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BURDEN OF INTESTINAL PARASITES AMONGST HIV/AIDS PATIENTS ATTENDING BAMENDA REGIONAL HOSPITAL IN CAMEROON

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ABSTRACT
Background: Intestinal parasitic infections cause severe diarrhea especially in debilitated subjects with clinical complications of dehydration, malabsorption and severe weight loss, complicating treatment schemes.
Materials and Methods: This was a cross-sectional, hospital based study during which data were collected by the use of questionnaires and laboratory tests of stool and blood samples respectively.
Results: A total of 200 volunteer patients participated in this study of which 132 (66.0%) were females and 68 (34.0%) males. Eight different intestinal parasites were identified in 69 (34.5%) participants. The most prevalent parasite was *Entamoeba histolytica* with 8.0% of infected cases. Opportunistic parasites were identified in 15.5% of the study population. Seven percent of patients were infected with *Cryptosporidium parvum*, 6.5% with *Isospora belli*, and 2% with *Microsporidium* species. Diarrhea was found in 38.5% of the study participants 62.3% of whom had at least a single intestinal parasite. Opportunistic parasites were significantly more prevalent in patients with diarrhea and participants with CD4+ T cell counts less than 200 cells/µl (P < 0.05). Diarrhea was significantly more prevalent in participants who were on antiretroviral drugs than in those who were not (66.5% vs. 33.5%, P<0.05).
Conclusion: Though opportunistic parasites were found in the majority of HIV/AIDS patients attending the Bamenda Regional Hospital, *Entamoeba histolytica* and other intestinal parasites represented a common burden. It was therefore recommended appropriate diagnosis before initiating the routine treatment which is usually practiced in our health settings.
Key words: Intestinal parasites, HIV/AIDS patients, Bamenda Regional Hospital

FARDEAU DES PARASITOSES INTESTINALES CHEZ LES PATIENTS INFECTES PAR LE VIH/SIDA ET CONSULTANT L'HOPITAL REGIONAL DE BAMENDA AU CAMEROUN

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RÉSUMÉ
Contexte: Les infections parasitaires intestinales provoque des diarrhées sévères particulièrement dans des sujets débilités avec les complications cliniques de déshydratation, malabsorption et la perte de poids sévère compliquant parfois le traitement de la maladie
Matériels et méthodes: Il s'agissait d'une étude transversale conduite en milieu hospitalier pendant laquelle les données étaient rassemblées par l'utilisation de questionnaires et les tests de laboratoire effectués sur des prélèvements de selles et de sang respectivement.
Résultats: Un total de 200 patients volontaires ont participé à cette étude parmi lesquels 132 (66.0 %) était de sexe feminin et 68 de sexe masculin (de 34.0 %). Huit parasites intestinaux différents ont été identifiés chez 69 participants (34.5 %). Le parasite le plus répandu était *Entamoeba histolytica* avec 8.0 % de personnes infectées. Les parasites opportunistes ont été identifiés dans 15.5 % de la population d'étude. Sept pour cent de patients étainet infectés par *Cryptosporidium parvum*, 6.5 % par *Isospora belli* et 2 % par des espèces de *Microsporidium*. La diarrhée a été présente chez 38.5 % des participants, 62.3 % de ceux ci ayant au moins un parasite intestinal. Ces parasites opportunistes étaient significativement plus répandus chez les patients souffrant de diarrhée et chez les participants ayant un taux de CD4 inférieur à 200 cellules / µ L (P< 0.05). La diarrhée était significativement plus répandue chez les sujets sous traitement par les antirétroviraux que chez ceux qui ne l'aient pas (66.5% vs. 33.5%, P<0.05)
Conclusion: Quoique des parasites opportunistes aient été trouvés dans la majorité de patients de VIH/SIDA consultant à l'hôpital Régional de Bamenda, *Entamoeba histolytica* et d'autres parasites intestinaux représentaient un fardeau commun. Il a été recommandé

le diagnostic approprié avant l'introduction du traitement ordinaire qui est d'habitude pratiqué dans nos institutions sanitaires
Mots-clés: Parasitoses intestinales, Patients infectés par le VIH/SIDA, Hôpital Régional de Bamenda

INTRODUCTION

Enteroparasitic infections constitute a serious public health problem in developing countries with inadequate sanitary conditions. Many different types of intestinal helminthes and protozoa infect man, provoking a wide range of symptoms that are generally associated with gastrointestinal tract disorders and are dependent on demographic, socio-economic, physiological and immunological factors [1]. Patients with some types of immunocompromised conditions and those subjected to immunosuppressive therapy have an increased probability of presenting with parasitic infections, generally manifesting with a high degree of severity [2]. The immune response of an immunocompetent host against parasites is a complex system in which both cellular and humoral defense mechanisms intervene [3].

HIV infections result in severe destruction of $CD4^+$ T cells as the virus undertakes lytic replication cycles in the infected $CD4^+$ T cells. The cellular arm of the immune system thus becomes weakened. It is therefore thought that in advanced stages of HIV infection (AIDS), the $CD4^+$ T cells are drastically diminished and as such, parasites which produce self limiting diseases in immunocompetent hosts tend to result in severe outcomes in immunocompromised subjects [4]. The main clinical manifestation of diseases resulting from intestinal tract parasitic infections is diarrhea [5]; this is an intestinal bowel movements accompanied by loose watery stools. Diarrhea has several etiologic agents, ranging from parasites, through viral, bacterial, to conditions that may inflame the intestinal mucosa such as HIV enteropathy. Diarrhea in immunocompromised patients is usually profuse, generally accompanied by weight loss, anorexia, malabsorption syndrome and in some cases fever and abdominal pain. In such patients, parasites such as *Cryptosporidium parvum, Enterocytozoon bieneusi*, *Encephalytozoon intestinalis* and *Strongyloides stercoralis* may disseminate to other organs such as the bronchia, bile and liver ducts, producing symptomatology specific to the organ affected [6].

Cyclospora cayetanensis, Cryptosporidium parvum, microsporidium species *such as Enterocytozoon bieneusi* and *Isospora belli* have been incriminated as causes of prolonged diarrhea, especially in AIDS patients, although they are thought to cause self limiting diarrhea in immunocompetent individuals. These are referred to as opportunistic parasites [6]. Different species of protozoa have been associated with acute and chronic diarrhea in HIV infection and AIDS. They include *Cryptosporidium parvum, Isospora belli, Microsporidium* species… and account for a significant number of cases of diarrhea [7,8]. Infective causes of chronic diarrhea may satisfactorily be managed because with the exception of cryptosporidiosis and HIV-related enteropathy, good response to treatment can be expected [9], but all etiologic agents are not easily diagnosed in Africa on routine basis because of limited diagnostic facilities and trained personnel [10]. It is known that the pathogens responsible for diarrhea are different according to geographical location; therefore laboratory diagnostic evaluations are required to determine prevalence in each population so that it can provide guidelines for therapy for treatable etiologic agents and necessary data for planning and evaluation of HIV-positive/AIDS patients' care. The objective of this study was therefore to determine the prevalence of these intestinal parasites burden among HIV/AIDS patients who came for medical attention at the Bamenda Regional Hospital.

MATERIALS AND METHODS

Study area and sampling

This study was carried out at the Bamenda Regional Hospital HIV treatment Centre. Bamenda is the North West Regional capital of Cameroon. Hygiene and sanitation within the municipality has been severely compromised by the rapid migration of students since the creation of the University of Bamenda four years ago. They usually reside in numerous clutters around the university, generating large quantities of domestic waste. The sudden population increase has also led to permanent water scarcity that is generating many public health problems. Study participants who were patients already known as HIV/AIDS carrier and seeking medical attention at the Bamenda Regional Hospital were briefed on the modalities of the study with the aid of an informed consent form. After receiving information on what participation entailed, the advantages and inconveniences involved as well as possibility for compensation for damages, those who freely accepted to donate blood and stool samples for the study were the effective study participants.

Study design and ethical considerations The study was a cross-sectional, hospital based study in which HIV/AIDS patients visiting the Bamenda Regional Hospital were provided information on the purpose/objectives of the study, the possible benefits and discomforts that were to go with their participation in the study. The samples collected from participants were identified and processed using codes. Names were not used through out the study. This ensured individual confidentiality. Participants in this study were not billed for the tests and the physicians were provided with the findings for necessary action. The samples collected were solely used for the defined purpose. The ethical clearance was approved by the Ethical Committee of the Hospital prior to sample collection. The study obtained ethical clearance and administrative authorization from the University Ethical Review Board and the Regional Delegation of Public Health for the North West respectively prior to sample collection. The minimum acceptable sample size was derived using the

formula for sample size determination as described by Eng [11]

Sample collection and processing

Stool samples were collected into clean wide mouth specimen containers from male and female patients. A single freshly voided stool sample was collected from participants. A portion of the stool was preserved in 10% formalin. Five mL of venous blood was collected into coded EDTA tubes for $CD4^+$ T cell counts. Stool specimens were processed with saline wet mount and examined microscopically using X10 and X40 objectives to detect motile trophozoites. Formol-ether concentration technique for stool parasites was used and the sediment examined as iodine wet mounts to detect ova, larvae and cysts of intestinal tract parasites. Smears were prepared from the sediments, air dried and stained by a modified acid-fast stain to detect *Crytosporidium* and *Isospora* species as earlier previously described elsewhere [12]. Blood samples c from the same participants who provided stool samples were analyzed for CD4+ T lymphocyte cell estimation using flow cytometry. On the basis of the CD4 + T cell counts, the participants were categorized by their immune status according to the 1993 revised classification system for the HIV infection by CD4 T-cell categories [13].

Laboratory Procedure

Preparing wet mounts of stool for direct microscopy

One gram of freshly voided stool sample, (accepted only when duration between collection and examination was less than or equal to three hours) was emulsified on 50ul of physiologic saline (0.85% NaC1 solution) on a glass slide. The preparation was covered with a coverslip and examined using the X10 and the X40 objectives. To facilitate identification of parasite ova, cysts or trophozoites, lugols iodine was glided under the coverslip to stain and differentiate parasite cytoplasm [14]. Attempt was made to go through all the fields of the preparation before samples were reported negative of parasites.

Formol ether concentration technique

One gram of faeces was emulsified in 3mls of 10% formol water. A further 4mls of formol water was added to the preparation and mixed. The emulsified preparation was sieved and filtrate collected in a beaker and transferred to a centrifuge tube.4mls of ethyl acetate was added to the preparation. The tube was stoppered and its content mixed for a minute by gently inverting the stoppered tube and returning it to its upright position. The stopper was gently removed and the preparation centrifuged at 300rpm for a minute. Using a stick, the layer of faecal debris from the side of the tube was gently loosened and the tube was rapidly inverted to discard the supernatant, consisting of fecal debris, fomol and ether. The sediments remaining consisted of cysts and ova of faecal parasites. The tube was then reverted to its upright position and allowed for the sediments to return to the bottom. To re-suspend the ova, the tube was gently taped and its content transferred to two slides, one was covered with a coverslip and observed using the X10 an X40 objectives. A drop of iodine was run under the slide to increase visibility of parasite ova [12]. The second slide was air dried and stained for opportunistic parasites by the modified Ziehl Neelsen staining technique.

Modified Ziehl Neelsen staining technique

A smear of stool sample sediment from formol ether concentration was made on a clean glass slide and allowed to air dry. The preparation was fixed with absolute methanol. The slide was then stained with carbol fuchsine for 10 minutes, followed by rising of stained slides with water. The preparation was decolorized with acid alcohol (99 mL of 96% alcohol and 1ml hydrochloric acid), followed by rinsing in tap water and counterstaining in methylene blue for one minute. The slide was then rinsed, dried and observed using the oil immersion objective [15].

Data analysis and management

Data management prior to analysis involved the use of workbooks for direct raw data entries prior to keying into Microsoft excel for separation and arrangement of raw data generated. Analysis was done using the SPSS Version 11.0, SPSS Inc statistical software package. Data was summarized using tables and and the strength of association measured by using the chi-square and its associated P value. Associations were considered to be statistically significant when $P< 0.05$.

RESULTS

A total of 200 volunteer patients participated in this study of which 132 (66.0%) were females and 68 (34.0%) males. Table 1 shows the distribution of study participants according to age and sex. The highest number of males (30.7%), and females (69.3%) were between 41 and 60 years of age. The difference between age groups and sex was not significant. By distributing study participants according to occupation, house wives constituted 11 (5.5%) of the study participants, business persons 54 (27.0%), employed persons 16 (8.0%), farmers 62 (31.0%), students 6 (3.0%), unemployed 26 (13.0%) and unskilled labourers 25 (12.5%) (Table 2).

TABLE 1: DISTRIBUTION OF STUDY PARTICIPANTS ACCORDING TO AGE AND SEX.

Age group, (years)	Number (%)* of participants		Total n (%)
	Males	Females	
20 – 40	30 (40.0)	45 (60.0)	75 (37.5)
41 -60	35 (30.7)	79 (69.3)	114 (57.0)
> 60	3 (27.3)	8 (72,7)	11 (5.5)
Total	68 (34.0)	132 (66)	200 (100.0)

*Percentages based on number of participants within age group

TABLE 2. DISTRIBUTION OF STUDY PARTICIPANTS ACCORDING TO OCCUPATION

Occupation	Number (%)* of participants
House wife	11 (5.5)
Business	54 (27.0)
Employed	16 (8.0)
Farming	62 (31)
Student	6 (3.0)
Unemployed	26 (13.0)
Unskilled labour	25 (12.5)
Total	200 (100.0)

* Percentages based on the total number of participants

Table 3 shows the Distribution of study participants with respect to residence. Most of the study participants 90 (45.0%) were from semi urban settings, while 63 (31.5%) came from rural communities and 47 (23.5%) from urban areas.

TABLE 3. DISTRIBUTION OF STUDY PARTICIPANTS WITH RESPECT TO RESIDENCE SETTING

Residence	Number (%)* of participants
Urban	47 (23.5)
Semi – urban	90 (45.0)
Rural	63 (31.5)
Total	200 (100)

* Percentages based on the total number of participants.

TABLE 4. PREVALENCE OF INTESTINAL TRACT PARASITES IN STUDY PARTICIPANTS

Parasites	Number (%)* of patients with parasites
E. histolytica	14 (7.0)
E. histolytica and *C. Parvum*	2 (1.0)
A. lumbricoides	14 (7.0)
C. parvum	12 (6.0)
I. belli	13 (6.5)
G. lamblia	7 (3.5)
Microsporidium spps	4 (2.0)
T. trichiura	2 (1.0)
D. fragilis	1 (0.5)
Total	69 (34.5)

* Percentages based on total study population.

Eight (8) different parasites were identified in 69 (34.5%) participants. The most prevalent parasite was *E. histolytica*, 16, (8.0%); [14 (7.0%) solely and 2 (1.0%) in co-infection with *C. parvum*]. In decreasing prevalence, the parasite trend was, 14 (7.0%) *A. lumbricoides,* 14 (7.0%), *C. parvum;* [2 (6.0%) solely and 2 (1.0%) in co-infection with *E. histolytica*], 13 (6.5%) *I. belli,* 7 (3.5%) *G. lamblia,* 2 (1.0%) *T. trichiura and* 1 (0.5%) *D. fragilis* (Table 4).

There were a total of 69 parasite positive stool samples. Diarrhea was diagnosed in 77 study participants. 21 (10.5%) 0f the study participants had parasitic infections without diarrhea, 48 (24.0%) had both intestinal parasites and diarrhea whereas 29 (14.5%) had diarrhea without intestinal parasites. Table 5 shows the association between parasites identified and diarrhea in study participants. *Entamoeba. histolytica* had a significantly higher prevalence in diarrheic participants, 16 (100.0%) compared to their non diarrheic counterparts, 0 (0.0%), ($P < 0.05$). *Ascaris lumbricoides* was significantly less prevalent in diarrheic, 2 (14.3%) than in non diarrheic patients 12 (85.7%) ($P = 0.05$). The prevalence of *C. parvum* was significantly higher in diarrheic, 13 (92.9%) than in non diarrheic participants 1 (7.1%) ($P < 0.05$). Eight, 8 (61.5%) of the participants infected with *I. belli* had diarrhea against 5 (38.5%) without diarrhea. The association between *I. belli* and diarrhea was not statistically significant ($P > 0.05$). *G. lamblia* and *Microsporidium* were significantly associated with diarrhea, ($P < 0.05$) as opposed to *T. trichiura and D. fragilis* ($P > 0.05$).

TABLE 5: ASSOCIATION BETWEEN PARASITES IDENTIFIED AND DIARRHEA IN STUDY PARTICIPANTS.

Parasite	Number of infected patients	Number (%)with diarrhea
+*E. Histolytica**	16	16 (100.0)
A. lumbricoides	14	2 (14.3)
+*C. parvum**	14	13 (92.9)
I. belli	13	8 (61.5)
*G. lamblia**	7	6 (85.7)
*Microsporidium sp**	4	4 (100.0)
T. trichiura	2	1 (50.0)
gilis		(0.0)
	1	50 (70.4)

+ Represents co-infections. *P<0.05

Table 6 shows the association between parasites solated, and level of immunesupression. Patients with $CD4^+$ T cell counts less than 200cells/ul had the highest parasite prevalence, 33 (46.5%), compared to 27 (38.0%), and 11(15.5%) for Participants with $CD4^+$ T cell counts between 200 and 499 cells/ul and greater than or equal to 500cells/ul respectively. *C. parvum* and *Microsporidium spps* were significantly more prevalent in severely immunosupressed subjects with $CD4^+$ T cell counts less than 200cells/ul ($P < 0.05$).

TABLE 6: ASSOCIATION BETWEEN PARASITES ISOLATED, AND LEVEL OF IMMUNOSUPPRESSION.

Parasite	$CD4^+$ T cell counts (cells/ul)			Total
	< 200 n (%)	200- 499 n (%)	≥ 500 n (%)	
$^+$*E. Histolytica*	8 (50.0)	5 (31.3)	3 (18.7)	16
A. lumbricoides	2 (14.3)	8 (57.1)	4 (28.6)	14
$^+$*C. Parvum**	11(78.6)	3 (21.4)	0 (0.0)	14
I. belli	4 (30.8)	6 (46.2)	3 (23.0)	13
G. lamblia	3 (42.9)	3 (42.9)	1 (14.2)	7
*Microsporidium sp**	4 (100.0)	0 (0.0)	0 (0.0)	4
T. trichiura	1 (50.0)	1 (50.0)	0 (0.0)	2
D. fragilis	0 (0,0)	1 (100.0)	0 (0.0)	1
Total	33 (46.5)	27 (38.0)	11 (15.5)	71

$^+$ Represents co- infections.
***P<0.05**

Table 7 shows the $CD4^+$ T cell counts and diarrhea profiles in study participants. The prevalence of diarrhea with respect to $CD4^+$ T cell counts was 44 (64.7%) for patients with $CD4^+$Tcell counts less than 200, 23 (26.7%) with $CD4^+$ T cell counts between 200 and 499cells/ul and 10 (21.7%) for patients with $CD4^+$ T cell counts greater than, or equal to 500 cells/ul ($P < 0.05$).

TABLE 7: $CD4^+$ T CELL COUNTS AND DIARRHEA PROFILES IN STUDY PARTICIPANTS.

Diarrhea status	number (%)* with $CD4^+$ T cell count			Total
	< 200	200-499	≥ 500	
Diarrheic	44 (64.7)	23 (26.7)	10 (21.7)	77 (38.5)
Non Diarrheic	24 (35.3)	63 (73.3)	36 (78.3)	123 (61.5)
Total	68	86	46	200 (100)

*** Percentages based on patients within same CD4 counts**

The occupations with the highest proportion of parasitic infections were business persons 21 (30.4%) and farmers, 17 (24.6%). The proportions of parasitic infections were least in employed persons 7 (3.5%), and students, 2 (1%), but the difference in parasite prevalence was not statistically significant between the different occupations ($P > 0.05$).

Intestinal parasites were significantly more prevalent in patients not yet on antiretrovirals than in those who were (59.1% Vs 14.5%, $P < 0.05$) and diarrhea was significantly more prevalent in patients on antiretrovirals, 51 (66.2%) than in those who were not, 26 (33.8%) ($P < 0.05$)

DISCUSSION AND CONCLUSION

In this cross-sectional study the overall prevalence of intestinal parasites from stool samples was 34.5% This finding show some degree of similarity with those earlier obtained in Chennai and Yaounde [16-17] respectively. It is however far below the 62.5% prevalence reported some years back by Zalem et al [18]. The relatively lower parasite prevalence in this study could be explained by the fact that the study was conducted in semi urban area where the levels of hygiene are far higher as compared to the rural setting in which the previous studies were carried out.

The parasite most associated with diarrhea was *Entamoeba histolytica, Microsporidium* species, *Cryptosporidium parvum* and *Isospora. beli.* This trend was similar to that reported by Haileeyesus and Beyene [19] in Ethiopia. These protozoa usually seek protection from human digestive enzymes in the intestinal lumen by causing the host's intestinal lining to form folds around them, leaving the impression that they are intracellular. Due to the fact that intracellular pathogens are destroyed by the cellular arm of the immune system they profit from the weakened

cellular immune system to cause diarrhea. These folds also reduce the intestinal surface area available for absorption, resulting in malabsorption. Opportunistic parasites were identified in 15.5% of the study participants. This finding was higher than the 9.7% reported in a similar study by Sarfati et al [17]. Diarrhea profiles followed the opportunistic parasite profiles, and were significantly associated with $CD4^+$ T cell counts, below 200cells/ul as earlier reported by Haileeyesus and Beyene [19] in Ethiopia. In our study, business persons and farmers constituted the highest proportions of parasite infected participants. The higher prevalence of infection in business persons could be explain by their regular feeding in with food provide by street food vendors, irrespective of sanitary conditions [20]. Farmers were equally on top of the parasite infection chart, probably due to their contact with the soil, which predisposes them to geohelminthes.

Intestinal tract parasites were more prevalent in patients with $CD4^+$ T cell counts less than 200cells/ul compared to patients with $CD4^+$ T cell counts between 200 and 499 and greater than or equal to 500 cells/ul respectively. This trend is similar to previous reports [19,21] in which increased parasite prevalence was found in patients with $CD4^+$ T cell counts less than 200cells/ul and could be explained by the fact that at lower $CD4^+$ T cell counts, the immune system is weakened; as such the host becomes unable to eliminate pathogens which thrive and cause diseases. Intestinal parasites were significantly more prevalent in patients who were not on antiretrovirals, compared to those who were on treatmentThis is in accordance with previous report from Haileeyesus and Beyene [19]. Antiretrovirals is known to improve on $CD4^+$ T cell counts, thus decreasing the incidence of opportunistic parasites. Diarrhea was significantly more prevalent in patients on antiretroviral drugs than in patients who were not on treatment. The trend was similar to that reported by Call et al.[21] while investigating diarrhea post highly active antiretroviral therapy (HAART) in HIV/AIDS patients in the United states. This trend could be explained by the fact that antiretrovirals such as protease inhibitors are thought to increase intestinal tract electrolyte Though opportunistic parasites were found in the majority of HIV/AIDS patients attending the Bamenda Regional Hospital, intestinal parasites represented a common burden**.** It was therefore recommended appropriate diagnosis before initiating the routine treatment which is usually practiced in our health settings.

concentration, thus increasing diarrhea prevalence. This perception is however not shared by Poles et al.[23] according to who the prevalence of in diarrhea is decrease with HAART.

The prevalence of intestinal parasites was highest in illiterates and persons who had only attained primary education followed by those who had attained secondary education. The least parasite prevalence was recorded in those who had attained university or other higher education level of studies. This was in line with previous other findings [19, 24] that related the prevalence of parasitic infections to the knowledge of the transmission pattern of the causative organism and the level of education of the respondents.

The present study has shown a prevalence of intestinal parasites among HIV/AIDS patients attending the Bamenda Regional Hospital at 34.5% portraying diarrhea as a common clinical manifestation of parasitic infections in these patients. The spectrum of enteric parasites causing diarrhea included opportunistic parasites such as *Cryptosporidium parvum*, *Isospora belli* and *Microsporidia* and conventional pathogens such as *Entamoeba histolytica*, *Giardia lamblia, Trichiuris trichiura* and *Ascaris lunbricoides*. Opportunistic intestinal parasites were more prevalent *in patients with* $CD4^+$ T cell counts less than 200cells/ul and they play a major role in causing chronic diarrhea. The prevalence of infection with a particular enteric parasite in HIV/AIDS patients was largely influenced by occupation, general hygiene practices and levels of education.

Competing interests

The authors declare they have no competing interests.

Authors' contributions

Bissong M. E. A. conceived and designed the study, conducted the literature search, drafted the manuscript and substantially revised it. Nguemain, N. F and Ng'awono, T. Epse Nkoa contributed to the design and substantially revised the manuscript. Kamga, F. H. L substantially revised the manuscript and prepared it for publication. All authors read and approved the manuscript.

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