.5	44.5	0.0	60	41.7	50	48.0	21	57.1	34	55.9	13	46.1	42	28.6	50	32.0
Village 6	109	25.7	23.9	1.8	68	23.5	41	29.3	19	21.1	24	45.8	10	50.0	56	14.3	36	27.8
Village 8	132	35.6	35.6	0.0	97	35.1	35	37.1	26	30.8	24	62.5	21	66.7	61	16.4	42	31.0
Mean	499	34.5	34.1	0.4	111	31.7	187	39.0	111	28.8	111	56.8	68	51.6	209	20.1	190	27.4
-	-	$\chi^2 = 4.6$; p>0.05			$\chi^2 = 4.6$; p>0.038						$\chi^2 = 52.8$; p=0.00						-	-

N = Sample size. *P.f* = *Plasmodium falciparum*. *P.m.*= *Plasmodium malariae.* F = females. M = males.

as spleen index 50 <SI≤ 75%; and holoenedmicity as spleen index SI > 75% (Spencer, 1963). Classification of intestinal helminhiasis into endemicity level and intensity of infection were made according to WHO guidelines (WHO, 2002, 2006, 2012). In either case, intensity of infection of the disease, the study could be classified as low-risk, moderate-risk or high risk.

Data were analyzed using the statistical package for the social sciences (SPSS) 18 statistical software and Chi-square test was used for statistical analysis. Differences were considered significant when p was less than 0.05.

RESULTS

Sample size examined

Recruitment of participants took place in four villages of the Hevecam plantation. A total 499 persons were recruited for malaria parasites detection in blood smears, 190 children aged between 2 years and 9 years were examined for spleen enlargement, and 312 subjects provided stool samples for helminth eggs and larva detection. In all villages, female participants were more represented than males.

Malaria infection trends at Hevecam

Prevalence of malaria infection

As indicated in Table 1, prevalence of malaria showed a meso-endemicity of malaria in the Hevecam agro-industrial complex as well as in either villages screened. There was a significant variation among malaria infection prevalence in the villages ($\chi2 = 4.6$; p=0.03). Infection prevalences ranged between 25.7 and 44.5% with an overall 34.5% in the area. Malaria infection cases were due to *Plasmodium falciparum* in 98.8% cases and *Plasmodium malariae* in 1.2%. *P. malariae* infections were recorded only in village 6. Participants who were infected by *P. falciparum* harboured either asexual stages (97.1%), either gametocytes stages (2.3%) or both (0.6%). There was no co-infection by the two Plasmodium species.

According to spleen enlargement at Hevecam

Considering the clinical feature based on spleen enlargement among children aged between 2 years and 9 years, malaria was also mesoendemic in the Hevecam villages. The overall spleen index was 27.4. As recorded with infection rates, spleen enlargement index also varied significantly between villages from 21 to 31 ($\chi2=63$; p=0.00) indicating a mesoendemicty of malaria in all villages. Sex did not influence significantly spleen indexes ($\chi2 = 0.37$; p=0.54).

Influence of demographic factors on malaria infection

Influence of sex

The sample study was made of 62.5% females and 37.5% males. Female were also more represented in all villages screened. As indicated in Table 1, sex signify-cantly influenced the prevalence of malaria infections in all villages investigated ($\chi2=4.6$; p = 0.03). Males were more infected than females (39 vs 31.7%) in the study area. However, predominant prevalence varied between sex varied from a village to another.

Influence of the age

Mean age of participants was 13.17 years (range 6 months to 75 years). There was a significant age-related influence on the Plasmodium infection prevalence in the whole area as well as in either study village ($\chi2 = 52.8$; p=0,00). In the Hevecam area, prevalence of *Plasmodium* infections showed a unique peak in parti-

Table 2. Prevalence of Plasmodium infection according to occupation at Hevecam.

Occupation	Pre-school children	School children	Housewives	Rubber farm workers	Others
Sample size	84	232	91	68	24
Prevalence (%)	27.4	48.3	22	22.1	8.3

Table 3. Prevalence of Plasmodium infection according to mosquito bite prevention tool use.

Prevention tool	Mosquito net		Insecticide sprays		Screen on windows		Smokes	
Answer	Yes	No	Yes	No	Yes	No	Yes	No
Sample size	299	200	17	482	96	403	70	429
Infected	109	63	6	166	32	140	23	149
Prevalence (%)	36.5	31.5	35.3	34.4	33.3	34.7	32.9	34.7

participants aged between 5 to 14 years whereas lowest prevalence occurred in older group. This tendency also occurred in studied villages excepting village 5 where prevalence of infection was highest in younger participants and decreased with age.

Influence of specific occupation

Specific occupations influenced significantly prevalences of Plasmodium infections in the Hevecam agro-industrial complex ($\chi2=39$; p=0,00). Plasmodium infection prevalences were higher in school-aged and preschool children than in other groups. Housewives and Hevecam rubber plantation workers had significantly high *Plasmodium* infection rates (22 and 22.1% respectively) (Table 2).

Influence of mosquitoe bites prevention tools

All study participants used at least a mosquitoe bites prevention tool. Participants used either a mosquitoe net (59.9%), an insecticide spray (3.4%), smokes (14%) or net on windows (19.2%) or a combination of two tools. According to Table 3, there was no significant influence of mosquitoe bites prevention tools on Plasmodium infection prevalence rates ($\chi2 = 0.04$; $p = 0.94$). Moreover, prevalences of Plasmodium infections were not significantly different between those who used mosquito bites prevention tools and those who did not.

Intensities of plasmodium infections

Mean plasmodium infection load among infected subjects was 1 876.23 *Plasmodium*/µl of blood (range: 135-112500 *Plasmodium*/µl of blood). Male subjects usually had higher infection intensities than females. According to age, 99.4% of parasitized participants had less than 4000 *Plasmodium*/µl of blood of whom 75% were less than 15 years old and 53.5% less than 10 years old. All participants older than 50 years had Plasmodium parasites load less than 1000 *Plasmodium*/µl of blood.

Intestinal helminth infections trends

Intestinal helminth infections prevalence

A. lumbricoides and *T. trichiura* were the helminth parasites identified in the investigated villages. As indicated in Table 4, the Hevecam area and all villages were found to be globally a low-risk area for intestinal helminth infections according to WHO classification. However, village 6 was still a moderate-risk area with prevalence higher than 20% (21.1%). The overall intestinal helminthes infection prevalence was 14.1%. Intestinal helminth infection prevalence varied but not significantly among the study villages ($\chi2 = 4.6$; $df = 3$; $p > 0,05$). Village 8 had the lowest infection prevalence rate (8.8%) whereas village 6 had the highest (21.1%). According to parasites species, *A. lumbricoides* and *T. trichiura* infected 12.5 and 3.5% of participants respectively. *A. lumbricoides* and *T. trichiura* infections occurred each as mono-infection in 77.3 and 9.1% respectively. Mix-infection by the two parasites occurred in 13.6% of infected subjects. Prevalences of *A. lumbricoides* infections were always higher than *T. trichiura* in each village.

Influence of age, sex, drinking water source and periodic anti-helminthic practice

As indicated in Table 4, intestinal helminth infection

Table 4. Helminth infections prevalence at Hevecam according to village, parasite, gender and age group.

Villages	Global		Specific infections and co-infections			Gender				Age group (years)							
						M		F		0-4		5-14		15-29		≥30	
	N	%	*A. l*	*T. t*	*A.l+T.t*	N	%	N	%	N	%	N	%	N	%	N	%
Village 2	89	14.6	12.3	5.6	3.4	41	12.2	48	16.4	27	11.1	32	18.7	19	10.5	11	18.2
Village 5	72	12.5	12.5	0.0	0.0	35	14.3	37	10.8	10	10.0	30	13.3	11	18.2	21	9.5
Village 6	71	21.1	18.3	7.0	4.22	23	21.7	48	20.8	13	7.7	24	37.5	13	15.4	21	14.3
Village 8	80	8.8	8.7	0.0	0.0	22	0.0	58	12.1	16	0.0	26	7.7	28	14.3	10	10.0
Overall	312	14.1	12.2	3.2	1.9	121	12.4	191	15.2	66	7.6	112	18.7	71	14.1	62	12.3

N= sample size. F: female. M: male. *A.l.= Ascaris lumbricoides*. *T.t.= Trichuris trichiura*.

prevalence showed a single peak with a sharp increase from 7.6% in preschool-aged subjects to 20.3% in subjects aged between 5 to 14 years then decreased at elder ages. Despite the differences, age was not associated with intestinal helminth prevalence in the Hevecam area ($\chi 2 = 4.7$; df = 3, $p > 0,05$). Prevalence of specific helminth infection (*A. lumbricoides* or *T. trichiura*) also showed the same trend with age of participants with a single peak which occurred in subjects aged between 5 to 14 years (Table 4). Female participants were more infected than males in the Hevecam area (Table 4). Sex was however not associated with intestinal helminth infection prevalence in the study area ($\chi 2 = 2.1$; df = 1, $p > 0,05$). The relationship between helminth infections and sex varied between villages. In villages 5 and 6 for example, males appeared more infected than females while all infected recorded in village 8 were females. Prevalence rates of *A. lumbricoides* infection were higher in females than males, inversely males had higher *T. trichiura* infection rate than females. According to specific occupation, school children had the highest helminth infection prevalence rate (18.4%). Hevecam workers and housewives had 9.8 and 12.7% prevalence rates respectively. Participants to this study drank piped water originating either from a well constructed borehole (98.8%) or the main water distributor society named Camwater (1.2%).

All cases of helminth infection recorded were among those who drank water from borehole. Considering deworming practices, 81.1% of study participants swallowed an adequate antihelminthic drug against STH at least once a year. Some of them dewormed up to four times in the year. Helminth infection prevalence was lower among subjects who systematically took an antihelminthic each year (13.8%) than those who did not (17.5%). According to drinking water source, 98.7 and 1.3% of participants reported drinking water from borehole and piped respectively. Helminth infections were found only among those who reported drinking water from borehole at a prevalence of 14.3%.

Intestinal helminth intensities of infection

Intensities of infection by each of the helminth infection ranged between light to heavy. Light-intensity of infections were predominant in both helminth infections. Egg count in *A. lumbricoides* infections ranged between 48 and 50400 egg per gram of faeces. Mean parasitic load recorded was 3161 eggs per gram of faeces. Light-intensity of infections represented 90% while moderate and heavy-intensity of infections accounted for 7.5 and 2.5% respectively. The heavy-intensity of infection was recorded in a one year old child while all moderate-intensity of infections were found among school-aged participants. Parasitic loads recorded in *T. trichiura* infections ranged between 48 and 1440 eggs per gram of faeces. Mean parasitic load was 6384 eggs per gram of faeces. There was no heavy-intensity of infection. Light and moderate-intensities of infections represented 80 and 20% respectively. Moderate-intensity of infection was recorded mostly among under-fifteen years old participants.

Occurrence of Plasmodium and helminth parasites co-infections

Some of the study participants harboured both intestinal helminth and *Plasmodium* parasites.

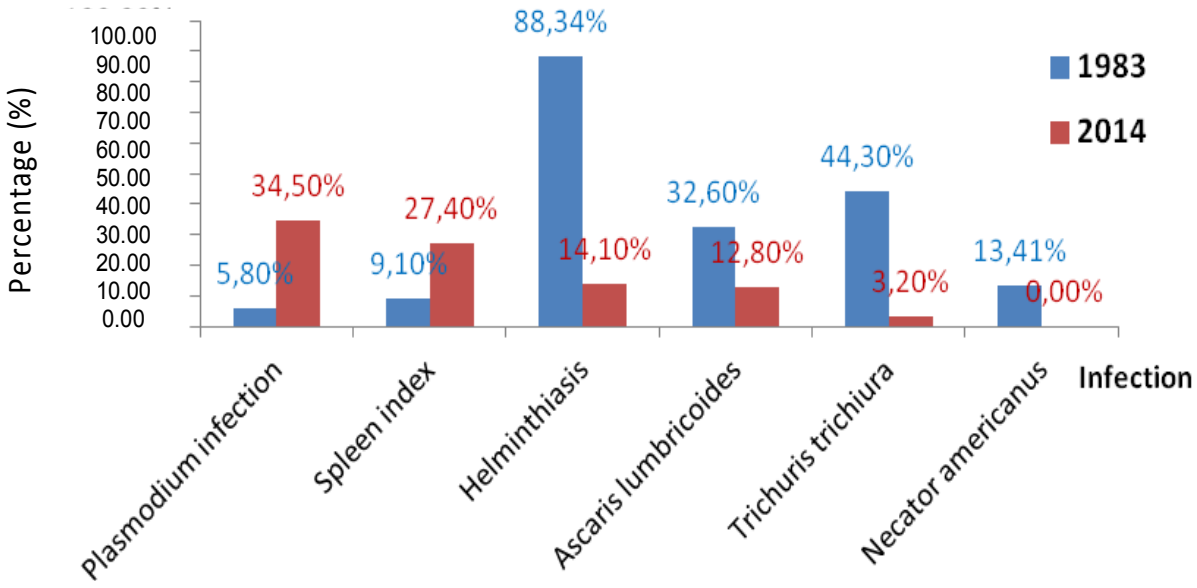

Figure 1. Prevalence of malaria and helminth infections at Hevecam in 1983 (Moyou et al., 1984) and 2014.

Such combination occurred in 4.5% subjects. Specific combinations recorded were *P. falciparum, A. lumbricoides* (3.2%), *P. falciparum, T. trichiura* (0.64%), *P. falciparum, A. lumbricoides* and *T. trichiura* (0.6%). Plasmodium plus intestinal helminth co-infections occurred most in female than male subjects, however differences were not statistically significant (p=0.98).

Evolution of malaria and intestinal helminth infections in the Hevecam villages from 1983 to 2014

As indicated in the figure below, comparison of data recorded in 1983 to actual data indicated a significant evolution in the health status of residents in the Hevecam villages either for malaria or intestinal helminthiasis. Concerning malaria, *P. falciparum* was the only species found in 1983. The occurrence of *P. malariae* together with *P. falciparum* in the area 31 years after indicated that malaria transmission trend is becoming complex in Hevecam settlements. Such complexity was confirmed by the significant increase in malaria prevalence rates from hypoendemic to mesoendemic according to WHO classification based on spleen index in 2 to 9 years old children. Unlike significant increase of spleen enlargement index, prevalence rates Plasmodium parasites carriage also significantly evolved within the same period increasing from 5.8% in 1983 to 34.5% in 2014 indicating also a move from hypoendemic to mesoendemic. The increase in Plasmodium carriage prevalence rate has also been significant in both sexes. The prevalence rate of Plasmodium infection in 2014 was almost three times the value in 1983 among male subjects (from 15.15 to 39%); and almost five times the prevalence rate in 1983 among female study participants (from 6.64% in 1983 to 31.7% in 2014) (Figure 1).

Evolution of intestinal helminth infections prevalence rates at Hevecam in 31 years

Overall intestinal helminth infections in Hevecam evolved in an opposite direction compared to malaria with a significant decrease in overall prevalence rate from 88.34 to 14.1% owing a 74.24% decrease in prevalence rate. This trend occurred also for each helminth parasites diagnosed in the area. Therefore, *A. lumbricoides* and *T. trichiura* infections prevalence rates dropped from 32.6 to 12.8% and from 44.3 to 3.2% respectively. Infections by hookworm which accounted for in 13.4% were not found in the 2014 investigations. The decrease in intestinal helminth infections was also significant in either sex group from 1983 to 2014.

DISCUSSION

This study aimed to assess the prevalence of malaria and intestinal helminthiasis in villages of the Hevecam agro-industrial company, and appreciate their evolutions in comparison to data obtained in 1983. The sample area included the same four villages which were also investigated in the 1983. Also, sampling methods used were the same used 31 years ago, namely spleen index measurement together with thin and thick blood smears for Plasmodium parasites detection for malaria diagnosis,

and the Kato Katz technique for intestinal helminth diagnosis. These assessment tools used are the foremost recommended in field work for diagnosis of malaria (WHO, 2012) and intestinal helminthiasis (Hotez et al., 2006; Glinz et al., 2010).

Data obtained in the present study indicated low prevalence rates of intestinal helminthes whereas malaria infections were of moderate prevalence rate in the Hevecam area. The prevalence rates observed in this study implicate and confirm an overall good standard of hygiene in the study area but a low implementation of malaria control tracking system in the area. Two helminth species all belonging to the Nematodes group *A. lumbricoides* and *T. trichiura* were identified in the faeces of residents of the investigated villages though mostly at low infection intensities. These two species were the most prevalent 31 years ago (Moyou et al., 1984). However, hookworms which were also present in the previous data were not found in the present work indicating either their disappearance or whenever they exist may be at very infection intensity to be detected by the Kato Katz technique. Using more sensitive parasitological techniques such as formol-ether concentration or FLOTAC techniques may have diagnosed some cases (Glinz et al., 2010). The significant reduction of helminth infection prevalence rates as well as intensity of infections were a proof of better hygiene conditions as shown by the access to improved drinking water sources, and also the significant anti-helmintic chemotherapy prevention coverage. In all villages investigated, water suitable human consumption (potable water) was collected exclusively from piped water. Providing households to access to piped water has been demonstrated to reduce markedly the risk of soil-transmitted infection by 43 to 60%, while wearing shoes reduces hookworm infection risk by 71%.

Two Plasmodium species namely *P. falciparum* and *P. malariae* were identified in blood smears with *P. falciparum* being the predominant specie. Occurrence of *P. malariae* in the area indicated a complication of malaria transmission in the Hevecam villages since this specie was not found 31 years ago (Moyou et al., 1984). Since malaria infection were diagnosed using both malaria Rapid Diagnostic Tests complemented by a specie confirmation with blood smears, the prevalence rate recorded in this study may be a true value. However, the moderate prevalence rates were in accordance with the low rate of malaria transmission prevention tools use. In the Hevecam villages, the percentage of dwellers who used a mosquitoe bednet was less than half the 80% target to be reached in 2015 as recommended by the World Health Organization and approved by the Cameroon Ministry of Public Health for the achievement of the Millenium Goal through reduction of malaria under public health importance (WHO, 2012, 2013, 2014).

While comparing data of the present study to those obtained 31 years ago in the same villages, intestinal helminth infections had significantly decreased in the area while malaria infections evolved in opposite way from hypoendemicity to mesoendemicity level. This evolution has also been characterized by apparition of *P. malariae* and absence or disappearance of hookworms thus simplifying the intestinal helminthiasis profile. In 1983, *P. falciparum* was the only malaria pathogen identified while *A. lumbricoides*, *T. trichiura* and hookworms were the causative agents of intestinal helminthiasis reported (Moyou et al., 1984). Appearance of *P. malariae,* and disappearance of hookworms in the Hevecam 31 years after indicated an occurrence of a complex malaria epidemiological profile whereas intestinal helminth infection profile was becoming less complex. Nevertheless, *P. falciparum* and *A. lumbricoides* have remained the predominant parasites for malaria and intestinal helminthiasis respectively. *P. malariae* and *T. trichiura* are therefore secondary causative agents while hookworms were not recorded.

The significant increase in malaria infection from hypoendemic to mesoendemic level was indicative of lack or poor implementation of malaria control guidelines. This increase of malaria cases contrasts the statistics released in the 2014 WHO report which indicated that up to 2013, the number of malaria infections dropped to about 26%, and the average malaria infection prevalence had a relative declined of 48% in children aged 2 to 10 years (WHO, 2012, 2014). A proper implementation of WHO recommended control measures was expected to lower by one third the malaria-related mortality rate among under five years children in the African region (WHO, 2012, 2014). The proportion of the participants who had an ITN and sleeping under one were assessed in the present study through an household survey. The possession rate of ITN was far less than half of the 80% WHO Millenium target (WHO, 2013). However, results of the present study corroborate previous entomological data which showed that environmental modifications due to agro-industrial activities in Niete might have influenced vector distribution and the dynamics of malaria transmission leading to transmission occurring both in the dry and rainy season with the intensities peaking in the dry season (Bigoga et al., 2012). Also, the densities of Anopheles vectors seemed higher in the dry season than the rainy season (Bigoga et al., 2012). The Hevecam company has been therefore incited to provide more mosquito nets to prevent malaria transmission.

Prevalence of intestinal helminth infections showed a significant decrease between 1983 and 2014 probably due to significant improve in personal hygiene practices as well as improved access to safe water. The reduction in prevalence rate was estimated at 69.24%. Intestinal helminthiasis are primarily caused by the absence of safe drinking water, lack of hygienic behaviour, improper sanitary habits, poor faecal disposal systems, poor socio-

economic status, and wide dispersion of parasites within human communities (Rai et al., 2000; Naish et al., 2012). The contribution of mass deworming and spontaneous practices was probably of greater influence in the decrease of helminthes infections in the Hevecam villages. In fact, the proportion of dwellers who swallowed one of the recommended anti-helminthic drugs at least once a year was greater than the 75% key target adopted by the World Health Organization for at risk-population by 2010 (WHO, 2002, 2012). Reduction of geohelminthiasis up to 73% has also been achieved in school-aged children through school-based survey in many health districts in Cameroon according (Tchuem et al., 2012, 2013).

Despite the low infection prevalence rate and overall low intensity of infection recorded in the present study, caution need to be taken in such clustered settlements where an outbreak of any communicable disease can occur at any time. Also, like heavy intensities of infections, a number of studies have suggested that even a moderate intensity of STH infections are considered a leading cause of sickness, absenteeism and disability adjusted life years (DALYs) lost as well as lost delayed physical growth and impaired cognitive development, particularly among school-children (Brooker, 2010; Curtale et al., 1998, 1999; Brooker et al., 2008; Bethony et al., 2006; Ostan et al., 2007; Murray et al., 2012). The magnitude of the burden of geohelminthiasis is often underestimated in most low income and middle income countries but it deserves to be given special attention because of its broad geographical distribution (WHO, 2012) and its negative health impact. Intestine inhabiting adult helminth have a significant health effect by interfering with the host's nutrition and inducing damage to the intestinal mucosa, therefore resulting in the host's reduced ability to extract and absorb nutrients from food which result in poor weight especially in children and women of child-bearing age (Bethony et al., 2006; Nokes and Bundy, 1994). Such impact implied that control system need to be sustained in the Niete area to lower the prevalence of intestinal helminth infections as well as other controllable communicable diseases. It is well known that preventive chemotherapy can eliminate infections of moderate and high intensity, but it does not prevent infection.

Therefore, reinfection often occurs rapidly after treatment as a result of poor sanitation, access to clean water and hygiene practices (Bartram and Cairncross, 2010; Jia et al., 2012). There is a need for combining preventive chemotherapy to other sustainable measures notably improvements to water, sanitation, and hygiene access and practices are highly recommended as a more effective elimination strategy of soil-transmitted helminth as public health problem in endemic areas (Bartram and Cairncross, 2010). Improving sanitation infrastructures coupled to good hygiene practices would ideally interrupt transmission of STH as well as other faecal-related infections and prevent the development of morbidity. Safe water access was shown to be operational in the Hevecam villages. Other efficient complementary measures such as sanitation access need to be implemented in the area.

The prevalence trends with age was in accordance to usually known data in endemic areas with a sharp increase from preschool-aged children to school-attending children followed by a marked decreased with adolescence. Such trend was also recorded in the data collected 31 years ago (Moyou et al., 1984). There were no standard sex-based differences in the prevalence of specific parasites infection among villages. Poly-parasitism by helminth parasites which occurred in 13.6% of participants followed a general rule in most intestinal helminthiasis endemic areas. Such co-infection was found in the study undergone in 1983 (Moyou et al., 1984). Also, the occurrence of co-infections by *Plasmodium* parasites, and one or two helminth parasites species as recorded were also reported 31 years ago in the Hevecam villages (Moyou et al., 1984). Such frequency of polyparasitic infections combining malaria parasites and helminth may have significant impact on the morbidity either by amplifying the clinical manifestations of each other diseases compared to mono-infection.

The evolution of malaria and intestinal helminth infections in the Hevecam scheme is certainly the consequence of the level of adhesion to recommended control strategies based on prevention and specific chemotherapy together with regular health education in the area.

Conclusion

This study raised that in 2014, malaria and intestinal helminth infections were mesoendemic and hypoendemic in the Hevecam agroindustrial complex respectively. When compared with previous data collected 31 years ago, malaria infection has significantly increased according to both parasitic infections as well spleen index, whereas intestinal helminth infections evolved in opposite direction decreasing from hyperendemic to hypoendemic. The present study indicated that *P. falciparum* was the leading malaria agent and *P. malariae* a secondary agent. Intestinal helminth infections were predominantly due to *A. lumbricoides* and secondary to *T. trichiura* with occurrence of co-infection by the two parasites species. These data appeal for implementation of an adequate integrated safeguard for disease surveillance in the Hevecam company which may need interventions of both the specific national control programs, the Hevecam administrations and health researchers.

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Conflict of interest

The authors of this manuscript declare that there is no conflict of interests regarding the publication of this research work.

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Full Length Research Paper

Molecular differentiation of *Entamoeba Spp.* isolated from Cameroonian human immunodeficiency virus (HIV) infected and uninfected patient

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***Entamoeba histolytica* is an utmost important cause of dysentery. *Entamoeba* spp. has been frequently reported in human immunodeficiency virus (HIV) positive individuals. Routine microscopic examination of stool sample is a most widely used technique but microscopy alone has low sensitivity and it is insufficient for differentiation among *Entamoeba* spp. Molecular techniques are newer methods which are currently used for the identification of *Entamoeba* spp. The present study was planned to differentiate the *Entamoeba* species by gene sequencing for the confirmation of microscopic findings in stool samples of HIV positive and negative patients of Cameroon. Out of 265 patients diagnosed microscopically for *Entamoeba,* 90 positive stool samples (28 from HIV patients) were collected and studied for the differentiation of *Entamoeba* species. DNA was extracted from infested stool samples and used to amplify a part of the genus *Entamoeba* small-subunit ribosomal RNA gene (SSU rDNA) as well as the serine rich *E. histolytica* protein gene and chitinase gene. The SSU rDNA were sequenced to identify the other species that could not be done by polymerase chain reaction (PCR), and for the differentiation of *E. histolytica* from *Entamoeba dispar* and *Entamoeba moshkovskii.* Sequence analysis identified seven different species of *Entamoeba* which were related to *Entamoeba*; *E. histolytica* (28.7%), *E. dispar* (25%), *E. moshkovskii* (10%), *Escherichia coli* (16.3%), *Entamoeba hartmanni* (6.2%), *Entamoeba polecki* (11.3%) and *Entamoeba struthionis* (7.5%), with the higher prevalence of *E. histolytica among* HIV infected patients than uninfected individuals. The phylogenetic analysis within the sequences of *E. histolytica* isolates suggested two distinguishable variants present among Cameroonian HIV patients. There is a possibility that specific genotypes may be more prevalent among HIV positive patients, and molecular diagnosis is important in establishing the correct diagnosis of amoebic dysentery.**

Key words: *Entanoeba* spp, HIV/AIDS, gene sequencing, Cameroon.

INTRODUCTION

Various *Entamoeba* species are often found in the stools of humans. Although, the majority of these *Entamoeba* spp. are considered to be harmless, care should be taken when *Entamoeba histolytica*, the causative agent of

amoebiasis, is involved. Infection with this gastrointestinal parasite may cause hemorrhagic dysentery, extra intestinal pathologies (example, liver abscesses) and death (Santos et al., 2010). Moreover, amoebiasis remains a significant cause of morbidity and mortality in the world. This infection is of major concern in public health, causing up to 100,000 deaths worldwide each year (WHO, 1997, 1997; Stauffer et al., 2006). In African countries, prevalence of *Entamoeba* spp. has been reported to vary from 1.4 to 12.4% (Gassama et al., 2001; Brink et al., 2002; Hailemariam et al., 2004; Samie et al., 2010).

Following the Human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS) pandemic, numerous studies demonstrated that intestinal parasites such as *Cryptosporidium* spp., *Microsporidia* spp., *Cystoisospora belli* and *Cyclospora cayetenensis* were frequently associated with episodes of severe, and often fatal diarrhea in both industrialized and developing countries (Stark et al., 2009; Nissapatorn et al., 2011, O'Connor et al., 2011). Currently, little is known about the occurrence of different *Entamoeba* spp. and their genotypes in co infection with HIV in Cameroon. However, some studies conducted in Mexico, South Africa and Taiwan on the *E. histolytica* and HIV co-infected patients demonstrated a high prevalence of infection with *E. histolytica* (Moran et al., 2005; Tsaï et al., 2006; Nkenfou et al., 2013). These studies were based on the detection of cysts or trophozoites in stool samples by using light microscopy or by detection of specific antibodies by serology in serum samples. However, differentiation between *E. histolytica* and other *Entamoeba spp.* (such as *Escherichia coli, Entamoeba hartmanni* and *Entamoeba polecki* like organisms) based on morphological features is difficult, and when *Entamoeba dispar or Entamoeba moshkovskii* is involved, it is impossible. Therefore, molecular methods, such as DNA-based tests, have aided in improving some of the sensitivity and specificity deficiencies associated with traditional methods for the detection of protozoan pathogens. A number of DNA-based assays like gene amplification with specific primers, multiplex polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and real-time PCR (RT-PCR) and gene sequencing have been developed for the identification of *Entamoeba* species infections (Fotedar et al., 2007; Samie et al., 2008; Bruijnesteijn van Coppenraet et al., 2009). To the best of this study knowledge, there is no study available from Africa in which PCR along with gene sequencing have been used for the identification of *Entamoeba* species and its subtypes isolated from HIV infected and uninfected patients. However, previously published studies are either based on serology (Jackson et al., 2000), microscopy or PCR (Zaki et al., 2003). The *Entamoeba* spp. that can be found in these patients remain unknown in Cameroon, and most of Sub-Saharan Africa. To fill this gap a molecular differentiation of *Entamoeba spp.* was performed among HIV positive and negative patients in two cities of Cameroon (Dschang and Ngaoundere).

METHODOLOGY

Ethics statement

This study was approved by the Cameroon National Ethic Committee (CNE) under the registration No. 131/CNE/SE/2012. The rules and regulations of good clinical laboratory practice were followed during the study. Participants consulting at the hospitals were kindly requested by the study team to participate in the study. All interested adult subjects provided written informed consent, and an interested parent or guardian of any child participant provided written proxy consent. All participants were offered professional counseling before and after HIV testing for those who had never done it before. All diagnostic results were kept strictly confidential. Anti-amoebic therapy treatments (metronidazole) were given to all participants who were found to be infected with *E. histolytica*.

Sample collection

A total of 265 patients (60 HIV positive patients) were recruited and diagnosed in the present study from July, 2012 to May, 2013 from two cities of Cameroon (Ngaoundere and Dschang) after obtaining their written informed consents. Out of 265 patients diagnosed for *Entamoeba* spp. infection, 90 stool samples (28 from HIV patients and 62 from HIV uninfected individuals) in which cysts or trophozoïtes of *Entamoeba* were detected by microscopic observation were further processed for confirmation by molecular method. Stool samples were kept in 2 ml Eppendorf tubes and stored at -20°C till further use.

Genomic DNA isolation from stool samples

For DNA extraction, stool samples of patients from Cameroon and *E. histolytica* strain grown on polyxenic medium at the Department of Medical Parasitology of Postgraduate Institute of Medical Education and Research, Chandigarh, India were used. Approximately, 200 mg of stool sample was taken to extract DNA using QIAamp DNA stool mini kit (Qiagen) according to the manufacturer's protocol with few modifications: all the centrifugations steps were carried out at 800 g except the final step of purification in which centrifugation was done at 1300 g. *E. histolytica* strain was harvested from culture at mid log phase and centrifuged at 3000 rpm. The pellet was washed with PBS buffer pH 6.8 and resuspended in the same buffer. A 200 µl volume of this suspension was used to extract DNA from cysts and trophozoites of *Entamoeba* as described above. The extracted DNA from culture was used as positive control for the amplification reactions. The purity of the extracted DNA was estimated from the absorbance ratio 260/280 and its concentration in all the samples was estimated from the 280 nm readings.

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PCR amplification of the targeted genes

Three loci have been targeted for the amplification reactions: small subunit of ribosomal DNA (SSU rDNA), chitinase gene and serine rich *E. histolytica* protein (SREHP). The SSU rDNA gene has been previously used for the identification of the species (Clark and Diamond, 1991; Novati et al., 1996; Verweij et al., 2001). In fact, the chitinase and SREHP genes have polymorphic DNA loci which have been used to study the molecular epidemiology and the geographical diversity among human isolates of *E. histolytica* (Ghosh et al., 2000; Haghighi et al., 2002; Takano et al., 2007). Specific primers used for the three set of genes were as follows: the Sense Primer known as Entam1 5'GTT GAT CCT GCC ATT ATA TG 3' and the Antisense Primer known as Entam2 5'CAC TAT TGG AAT TAC 3'for the small subunit of ribosomal RNA (Ghosh et al., 2000), Sense Primer or SREHP1 5'GCT AGT CCT GAA AAG CTT GAA GAA GCT G and the Antisense Primer or SREHP2 5'GGA CTT GAT GCA GCA TCAAGG T 3'for the amplification of SREHP gene, the Sense Primer or EHF 5' GGA ACA CCA GGT AAA TGT ATA 3' and the Antisense Primer or EHR 5'TCT GTA TTG TGC CCA ATT 3' for the chitinase gene (Haghighi et al., 2002; Takano et al., 2007). PCR amplification of the SSUrDNA gene was performed in a total volume of 40 µl containing 2.5 µl of 10× PCR buffer, 3 µl of 25 mM $MgCl_2$, 250 µM of each dNTP, 25 pmol of each primer (Entam1 and Entam2), 2.5 U of Taq DNA Polymerase (Promega) and 3µl of genomic DNA sample. PCR mixture was submitted to denaturation at 94°C during 5 min, then to 35 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for 1 min followed by the final step of extension at 72°C for 10 min. For the amplification of the SREHP gene and chitinase, only the samples found positive for the genus *Entamoeba* (SSUrDNA) gene amplification were chosen. PCR amplification reactions with SREHP and chitinase primers were performed in a total volume of 40 µl containing 2.5 µl of 10× PCR buffer, 6 µl of 25 mM $MgCl_2$, 250 µM of each dNTP, 25 pmol of each primer (EHF and EHR), 2.5 U of Taq DNA Polymerase (Promega) and 3 µl of genomic DNA sample. PCR mixture was submitted to denaturation at 94°C during 5 min, then to 45 cycles at 94°C for 1 min, 60°C (SREHP) or 50°C (chitinase) for 1 min and 72°C for 1 min followed by the final step of extension at 72°C for 7min. To visualize the amplified genes, 5 µl of the PCR mixture were submitted to 1.5% agarose gel electrophoresis containing ethidium bromide. The migration was done under a voltage of about 78 to 80 mV and a current of 34 mA. This migration was followed by mixing the sample with the loading buffer containing bromophenol blue dye. After migration, the gel was visualized by Transluminscence (UVITEC Transluminator, Cambridge CB4 1QB-England) and photographed.

Gene sequencing and sequence analysis

The 550 bp PCR products containing the SSUrDNA locus were directly sequenced with appropriate primers in both directions. All of the PCR samples that were found to contain single bands on the agarose gels were treated with a Pre-Sequencing kit (USB Corporation, Cleveland, Ohio) before sequencing. Each 550 bp DNA fragment of the PCR samples that showed double or triple bands by agarose gel electrophoresis were excised and treated using a QIAquick gel extraction kit (Qiagen, Hilden-Germany). Individual PCR products were then sequenced using an ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems), according to the manufacturer's directions. The SSUrDNA PCRs products that generated multiple sequencing products which appeared as mixed profile in sequencing reaction, were purified with Qiaquick gel extraction Kit (Qiagen, Hilden, germany) and cloned using pCR2.1-TOPO vector as described in the protocol from the TOPO TA cloning Kit (Invitrogen, Carlsbad, CA,USA) (Santos et al., 2010). The sequences obtained were manually edited and aligned using ClustalW2. The phylogenetic tree based on the partial 16S like SSUrDNA sequences showing the distance among clinically important species of *Entamoeba* (*E. histolytica, E. dispar* and *E. moshkovskii*) were constructed (Saitou and Nei, 1987). Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2004; Tamura et al., 2013). The accession numbers of the nucleotide sequences used as reference in that construction were as follows: *E. dispar* (Z49256.1) *E. histolytica* (AB197936.1) and (X64142), *E. struthionis* (AJ566411.1), *E. coli* (ST1 or AF149915, and ST2 or AF149914), *E. polecki* (EF110881.1), *E. hartmanni* (AF149907.1), *E moshkovskii* (AF149906.1), *Entamoeba invadens* (AF149905) and *E. chattoni* (AF149912).

Nucleotide sequence accession numbers

The nucleotide sequence data reported in the present work have been submitted to the GenBank/EMBL/DDBJ database under accession numbers AB845670 to AB845674; AB851494 to AB851500; KF515235 to KF515253 and KF870200 to KF870233.

Statistical analysis

Data were registered in Microsoft excel 2010 and analyzed with Statistical Package for the Social Science (SPSS) version 11.0 statistical software. Chi square (χ^2) test allowed us to compare the prevalence of *Entamoeba* infection according to HIV status. Associations were tested at 95% confidence.

RESULTS

A total of 265 patients were recruited and their stools were examined microscopically for *Entamoeba* spp. Of these, 90 samples were diagnosed as *Entamoeba* positive (28 samples from HIV patients and 62 from non-HIV patients) were collected and studied for the differentiation of *Entamoeba* species. Out of 90 stool samples positive for all the *Entamoeba* species, 45 (50%) samples were positive for *E. histolytica* as initially diagnosed by microscopy; 80 (88.9%) (Table 1) were positive for PCR of the genus specific *Entamoeba* with the SSUrDNA primers set and 23 (28.7%) were positive for *E. histolytica* with the chitinase and SREHP primers set (Table 1). Ten samples (11.1%) initially diagnosed microscopically positive for *Entameoba* spp. were negative by PCR (Table 1). After performing sequencing and Basic Local Alignment Search Tool (BLAST) similarity of the different sequences, the result (Table 2) showed that 7 different species of *Entamoeba* that is, *E. histolytica* (28.7%), *E. dispar* (25%), *E. moshkovskii* (10%), *E. coli* (16.3%), *E. hartmanni* (6.2%), *E. polecki* (11.3%) and *E. struthionis* (7.5%) were found in 80 PCR confirmed stool samples. *E. histolytica*, *E. coli*, *E. hartmanni* and *E. struthionis* were found to be more prevalent in HIV infected patients (33.3, 20.8, 8.3 and 8.3% respectively) than in negative cases (25, 14.3, 5.4 and 7.1% respectively). *E. dispar*, *E. moshkovskii* and *E. polecki* (25, 11.7 and 11.3%) were more prevalent within the HIV uninfected individuals. However, multispecies

Table 1. Overall table showing the species of *Entamoeba* isolated from stool samples of Cameroonian HIV infected and uninfected patients by PCR and gene sequencing after microscopy diagnosis. EH= *E. histolytica* cyst; EC = *E. coli* cyst; NIA= Non Identified Amoeba cyst; += positive; - = negative. NB; three cases of double species infection occurred and are mentioned in the table.

Samples code	HIV status	Microscopy	PCR amplification			Sequencing of SSUrDNA gene
			SSUrDNA	SREHP	Chitinase	
TA	-	NIA	+	-	-	*E. coli*
DS	-	NIA	+	-	-	*E. polecki*
NS	-	EH	+	-	-	*E. dispar*
TE	-	EH	+	+	+	*E. histolytica/E.moshkovskii*
DM	-	NIA	+	-	-	*E. coli*
TM	-	NIA	+	-	-	*E. coli*
SD	-	EH	+	-	-	*E. dispar*
EM	-	EH	+	-	-	*E. dispar*
CN	-	NIA	+	-	-	*E. coli*
KC	+	EC	-	*Not done*	*Not done*	*Not done*
DR	-	EC	+	-	-	*E. coli*
LM	-	EH	+	+	+	*E. histolytica*
LF	-	EC	+	-	-	*E. hartmanni*
AF	-	NIA	+	-	-	*E. coli*
AI	-	EH	+	+	+	*E. histolytica/E. dispar*
AJ	-	EH	+	-	-	*E. dispar*
LA	-	EH	+	-	-	*E. moshkovskii*
ME	-	EH	+	-	-	*E. dispar*
1010	-	NIA	-	*Not done*	*Not done*	*Not done*
1011	-	EH	+	+	+	*E. histolytica*
1012	+	EC	+	-	-	*E. coli*
1013	-	EH	+	+	+	*E. histolytica*
1014	-	EH	+	+	+	*E. histolytica*
1015	-	EC	+	-	-	*E. struthionis*
1016	+	EH	+	-	-	*E. dispar*
1017	+	NIA	+	-	-	*E. hartmanni*
1018	+	EH	+	-	-	*E. moshkovskii*
1019	-	EH	+	-	-	*E. dispar*
103	-	EC	+	-	-	*E. polecki*
109	-	EC	+	-	-	*E. coli*
1234	+	NIA	+	-	-	*E. polecki*
1145	+	EC	-	*Not done*	*Not done*	*Not done*
1211	+	EH	+	+	+	*E. histolytica*
230	+	EH	+	-	-	*E. dispar*
235	+	EH	+	+	+	*E. histolytica*
434	+	EC	+	-	-	*E. struthionis*
S1	-	EH	+	-	-	*E. moshkovskii*
S2	-	EC	-	*Not done*	*Not done*	*Not done*
S3	-	EC	-	*Not done*	*Not done*	*Not done*
113	-	EC	+	-	-	*E. coli*
121	+	EH	+	+	+	*E. histolytica*
1262	+	EH	+	+	+	*E. histolytica*
1277	-	EC	+	-	-	*E. polecki*
1273	-	EC	+	-	-	*E. polecki*
114	+	EH	+	+	+	*E. histolytica*
139	+	EH	+	+	+	*E. histolytica*
67	-	EH	-	*Not done*	*Not done*	*Not done*

Table 1. Contd.

94	-	EH	+	+	+	*E. histolytica*
833	-	EC	+	-	-	*E. polecki*
172	-	NIA	-	*Not done*	*Not done*	*Not done*
1083	-	EC	+	-	-	*E. polecki*
MO	-	EH	+	-	-	*E. dispar*
1040	-	EC	+	-	-	*E. coli*
4121	-	EH	+	-	-	*E. dispar*
0 3702	-	EH	+	+	+	*E. histolytica*
43121	+	EC	-	*Not done*	*Not done*	*Not done*
1874	-	EC	+	-	-	*E. dispar*
1062	+	EH	-	*Not done*	*Not done*	*Not done*
11664	-	EH	+	-	-	*E. dispar*
11804	+	EH	+	-	-	*E. dispar*
7335	+	NIA	+	-	-	*E. struthionis*
1073	+	NIA	+	-	-	*E. struthionis*
4142	-	NIA	+	-	-	*E. hartmanni*
11673	-	EC	+	-	-	*E. coli*
FC	-	EH	+	-	-	*E. dispar*
KH	+	EC	+	-	-	*E. coli*
AO	+	EH	+	+	+	*E. histolytica*
MJ	+	NIA	+	-	-	*E. polecki*
NM	-	NIA	+	-	-	*E. struthionis*
DF	-	NIA	+	+	+	*E. histolytica*
NR	-	EH	+	-	-	*E. moshkovskii*
TB	-	EH	+	+	+	*E. histolytica*
NT	-	NIA	+	-	-	*E. moshkovskii*
TH	-	NIA	-	*Not done*	*Not done*	*Not done*
SV	-	NIA	+	-	-	*E. struthionis*
TI	-	EH	+	+	+	*E. histolytica*
SR	-	EH	+	-	-	*E. dispar*
DO	-	EH	+	+	+	*E. histolytica*
VJ	-	NIA	+	-	-	*E. hartmanni*
NC	+	EC	+	-	-	*E. Coli*
MB	-	EH	+	+	+	*E. histolytica*
ER	-	NIA	+	-	-	*E. hartmanni*
SO	+	EH	+	+	+	*E. histolytica*
YU	+	EH	+	-	-	*E. dispar*
ML	-	EH	+	-	-	*E. moshkovskii*
TL	-	NIA	+	-	-	*E. moshkovskii*
TF	-	EH	+	+	+	*E. histolytica/E. dispar*
BB	-	EH				*E. dispar*
DJ	-	EH	+	-	-	*E. dispar*
DB	-	NIA	+	-	-	*E. polecki*

infection was rare in this study population. HIV individuals were infected with only one species while in HIV uninfected group, 3 individuals (3.2%) were infected with two species (*E. histolytica*/, *E. moshkovskii* and *E. histolytica*/*E. dispar*).

The phylogenetic tree was constructed using the Neighbor-Joining Method (Figure 1), which shows the distances between sequences of three clinically important *Entamoeba species* (*E. histolytica*, *E. dispar* and *E. moshkovskii*) isolated from both HIV infected and uninfected individuals. This phylogenetic tree presents four clades (group of clusters different each to other) of

Table 2. Prevalence of *Entamoeba spp* differentiated in stool samples of Cameroonian HIV infected and uninfected patients tested by PCR and gene sequencing (p<0.002).

Species	HIV/AIDS patients (%)	HIV negative (%)	Overall population infected by each species (%)
E. histolytica	8 (33.3)	15 (26.8)	23 (28.7)
E. dispar	3 (12.5)	15+2* (30.3)	18+2* (25)
E. moshkovskii	1 (4.2)	6+1* (12.5)	7+1* (10)
E. coli	5 (20.8)	8 (14.3)	13 (16.3)
E. hartmani	2 (8.3)	3 (5.4)	5 (6.2)
E. polecki	3 (12.5)	6 (10.7)	9 (11.3)
E. struthIonIs	2 (8.3)	4 (7.1)	6 (7.5)
totaux	24 (100)	56+3* (100)	80+3* (100)

NB: *= double species infection.

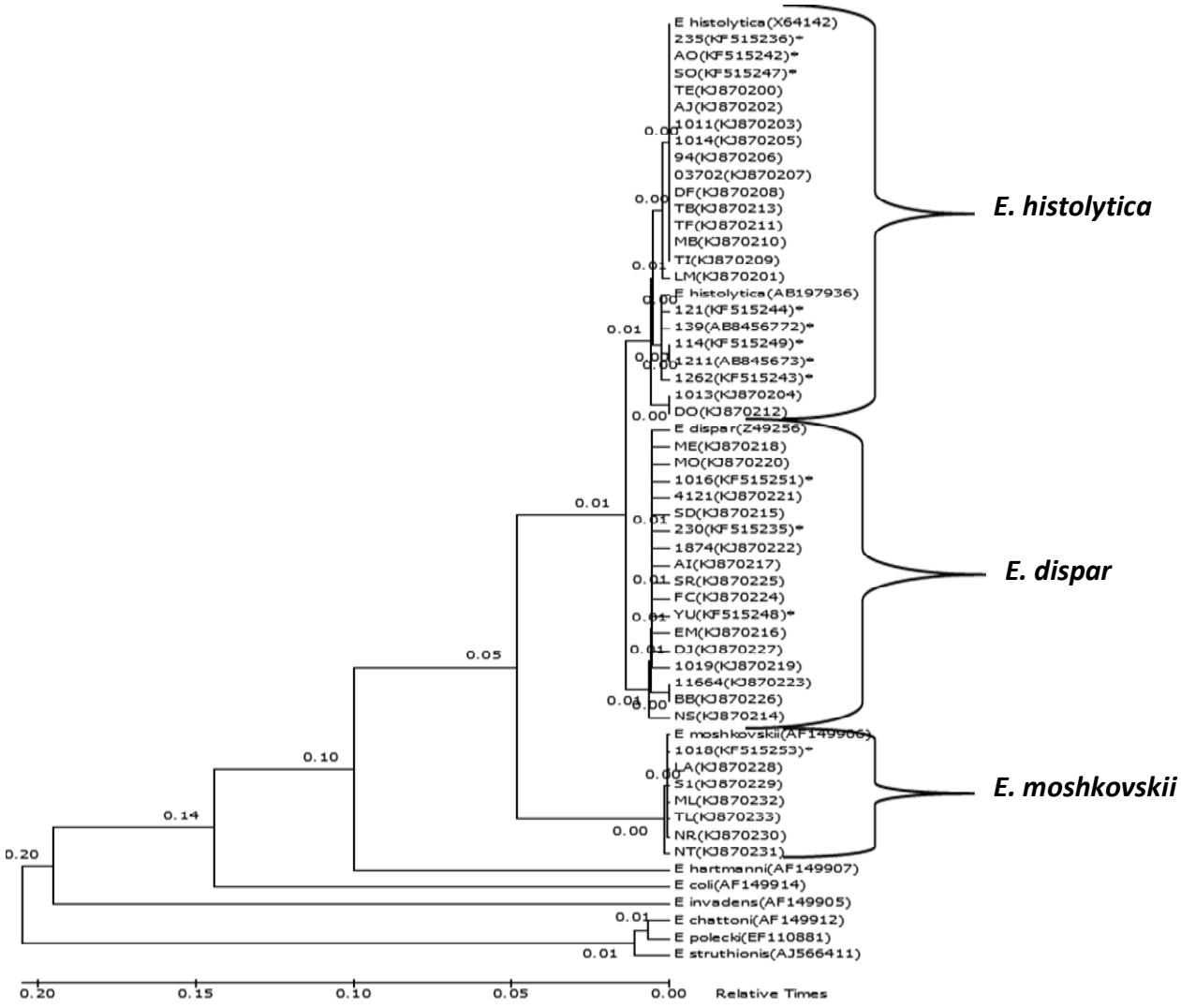

Figure 1. Phylogenetic tree based on partial SSUrDNA sequences, showing the relationships among clinically important identified species of *Entamoeba* (*E. histolytica*, *E. dispar* and *E. moshkovskii*). Phylogenetic analysis used two different approaches, distance-based analysis and maximum-likelihood (ML), produced trees with identical topologies of which only ML tree is presented. GenBank accession numbers are given in parentheses after the taxon name. Sequences without taxons were obtained during this study. Numbers above branches are relative time values from 1,000 replicates. N.B:* =sequences isolated from HIV positive patients.

sequences of clinically important *Entamoeba species* (2 clades of *E. histolytica*; 1 clade of *E. dispar* and 1 clade of *E. moshkovskii*). *E. histolytica* sequences isolated from all the patients is represented in two clades closely related respectively to the reference sequences of *E. histolytica* (AB197936) and (X64142). *E. histolytica*, *E. dispar* and *E. moshkovskii* were aligned with reference sequences of corresponding *Entamoeba* species retrieved from the gene bank (X64142, Z49256, and AF149906). Comparison of these sequences revealed that out of 8 sequences of *E. histolytica* isolated from HIV patients, 5 have 99.1% identity with the reference sequence X64142 whereas 3 isolates have 100% similarity with reference to the same sequence (Figure 2). *E. dispar* sequences isolated from HIV patients have 100% similarity with reference sequence Z49256. Among HIV negative patients, the majority of *E. histolytica* sequences have 100% similarity with reference sequence X64142 except three sequences (KJ870201; KJ870204 and KJ870212) that have 98.9% similarity with the same reference sequence. Only one sequence of *E. dispa*r (KJ870214) has 99.2% similarity with reference sequence Z49256 (Figure 3), and majority of the other sequences of *E. dispar* have 100% similarity with the same reference sequence. Same observation is made with *E. moshkovskii* sequences (Figure 4) among the sequences that have 99.2% similarity with reference sequence AF149906, and one sequence (KJ870231) has 100% similarity with the same reference sequence.

DISCUSSION

Intestinal opportunistic parasites such as *Cryptosporidium* spp., Microsporidia spp., *Cystoisoisospora belli* and *Cyclospora cayetanensis* are utmost importance cause of diarrhea among HIV positive individuals (Stark et al., 2009). *Entamoeba spp*. has been reported to colonize with increased frequency among HIV positive individuals (Hung et al., 2005; Watanabe et al., 2011). Recent data have shown an increase in the occurrence of *E. histolytica* among HIV patients in countries such as Japan, Mexico, Taiwan and South Africa (Moran et al., 2005; Hung et al., 2008; Samie et al., 2008; Watanabe et al., 2011). With the hall mark of HIV infection being the depletion of CD4+ T cells count (below 200 cells/µl) and the progressive decline of the mucosal immunologic defense mechanisms, HIV/AIDS patients become more prone to life-threatening gastrointestinal infections such as diarrhea due to opportunistic pathogens (Stark et al., 2009).

E. histolytica is an important cause of dysentery, and can also manifest as extra-intestinal invasive form. Majority of the infections are asymptomatic and in about 10% of the cases it is symptomatic (WHO, 1997). Laboratory diagnosis of the etiological agent of diarrhea/ dysentery is of utmost important for the timely management of dysentery cases. Routine microscopic examination of stool sample is the most widely used technique for identifying the parasitic cause of diarrhea. However, microscopy alone is insufficient for differentiation between *E. histolytica*, *E. dispar* and *E. moshkoweskii*. It also suffers from low sensitivity (<10%) and specificity (Huston et al., 1999; Fotedar et al., 2007). There are other diagnostic methods such as zymodeme analysis which is cumbersome to perform (Sargeaunt et al., 1978). Molecular techniques such as PCR (Tanyuksel et al., 2003; Solaymani et al., 2006), RFLP (Hooshyar et al., 2003), real time PCR (Hamzah et al., 2010) and genotyping (Ali et al., 2005; Kumari et al., 2013) are newer methods which are currently being used for the identification of *Entamoeba* speciesc.

The results of PCR amplification showed that only 51.1% (23/45) of the stool samples initially diagnosed as positive for *E. histolytica* by microscopy were found to be positive by PCR. The present study also highlights the limitation of microscopy in correctly diagnosing the *Entamoeba* spp. as compared to the molecular identification as reported by previously published studies (Krogstad et al., 1978; Tannich et al., 1989; Acuna-Soto et al., 1993; Diamond and Clark, 1993). Among Cameroonian patients, *Entamoeba* spp. other than *E. histolytica* was found to be present in higher number. These results are consistent with earlier observations that *Entamoeba* infection in Africa is more frequently due to other species of *Entamoeba* as compared to *E. histolytica* (Ekou et al., 2013). Similar observations have been made in Brazil, Nicaragua and Italy (Fotedar et al., 2007). Australia exhibits the highest frequency of *E. dispar* (73.3%) and *E. moshkovskii* (60.7%) infections, detected by molecular techniques in microscopic positive for *Entamoeba* cysts in general population (Fotedar et al., 2007). Thus, in immune compromised individuals also other species of *Entamoeba* may be mistaken for *E. histolytica* if only microscopy is used for diagnosis. Though, molecular techniques are much more sensitive and specific than microscopy but these are expensive to perform in routine clinical setting in developing countries.

The SSUrDNA was sequenced to identify the other species that could not be done by PCR because only the primers specific for *E. histolytica* were used in the amplification reaction. The reason of choosing SSUrDNA gene for sequencing and further analysis is attributed to the fact that it has polymorphic DNA loci and successfully used in previously published literature for phylogenetic study of *Entamoeba* spp. (Clark and Diamond, 1997; Silberman et al., 1999; Clark et al., 2006). After sequencing, 24 samples (52.2%) initially diagnosed as *E. histolytica* by microcopy and negative by PCR for *E. histolytica* were found to be positive for *E. moshkovskii* (15.2%) and *E. dispar* (37%). Some samples initially diagnosed microscopically as *E. coli* were found to be *E. struthionis*, *E. hartmanni*, or *E. polecki*. Earlier, *E. struthionis* was isolated from farmed ostriches in Spain

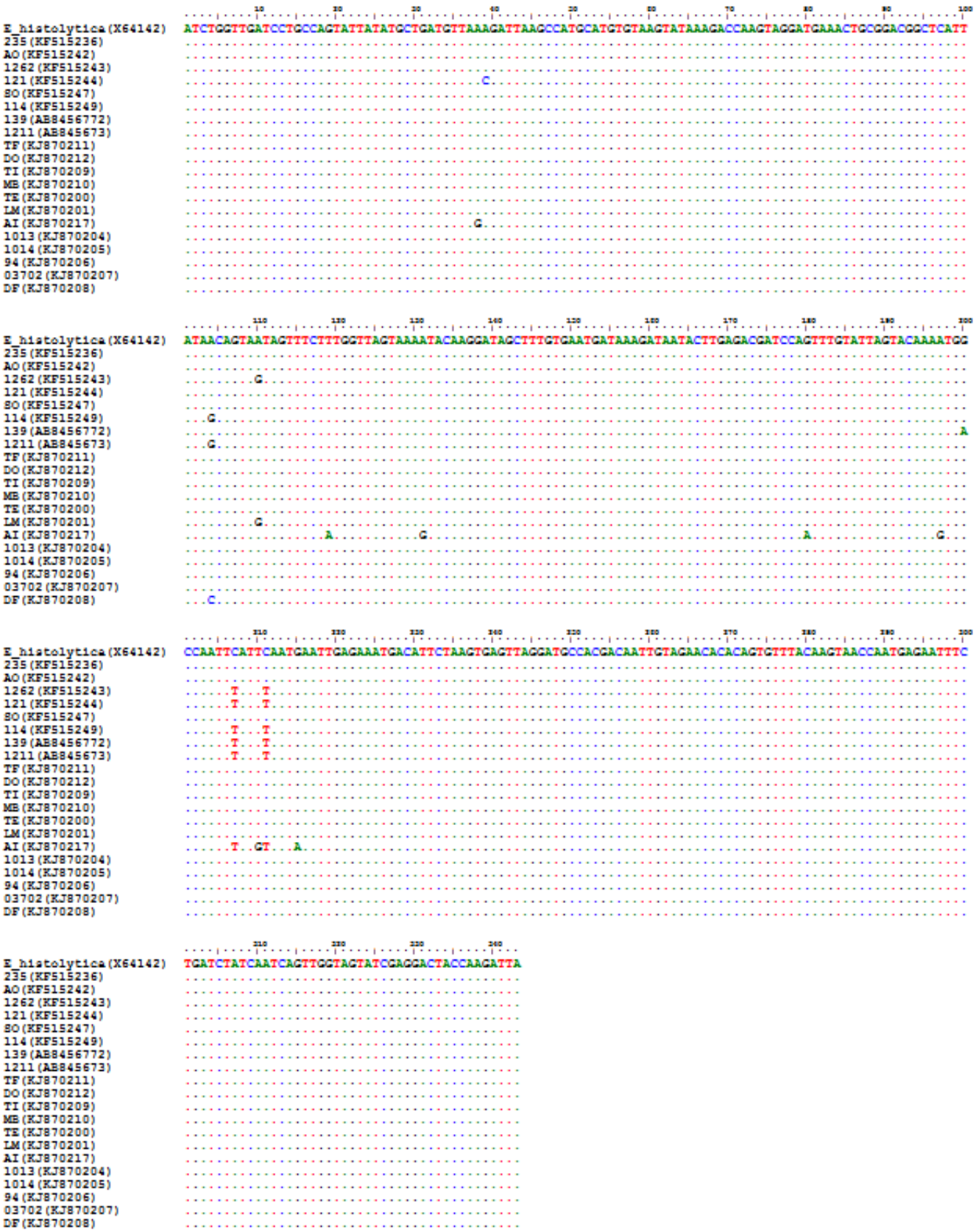

Figure 2. Multiples sequences alignment of *E. histolytica*, 16S like SSUrDNA gene sequences from Cameroonian HIV positive and negative patients with reference sequence of *E. histolytica* retrieved from the genbank.

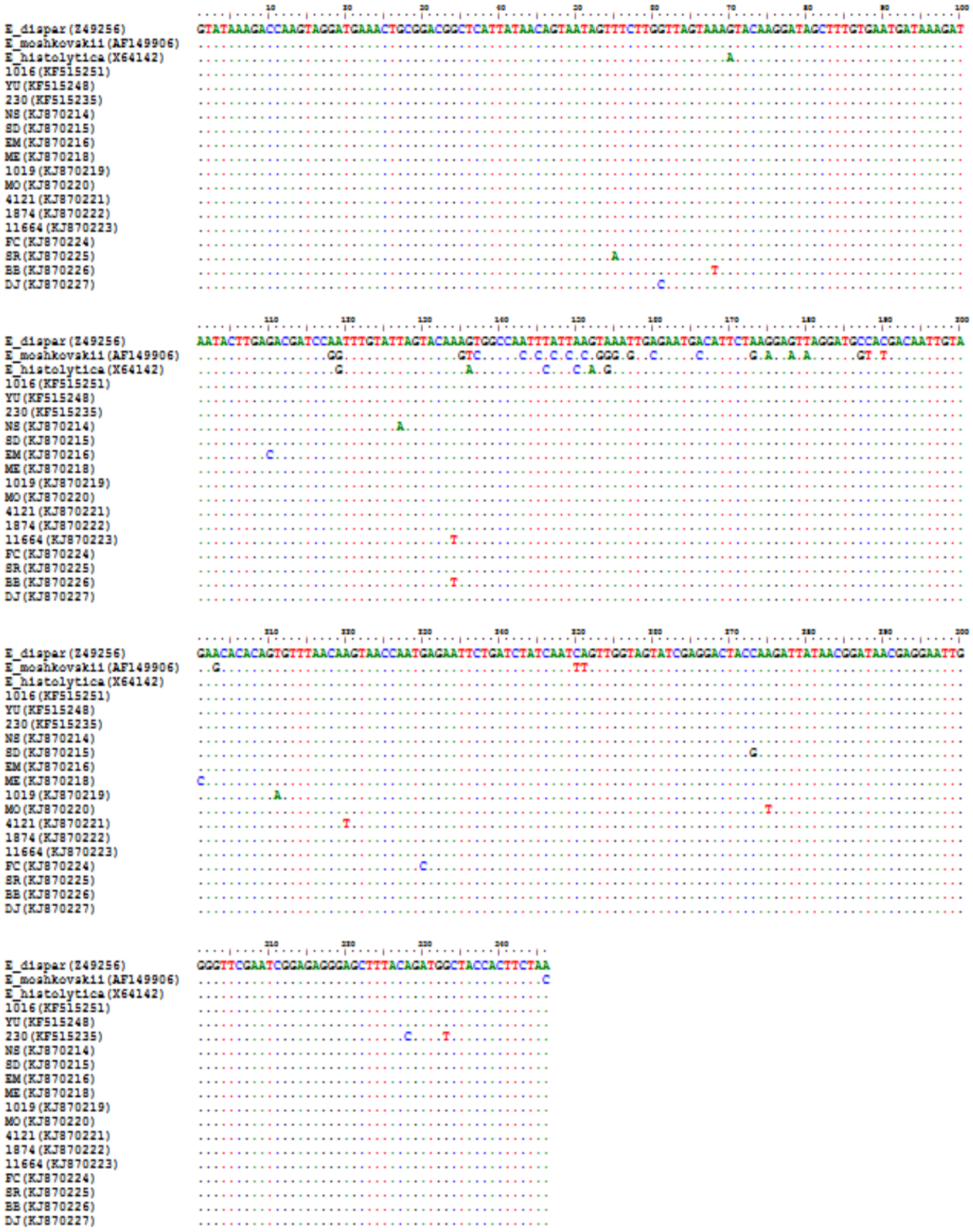

Figure 3. Multiples sequences alignment of *E. dispar* 16S like SSUrDNA gene sequences from Cameroonian HIV positive and negative patients with reference sequences of *E. histolytica*, *E. dispar* and *E. moshkovskii* retrieved from the genbank.

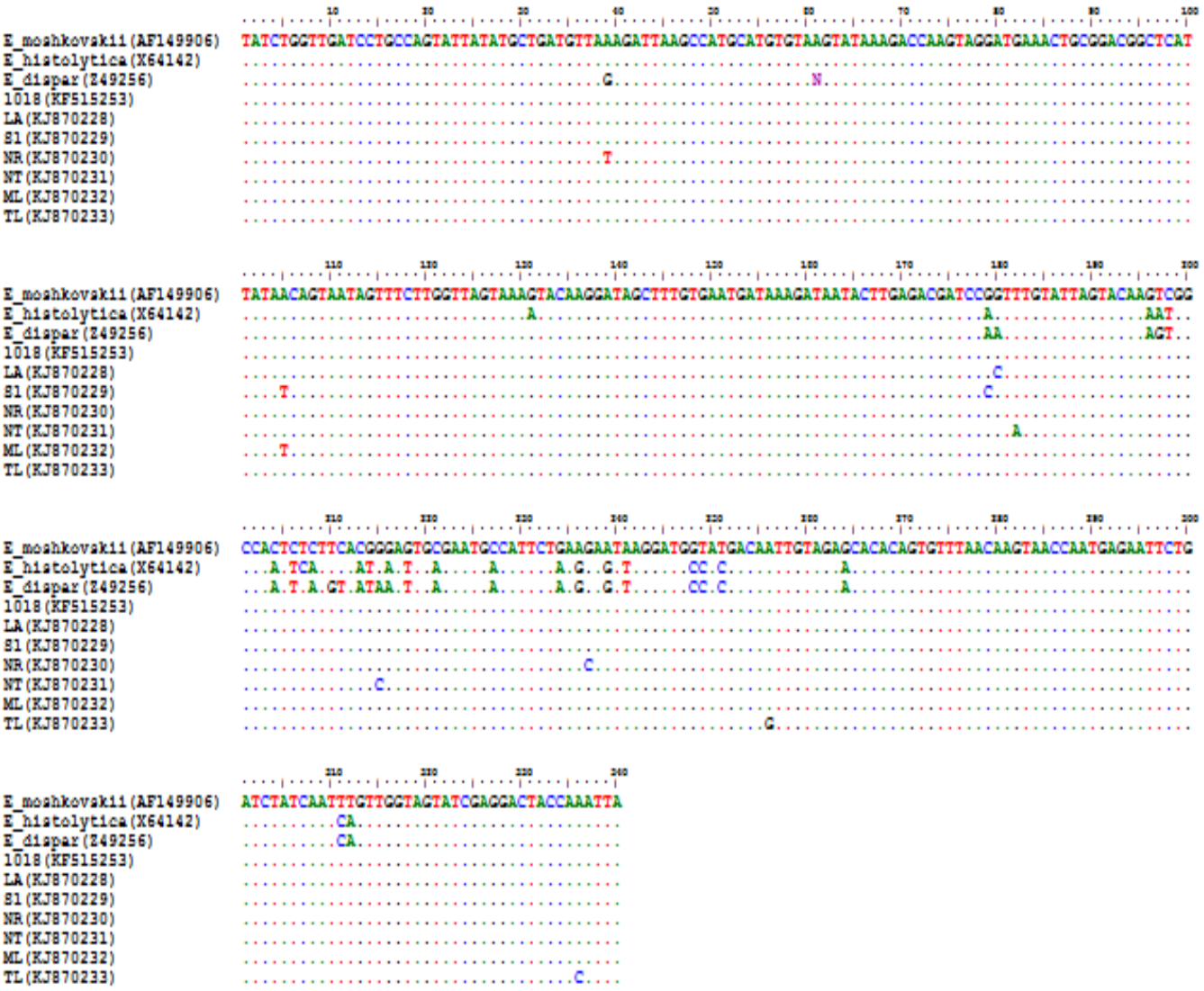

Figure 4. Multiples sequences alignment of *E. moshkovskii* 16S like SSUrDNA gene sequences from Cameroonian HIV positive and negative patients with reference sequences of *E. histolytica*, *E. dispar* and *E. moshkovskii* retrieved from the genbank.

and is known to be closely related to *E. polecki* (Ponce et al., 2004). It has been documented that it is not restricted to pigs and birds but can also infect humans (Clark et al., 2006). The cities where the samples used in this study were obtained, are well known for domesticating pigs and poultry where hygiene conditions are not very good. Thus, poor hygienic conditions may have led to the cross infection from pigs and birds to humans. Though, *E. struthionis* has been identified for the first time in human stool sample from Cameroon but its significance in humans is still unknown.

The phylogenetic analysis is in concordance with the previously published study as cluster of medically important *Entamoeba* spp. (*E. histolytica*, *E. dispar* and *E. moshkovskii*) is quite different from the other species of the *Entamoeba* (Clark et al., 2006). It also showed a difference among the closely related cluster of medically important *Entamoeba* species which were not identified correctly by microscopy. Analysis of the SSUrDNA suggested that nucleotide sequences of *E. histolytica* isolated from three HIV patient's samples (SO, AO and 235) belong to the clade closely related to the reference strain of *E. histolytica* (X64142) in which sequences isolated from HIV negative individuals are more predominant. Whereas, sequences isolated from HIV positive patients are more predominant in the clade comprised by reference strain of *E. histolytica* (AB197936). Thus, there is a possibility that two different variants of *E. histolytica* are more prevalent among HIV patients of Cameroon (Figure 1). Though, different variants have not been studied in *E. histolytica* but similar observation was made by Verweij et al. (2001) with

Entamoeba chattoni species in the phylogenetic tree presenting the distances between human isolates of uni and tetra nucleated cyst producing amoeba (Verweij et al., 2001; Ponce et al., 2004).

In the present study, PCR and gene sequencing to differentiate between various species of *Entamoeba* that infect HIV positive and negative patients in Cameroon were used. Therefore, further genotyping using *E. histolytica* specific primers as well as the correlation of the severity of *E. histolytica* infection and level of CD4+ T cells in AIDS patients are needed to highlight the relationship between HIV/AIDS and amoebiasis.

Conclusion

Cameroonian HIV patient stool samples tested present 7 species of *Entamoeba*; *E. histolytica* (28.7%), *E. dispar* (25%), *E. moshkovskii* (10%), *E. coli* (16.3%), *E. hartmanni* (6.2%), *E. polecki* (11.3%) and *E. struthionis* (7.5%). The phylogenetic analysis within the *E. histolytica* sequences isolated from Cameroonian HIV patients presented two distinguishable variants. Thus, there is a possibility that specific genotypes may be prevalent among HIV positive patients.

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Conflicts of interest

There is no competing interest between the authors.

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Effect of the larval habitat depth on the fitness of the malaria-vector mosquito, *Anopheles gambiae s. s.*

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This work was carried out with the aim to evaluate the impact of the lodging depth of *Anopheles gambiae* larvae on life features that characterize the population's fitness of this significant vector of human malaria. The study noted that depth is a significant factor that can considerably influence dynamics of the *A. gambiae* populations. Thus, on one hand depth increases the duration of larval stages from 8.16 days at 3 cm to 11.23 days at 25 cm depth, and on the other hand depth does not affect the survival of larvae. Depth reduces nymph mass from 2.1 mg at 0.5 cm to 1.4 mg at 25 cm depth. The size of adults is also influenced by this factor in both sexes. Among females, wings length passes from 3.6 mm at 1 cm to 2.8 mm at 25 cm depth, while width varies from 1 mm at 1 cm to 0.6 mm at 25 cm depth. For males, the wings length passes from 3.4 mm at 1 cm to 2.6 mm at 25 cm depth. The wings width passes from 0.97 mm at 1 cm to 0.79 mm at 25 cm depth. Depth acts even on the fecundity of females. Size of oviposition decreases from 117.61 eggs per female at 6 cm to 72.00 eggs per female at 25 cm and the hatching rate varies from 99.54% at 6 cm to 62.03% at 25 cm depth.

Key words: *Anopheles gambiae*, habitat depth, larvae, fecundity, fitness.

INTRODUCTION

Malaria is a plague in countries located in the intertropical zone. This disease is responsible for 207 million new clinical cases and 627 000 deaths per year (WHO, 2013). In Cameroon, health data indicates that this disease is responsible for 24% of death in health units, 52% of morbidity in children of age less than five years (MINSANTE, 2014). One of the most significant vectors of malaria in Africa is *Anopheles gambiae* (CDC, 2004). The fight against vectors should take into account processes controlling the population dynamics (Lyimo et al., 1992). Studies on the dynamic of mosquito populations should take into account mechanisms that affect life cycle, and thus growth rate of the population (Stearns, 1976). For some mosquitoes species, duration of larval development and size of adults, influence the dynamics of the population strongly (Lyimo et al., 1992). Peaks of *A. gambiae* pupating was observed to be coinciding with rainy seasons (Manga et al., 1992). Some ecological studies revealed that larvae of *A. gambiae* are generally present in temporary aquatic sites with very low depth and scarce in larval habitat of more or less depth (Manga et al., 1992; Timmermann and Briegel, 1993; Mwangangi et al., 2006).

Several factors such as temperature, water depth and

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larval density affect development, accumulation of reserves (Timmermann and Briegel, 1993), larval mortality, body size, fecundity (Lyimo et al., 1992) and adult longevity (Briegel, 1990 ; Kitthawee et al., 1992) of mosquitoes. Only *Aedes aegypti* larvae could dive, feed and successfully develop down to at least 14 cm (Timmermann and Briegel, 1993).

If the study considers the large adaptational character of insects, is it possible to find *Anopheles* larvae in the greater deep waters? Thus, this study have performed experiments with *A. gambiae* which is the most important vector of malaria in Africa whose larvae were reared in several levels of depth. The aim of this study is to evaluate the impact of the depth of water on development of aquatic stages of *A. gambiae* s.s. and on adult size and female fecundity which determine the dynamics of mosquito populations.

MATERIALS AND METHODS

In the present study, larvae came from an *A. gambiae* s.s. strain from Yaounde (Cameroon) colonized continuously in the laboratory for more than five years. This work was carried out at the Biotechnology Centre of University of Yaoundé I, (Yaounde, Cameroon). These experiments proceeded under temperatures ranging between 26 and 30°, RH within 70 to 80% and a photoperiod of L/D: 12/12.

Effects of depth on larval development

Larvae were reared in bottles of 10 cm diameter containing spring water. Eight different levels of depth (0.5 cm, 3 cm, 6 cm, 10 cm, 15 cm, 20 cm and 25 cm) were chosen for this work. One hundred larvae were introduced into each depth. Each larva potentially receives 0.01 mg of fish food (Tetramin) during the first four days and 0.02 mg for the following days in order to avoid pollution of the water. Each setting of the depths was repeated four times. Pupae were counted daily using a pipette in order to evaluate the larval phase duration and larval mortality. The larval phase duration was determined by the method of Dempster (1961), who defined it as the time of transformation of the 2/3 of the larvae into pupae. Pupae from each level depth were then introduced into a cage of 40 cm covered with mosquito net. After eclosion, dead pupae were counted.

Effects of depth on pupal biomass

One hundred pupae of the four replicates for each depth treatment were submerged into 70% alcohol for one day. These pupae were dried up from the alcohol and weighed on an electronic balance (Sartorius, dd = 0.1 mg).

Effects of depth on wing size of male and female adults

One wing of 30 adults from each depth is measured. Measurements were performed on the length (distance between insertion from the wing on the body with the silk fringe of the distal end) and the width (taken on the level of the median area of the wing) of adults wings according to Lyimo et al. (1992) method. A wing of each adult was removed using two needles and measured using a magnifying glass equipped with an ocular micrometer.

Effects of depth on female fecundity

Adults were feed with a saccharine solution to 10% *Ad libitum*. Three days after emergence, females were provided a blood meal on a rabbit. During the meal, females draught in each cage were counted in order to determine average egg number per female. Eggs were laid on a piece of filter paper soaked with spring water, which was set at the bottom of a Petri dish. Eggs from each cage were counted using a magnifying glass. One hundred eggs from each cage were introduced into another Petri dish containing spring water for hatching. The first-instar larvae were counted.

Statistical analysis

The ANOVA test was performed to compare variables of larval development duration, pupae mass or adult wing size at different depth, and the correlation test to see the effect of increasing depth on this parameters. The coefficient of correlation was also used to estimate relation variation between the wings length and width in both gender, the wings length of female and the oviposition (average egg number per female), then between the oviposition and the hatching rate. The different means of the larval and the pupae mortality, the oviposition and the hatching rate were compared by the Kruskall-Wallis test. The software statistical package for social science (SPSS) (Windows version 12.0) was used to perform the above statistical analyses.

RESULTS

Larval and pupae development duration

The duration of larval and pupae phases varied significantly according to the depth of breeding medium (Table 1). Duration of larval and pupae development also increased with depth. The duration of the larval phase was shortest at 3 cm depth with average duration of 10.16 days. It became longest at 25 cm depth with average duration of 13.23 days. There is higher positive correlation between aquatic stages duration and increasing depth (Table 4). Comparisons of the durations of the larval phase at various depth revealed that duration of larval development did not change significantly for mediums with depth under 10 cm (Table 4). On the other hand, at a depth of above 10 cm, the effect of depth on the duration of larval phase became perceptible and was accentuated for high depths (20 and 25 cm). The results indicated that the most favourable medium depth for larval development was around 3 cm. The depth of the medium did not significantly affect the survival of the larvae and the pupae. Larval and pupae mortality were low whatever the depth of the medium.

Pupal mass

Mass of the *A. gambiae* pupae varied very significantly with the depth of the breeding medium (Table 1). Pupal

Table 1. Variation of larvae and pupae duration, pupae mass, size of adult's wings of *A. gambiae* with habitat depth (ANOVA Test).

Parameters	0.5 cm	3 cm	6 cm	10 cm	15 cm	20 cm	25 cm	F	P<
Larval duration (days)	11.05±0.52a	10.16±0.45ab	10.64±0.28a	11.03±0.35b	12.37±0.84c	12.24±0.51d	13.23±0.65d	12. 28	0. 0001
Pupae mass	0.21±0.03 h	0.20±0.03 hg	0.20±0.03 h	0.18±0.04 g	0.16±0.03 k	0.15±0.04 k	0.17±0.04 e	24. 68	0. 0001
Length of male wings	3.13±0.09r	3.15 ± 0.17 rt	3.11±0.18 rt	3.11±0.12 rt	2.95±0.11 o	2.84±0.10 p	3.03 ±0.18 f	15. 27	0. 0001
Width of male wings	0.75±0.04 q	0.71±0.04 qj	0.74±0.08 qj	0.68±0.05 qj	0.66±0.04 jv	0.65±0.03 vw	0.70±0.07 e	15. 92	0. 0001
Length of female wings	3.25±0.11 n	3.30±0.18 nm	3.37±0.18 nm	3.30±0.16 nm	3.19±0.11 i	3.16±0.16i	3.23±0.17 yf	24. 64	0. 0001
Width of female wings	0.81±0.04 x	0.83±0.08 x	0.86±0.08 x	0.83±0.07 x	0.77±0.08 xl	0.74±0.06 l	0.79±0.07 z	24. 03	0. 0001

Same letters on same line: difference not significant.

mass decreased when the depth of the medium increased. The highest mass, 2.1 mg was obtained with depths of 0.5 and 1 cm while the lowest mass of 1.4 mg was at 25 cm depth. The comparison of the pupal mass at various depth revealed that mass did not vary significantly between the pupae for the mediums of depths lower than 10 cm, indicating that the depth acted on the pupal mass at 10 cm and above. The difference in the mass was highly significant between the pupae at ≤ 6 cm and those at ≥ 15 cm.

Wings size of male and female mosquitoes

The length of male and female wings of *A. gambiae* varied significantly with the depth of the breeding medium of larvae (Table 1). The length of male and female wings decreased with the medium depth. Although, the female wings were longer than the male's whatever the depth. The variations in the wing lengths were the same in both gender. Wings of the females and males resulting from 1 cm depth were the longest (3.6 mm for females and 3.4 mm for males) comparatively to those for adults in both gender from 25 cm depth (2.8 mm for females and 2.6 mm for males). Length of wings did not vary significantly between the females resulting from depth ranging in 0.5 to 10 cm (Table 1). Difference in length became significant between wings of females from depth higher than 10 cm and those of females from lower depth. The study had the same groups in males. Wing width showed the same trend in both gender. The differences in the width were highly significant with the various depths (Table 1). Width of wings decreased when the depth of the medium increased. The greatest widths (for females, 1 mm and among males, 0.9 mm) were recorded at 1 cm and the smallest ones (for females, 0.6 mm and 0.6 mm for males) were at 25 cm depth.

Within a gender, the wing lengths were separated into 2 significantly different groups from different ranges of the medium depth (Table 1). Among the females, those at depths ≤ 10 cm formed the first group. Within this group, width of wings did not differ significantly from one level of depth to the other. The second group consisted of the females from depth > 10 cm. In this group, the effect of depth was perceptible; the difference in width was significant from one level of depth to the other. In the males, the first group was those at 1 cm depth, whose wings were significantly larger than the rest from deeper medium. The second group included individuals coming from the mediums of depth lower than or equal to 6 cm. In this group, wing width did not differ significantly from one level of depth to the other. Finally, the third group formed by individuals proceeding mediums of depths higher than 6 cm, width of wings in general varied from one level of depth to another.

Fecundity of females

Average number of eggs per female significantly varied from one level of depth to another (Table 2). The number of eggs laid per female increased from lowest depth of breeding mediums to reach the maximum value, 117.61 eggs per female at 6 cm. Then, size of oviposition decreased when the depth increased; the lowest value, 72.00 eggs per female was recorded at 25 cm of depth (Table 2).

There was a significant correlation between female wing size (length or width) and average number of eggs per female, and between the wing length and width (Table 3). Hatching rate varied significantly with the depth of the medium (Table 2). This rate was high for eggs laid by females from lowest depth and decreased for the depths higher than 15 cm. The maximum rate of hatching, 99.54% was recorded at 6 cm depth and the minimal value, 62.03% at 25 cm of depth. A positive correlation existed between the size of

Table 2. Variation of oviposition and hatching rate of *A. gambiae* with habitat depth.(Kruskall-Wallis test).

Parameters	0.5 cm	3 cm	6 cm	10 cm	15 cm	20 cm	25 cm	H	P <
Oviposition	80.50±6.99 a	97.80±6.81 c	117.60±13.56 d	98.60±5.88 e	88.96±2.35 f	75.00±7.18 gh	71.92±5.83 h	185.17	0. 0001
Hatching rate	94.86±1.51 f	97.68±1.66 d	99.54±1.01 c	98.08±1.41j	88.75±1.35k	73.51±2.41 l	62.03±2.76 n	212. 57	0. 0001

Same letters on same line: difference not significant.

Table 3. Correlations of pearson.

Parameter	Wings length and wings width (female)	Wings length and wings width (male)	Wings length (female) and oviposition	Oviposition and hatching rate
r	0.972	0.953	0.301	0.673
-	P< 0.0001	P < 0.0001	P <0.0001	P <0.001

Table 4. Linear correlation.

Parameter	Increasing depth and aquatic stages duration	Increasing depth and pupae mass	Increasing depth and length of male wing	Increasing depth and length of female wing
r	0.814	-0.643	-0.616	-0.509
-	P< 0.0001	P < 0.0001	P < 0.0001	P <0.001

the oviposition and the hatching rate (Table 3).

DISCUSSION

This study showed that larvae of *A. gambiae* were sensitive to the depth environmental conditions during their growth. Thus, duration of the larval phase varies when the depth of the lodging changes. Larvae of *A. gambiae* present a short growth duration when the depth of the breeding medium is lower or equal to 10 cm. Beyond this level, increase of depth also involves an increase of larval phase duration. Duration of larval development that was obtained remain within the time limit found by Holstein (1954), Diop et al. (1998) and Foko et al. (2007) who found out that duration of larval phase ranges between 8 days and 12 days. On the other hand, Timmermann and Briegel (1993) noted that beyond 2 cm of depth, the duration of larval phase of *A. gambiae* becomes higher than 13 days. The effect of depth on the larval phase would be related to the energy expenditure caused by displacement of larvae. Indeed, in the natural habitats, larvae consume the food particles coming from the microbial decomposition of organic matter at the bottom of their habitats (Fish and Carpenter, 1982).

In the case of breeding in laboratory, it is the food which forms a deposit in the bottom of the boxes of breeding. These larvae must each time go up to the surface to breathe. Thus, energy expenditure related to displacement increases when the place of provision of food is far from the place of supply of oxygen. Under natural conditions, the authors noted that the larvae of *A. gambiae* met only in temporary lodgings of low depth (Ginning et al., 2001).

Timmermann and Briegel (1993), showed that development of *A. gambiae* larvae was only possible when the depth of medium is lower or equal to 5 cm. On the other hand, within the framework of the study, larval and pupae mortality were low whatever the depth. This shows that, *A. gambiae* is able to develop in mediums wherein depth is largely (5 times) higher than the limited depth found by these last authors. The fact that, in nature, *A. gambiae* is found only in very small deep aquatic sites could be then an adaptive preference selected by this mosquito's species.

Pupae is a stage after larval maturity, its mass

represents the biomass accumulated during the larval phase. Indeed, growth of the larvae is accompanied by an accumulation of reserves, in particular, proteins (Van Handel, 1986) and lipids (Timmermann and Briegel, 1993). Thus, pupal mass could reflect the quality of environmental conditions of larval growth. The study results showed that depth of breeding medium affected accumulation of biomass in the larvae of *A. gambiae.* Larvae in the conditions of lower depth medium could accumulate more reserves than those from deeper medium. The effect of depth on the accumulated biomass becomes perceptible as from 10 cm of depth. On the other hand, according to Timmermann and Briegel (1993), accumulation of reserves in larvae of *A. gambiae* grows with the depth, particularly in the accumulation of lipids. Depth of the breeding medium of larvae also affects size of adults.

Indeed individuals of bigger adult came from the mediums of depth lower than 10 cm. The highly-positive correlation between pupal mass and wing sizes of adults in both sexes supports this hypothesis. Adult body size is decided by the amount of biomass accumulated during the larval stage because pupae do not feed (Timmermann and Briegel, 1993).

Size of ovipositions and hatching rate of eggs were also affected by depth of breeding medium of *A. gambiae* larvae. Fecundity is high for females resulting from the mediums of depth lower or equal to 6 cm and low when the depth of medium increases. Effect of medium depth on fecundity would be rather indirect because of the positive correlation between fecundity and size of individual adults found in this study and others (Briegel, 1990; Karino et al., 2004). Indeed, bigger female mosquitoes take greater quantities of blood as Steinwaschen (1982) found in *A. aegypti*.

CONCLUSION

The depth of the lodging influences development of larvae of *A. gambiae S. S.* at the laboratory situation. The high depths lengthen the larval development time, reduce the size of adults and their reproduction, and then corrupt fitness of populations of this important malaria vector in sub-Saharan Africa. However, the depth of water is not a selection factor for the development of *A. gambiae S. S.* because it has no impact on the mortality of larvae of this vector.

RECOMMENDATION

During the collection of larvae of *A. gambiae S. S.* in the field study, in the case of lack of temporary lodging and then shallow, larvae of this vector must be checked also in the permanent lodging like lakes.

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Conflicts of interest

Authors have none to declare.

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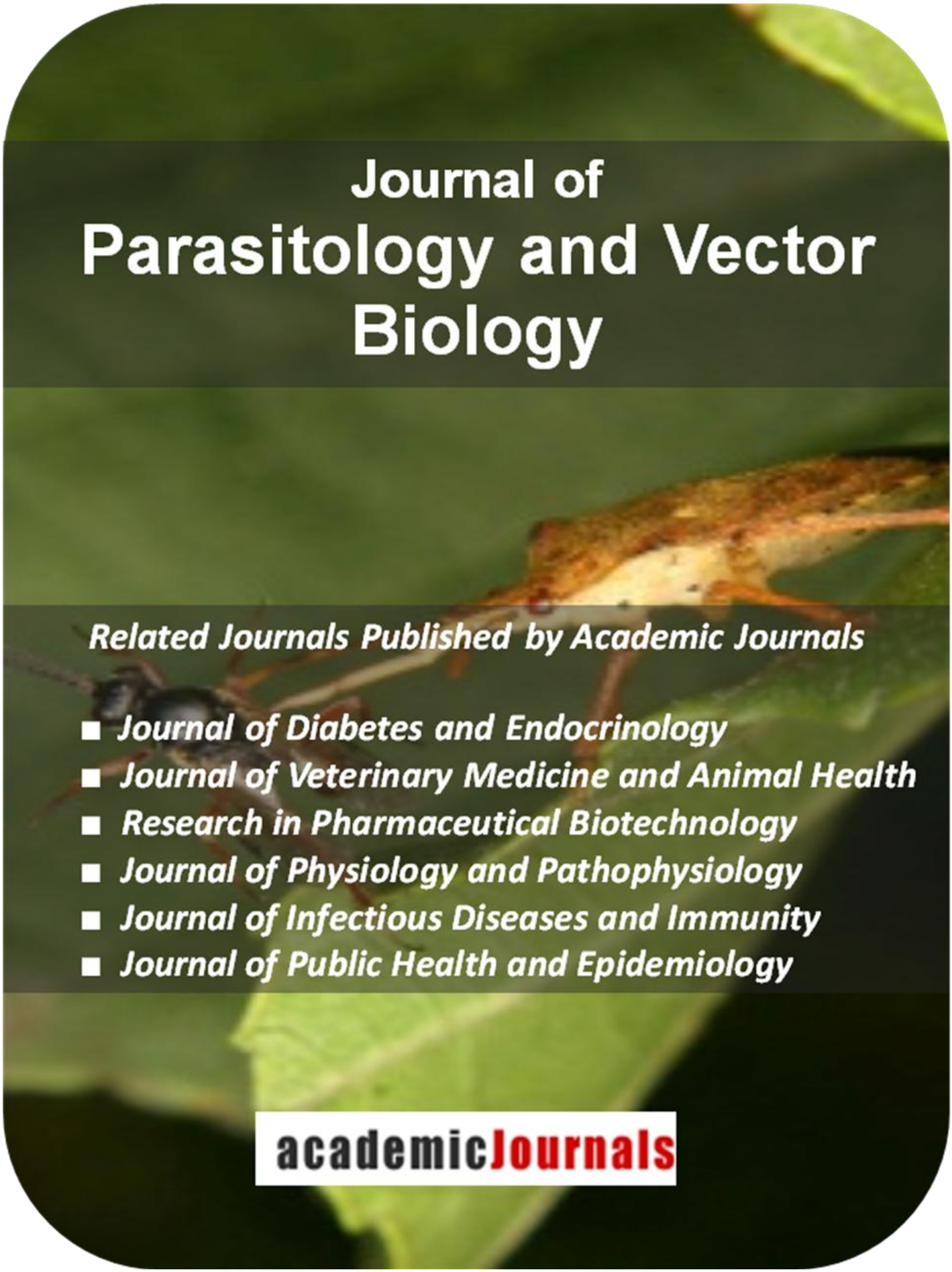
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