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Bacterial resistance and immunological profiles in HIV-infected and non-infected patients at Mbouda AD LUCEM Hospital in Cameroon

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Summary This study investigated the variations in some cells of the immune system, as well as the antibiotic resistance of the bacteria responsible for enteric infections among HIV+ patients compared to HIV− patients in Mbouda AD LUCEM Hospital, Cameroon.

A cross-sectional study was performed from September 2014 to February 2015 in 67 human immunodeficiency virus (HIV)-seropositive (HIV+) and 37 HIV-seronegative (HIV−) patients. Blood collected from these patients was used to perform cluster of differentiation 4 (CD4) and cluster of differentiation 8 (CD8) lymphocyte blood counts and a white blood cell count, as well as to measure C-reactive protein (CRP) blood by flow cytometry and perform optical and immuno-turbidimetric detection. Enteric bacteria were isolated from the stool of patients, and their antibiotic susceptibility profiles were determined using agar diffusion methods.

The results showed that *Escherichia coli* was the main pathogenic bacteria in the digestive tracts of HIV+ (85.3%) and HIV− (81.1%) patients, and infections with *Klebsiella* sp. were also predominant among HIV− patients (29.4%). Resistance of *Klebsiella* sp. to ceftriaxone (CRO; $P = 0.001$), gentamicin (GEN; $P = 0.005$), chloramphenicol (CHL; $P = 0.0004$), ciprofloxacin (CIP; $P = 0.005$) and doxycycline (DOX; $P < 0.0001$) was significantly higher in HIV+ patients than in HIV− patients.

Abbreviations: AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; HIV+, seropositive for HIV; HIV−, seronegative for HIV; CD, cluster of differentiation; CRP, C-reactive protein; AMO, amoxicillin; AMC, amoxicillin + clavulanic acid; CRO, ceftriaxone; GEN, gentamicin; CHL, chloramphenicol; CIP, ciprofloxacin; LEV, levofloxacin; TET, tetracycline; DOX, doxycycline; NT, not tested; S, significant; NS, non-significant.

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Enterobacter sp. showed high resistance to GEN ($P = 0.009$) and CIP ($P = 0.001$) in HIV+ patients compared to HIV− patients. *Citrobacter* sp. was resistant to GEN ($P = 0.009$) in HIV+ patients compared to HIV− patients. *Salmonella* sp. showed high resistance to CHL ($P < 0.0001$) and DOX ($P < 0.0001$) in HIV+ patients compared to HIV− patients. Resistance of *Serratia* sp. to AMO ($P = 0.005$), AMC ($P = 0.005$) and CHL ($P = 0.005$) was significantly higher in HIV+ patients than in HIV− patients. Lymphopenia was higher in HIV+ patients (36.8%) than in HIV− patients (2.7%). In 45.9% of the HIV− patients, the CRP rate was higher than 6 mg/L compared to 16.2% in HIV+ patients. In general, bacterial multi-drug resistance in HIV+ patients (79.4%) was significantly higher ($P < 0.0001$) than in HIV− patients (29.7%).
The present study revealed that the resistance profiles of bacteria should be considered in HIV-infected patients to improve their health care.

Introduction

Enteric diseases are among the primary causes of morbidity and mortality in low income countries. Acquired immunodeficiency syndrome (AIDS), which is responsible for immune depression, increases the incidence of enteric diseases. Interest in topics related to infection with human immunodeficiency virus (HIV) and opportunist diseases continues. Indeed, HIV infects T4 lymphocytes, monocytes, macrophages, Langerhans cells, dendritic follicular cells of the ganglia and the cerebral astrocytes due to the CD4 protein receptors of this virus [1]. The principal consequence of infection with HIV is immune depression of the infected person, rendering him or her vulnerable to opportunistic infections.

Resistance of the enteric bacteria to antibiotics remains a major cause of morbidity and mortality worldwide. In people living with HIV/AIDS in developing countries, enteric diseases represent the second most common cause of death after tuberculosis [2]. As immunodeficiency progresses due to HIV infection, the most opportunistic diseases are observed when CD4 counts fall to less than 200 cells/μL of blood [3]. Prophylactic antibiotherapy is administered when the immunodeficiency becomes severe. This practice can lead to the development of bacterial resistance. Immunodeficiency and the presence of the bacteria resistant to standard antibiotics among patients endanger their prognoses. In light of the disordered state of the defense mechanisms caused by HIV infection, we believed that it could have a connection with colonization of the cells of the immune system and the development of bacterial resistance responsible for enteric infections. Many authors have been interested in evaluating enteric infections from various aspects such as epidemiology and clinical occurrence, as well as therapeutic interventions, to fight against them. To the best of our knowledge, only a few studies have combined the immunological aspects of these infections, HIV and bacterial resistance. Thus, we proposed, in this work, to study the variations in some cells of the immune system, as well as the sensitivity of the bacteria responsible for enteric infections among HIV+ patients compared to HIV− patients in Mbouda AD LUCEM Hospital, a reference heath care unit in the West Region of Cameroon.

Methods

Study area

This work was performed in the Biochemistry, Haematology and Bacteriology Laboratories of Mbouda AD LUCEM hospital located in the Bamboutos division, more precisely in the downtown area of Mbouda, West Region of Cameroon. This area was selected for the study based on its good reputation in the management of HIV-infected patients.

Population of the study

In this study sixty-eight HIV+ and thirty-seven (control group) HIV− patients were enrolled. The consenting participants had physicians who prescribed stool examinations, and also included in this study were patients who had not received any specific antibacterial therapy in the previous two weeks. Not included in this study were pregnant

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women, diabetic patients, victims of burns, patients receiving estrogens and smokers.

Study design

This study was a cross-sectional analysis to determine the immunological and microbiological profiles of bacteria responsible for enteric infections in HIV+ patients and HIV− control patients.

Ethics approvals

Ethical clearance for this study was obtained from the Ethics Review and Consultancy Committee of the Cameroon Bioethics Initiative (CAMBIN) under reference number CBI/295/ERCC/CAMBIN of 16 September 2014. Authorization to collect and analyze blood samples was also obtained from the Mbouda AD LUCEM hospital. All of the participants were duly informed of the study goals, procedures, potential harm and benefits, and cost, as well as the finality of the study. They willingly provided informed consent, either by signing or placing their thumbprint on the consent form after being satisfied with the responses to all questions asked of the investigators. Information was provided in English or French or interpreted in the local dialect by a volunteer independent of the study team. Participants' blood samples and results were anonymized. Leftover blood and stool samples were destroyed, according to hospital biosafety procedures.

Laboratory examination

HIV testing

Screening for HIV sero-status was performed using OraQuick HIV (Ora Sure Technology, USA) test kits, as described by the manufacturers and as previously reported [4].

Collection of blood and stool samples

The skin surface was cleaned with 70% ethanol and then 10 mL of blood were collected and distributed immediately after collection into three tubes, including two EDTA tubes for white blood cells and CD4/CD8 lymphocytes counts and a dry tube for the CRP level. For the stool samples, the hands were washed in an aseptic manner and then rinsed with tap water. Subsequently, we obtain approximately 10 g of stool in a sterile bottle, taking care not to touch the upper edge of the bottle. The bottle was then closed hermetically and labeled and transported in a cooler to the laboratory for bacterial analyses within 5—15 min of collection.

Culture media and antibiotic disks

Isolation media commonly used for the isolation of enteric bacterial pathogens were employed in this study. They consisted of Shigella-Salmonella agar (S-S agar), eosin methylene blue agar (EMB), Hektoen agar, nutritive agar, Muller Hinton agar, and Usual's antibiotic disks. The antibiotic disks were purchased from Verna Industrial Estate, Verna Goa, India. They were amoxicillin (AMO, 25 μg), amoxicillin + clavulanic acid (AMC, 20/10 μg), ceftriaxone (CRO, 30 μg), gentamicin (GEN, 15 μg), chloramphenicol (CHL, 30 μg), ciprofloxacin (CIP, 5 μg), levofloxacin (LEV, 5 μg), tetracycline (TET, 30 IU), and doxycycline (DOX, 30 IU).

Isolation of bacterial enteric pathogens

Stool specimens were collected and processed in the same manner in all of the HIV+ and HIV− patients. For the isolation of pathogenic enteric bacteria, stool suspension was prepared in 5 mL of sterile physiological water. Each suspension was sown thereafter on selective and differential culture media in Petri dishes for the isolation of enteric bacteria (EMB agar, Hektoen and SS agar). The petri dishes were then incubated at 37 °C for 24 h. Colonies of *Escherichia coli* were dark purple with a metallic surface on EMB agar, yellow on Hektoen and more or less mobile with a fresh state on microscopic observation. Colonies of *Klebsiella* sp. were pink with a violet center, convex without metallic surfaces on EMB agar, yellow on Hektoen and not mobile with a fresh state on microscopic observation. Colonies of *Citrobacter* sp. were pale violet with a center, and a metallic characterized it on EMB agar, while it was yellow on Hektoen and mobile with a fresh state on microscopic observation. Colonies of *Enterobacter* sp. had a bluish aspect on EMB agar and were yellow on Hektoen and mobile with a fresh state on microscopic observation. Colonies of *Salmonella* sp. were colorless with a black center on SS agar, blue-green with a black center on Hektoen, and grayish transparent on EMB. These colonies were mobile with a fresh state on microscopic observation. The colonies of *Serratia* sp. were colorless with a gray center on SS agar and mobile with a fresh state on microscopic observation. Colonies of *Proteus* sp. were yellow with a black center on Hektoen agar, grayish with a film around the colony on EMB agar and very mobile with a fresh state on microscopic observation. Each colony was isolated and purified by culture on nutritive agar at 37 °C for 18 h, and confirmation was realized by the study of biochemical profiles on the API 20E gallery conform, as described by the manufacturer (Bio Merieux, Lyon, France) [5]. The confirmation of the bacterial

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species was performed according to the established biochemical characteristics (see Table S1; supporting information).

Antibiotic susceptibility testing

Agar diffusion in solid medium was used to evaluate the susceptibility of the isolates to antibiotics, as described by CLSI (2013). An inoculum of opacity of 0.5 Marc Farland (10^8 bacteria per mL) was inoculated on Muller Hinton agar for all of the isolates. On a dry surface of Muller Hinton agar 4 mm thick, bacteria were inoculated by flooding. The antibiotic disks were deposited thereafter on the surface of the agar using a sterile grip (5 disks of antibiotics in one Petri dish of 90 mL). Each disk was pressed to ensure complete contact with the agar and was distributed so that the center of each disk is located at 25 mm from the others. Each Petri dish was allowed for 3—5 min to undergo diffusion of antibiotics and then was reversed and placed in an incubator (Prolabo, Medical international, Rochin, France) at 37 °C for 24 h. The resulting zones of inhibition were measured to the nearest whole millimeter using a meter ruler and were interpreted using the standards of CLSI. Performance standards for antimicrobial susceptibility testing and the organisms were reported as susceptible (S), intermediate (I) or resistant (R) to the antibiotic being tested (see Table S2; supporting information).

CD4 and CD8 counts and C-reactive protein levels

The CD4 and CD8 T-lymphocytes counts of all of the participants were determined using flow cytometry applied in clinical immunology by the Becton Dickinson's' FACS count method [6] The level of C-reactive protein (CRP) was determined by immunoturbidimetry techniques [7].

Blood count

Blood was collected in pre-labeled EDTA tubes. Then, the tubes were gently agitated to avoid the formation of clots. Each sample was then introduced into an automat (Hemascreen 18; Hospitex diagnosis; Florence, Italy). After 9 s, the automat calculated and automatically reported white blood cell, lymphocyte, monocyte and granulocyte counts.

Statistical analysis

Summary statistics of demographics and clinical characteristics were calculated for patients with and without HIV. To examine the association HIV status with patients' demographic and clinical characteristics, we used the chi-square test for categorical variables and the *t* test for continuous variables. A *P*-value of ≤ 0.05 was considered to be statistically significant.

Results

Of the 68 HIV+ patients, 44 (64.70%) were women, and 24 (35.30%) were men. Of the 37 HIV− patients, 21 (56.76%) were women, and 16 (43.24%) were men. Compared to the HIV+ patients, the HIV− patients were younger, with ages between 31 and 50 years old more represented in HIV+ patients, and there was a significant association between HIV status and age group (χ^2 (1) = 25.054, $P < 0.0001$; Table S3; supporting information). Table S3 also shows significantly higher multi-drug resistance ($P < 0.0001$) in HIV+ patients than in HIV− patients.

The distribution of the bacterial species responsible for enteric infections in the two populations is represented in Fig. 1. It appeared that the distribution of infections with *E. coli* was higher in HIV+ patients than in HIV− patients in this study. The prevalence of infections with *Klebsiella* sp. was

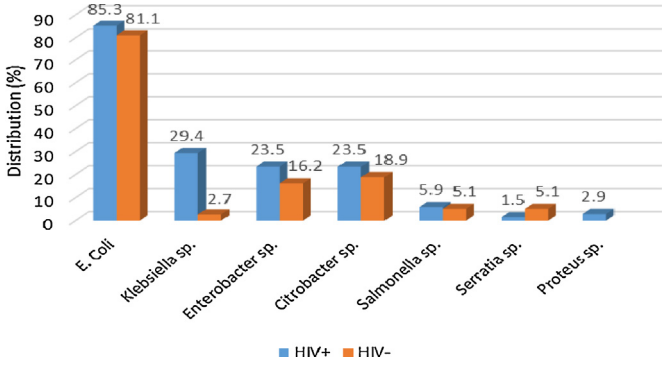

Figure 1 Distribution of the bacterial infections in HIV− and HIV+ patients: 37 (HIV−) and 68 (HIV+).

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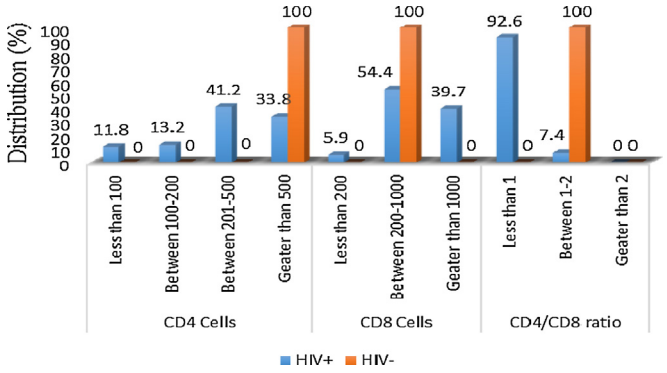

Figure 2 Distribution of CD4 cells, CD8 cells and CD4/CD8 ratios in HIV− and HIV+ patients: 37 (HIV−) and 68 (HIV+).

higher in HIV+ (29.4%) patients than in HIV− (2.7%) patients.

The distribution of resistance of the various bacterial isolates to antibiotics was also determined. Resistance of *Klebsiella* sp. to CRO ($P = 0.001$), GEN ($P = 0.005$), CHL ($P = 0.0004$), CIP ($P = 0.005$) and DOX ($P < 0.0001$) was significantly higher in HIV+ patients than in HIV− patients. *Enterobacter* sp. showed highly significant resistance to GEN ($P = 0.009$) and CIP ($P = 0.001$) in HIV+ patients, compared to HIV− patients. *Citrobacter* sp. was resistant to GEN ($P = 0.009$) in HIV+ patients, compared to HIV− patients. *Salmonella* sp. showed highly significant resistance to CHL ($P < 0.0001$) and DOX ($P < 0.0001$) in HIV+ patients, compared to HIV− patients. Resistance of *Serratia* sp. to AMO ($P = 0.005$), AMC ($P = 0.005$), and CHL ($P = 0.005$) was significantly higher in HIV+ patients than in HIV− patients (Table S4; supporting information).

The blood cells (total white globules) involved in the defense of the organism, particularly the lymphocytes, the monocytes and the granulocytes, were counted. Table S5 compares the principal anomalies of the white globules observed among HIV+ and HIV− patients. It could be observed that the HIV+ individuals had more pronounced lymphopenia than the HIV− population.

The CD4 and CD8 lymphocytes and their ratios were determined. Their blood levels were determined according to the published values [8–10] represented in Fig. 2. It appeared that a bacterial infection does not involve a reduction in the blood rates of CD4 lymphocytes (normal value: 500–1500 cells/μL) and CD8 lymphocytes (normal value: less than 1000 cells) in the HIV− population. In 66.2% of HIV+ patients, the CD4 lymphocyte rate was less than 500 cells/μL of blood (with 11.8% less than 100 cells, 13.2% ranging between 100 and 200 cells and 41.2% ranging between 201 and 500). A proportion of 92.6% of HIV+ patients had a CD4/CD8 ratio less than 1 (normal value: 1–2), compared to the HIV− patients, who had blood CD4 lymphocyte levels of 500 cells/μL, which was higher than that of CD8 lymphocytes, which ranged between 200 and 1000 cells/μL, and their ratio (CD4/CD8 ratio) ranged between 1 and 2, corresponding to the normal values.

C-reactive protein was also quantified, and the results are depicted in Fig. 3. It appeared that a normal C-reactive protein blood level (less than

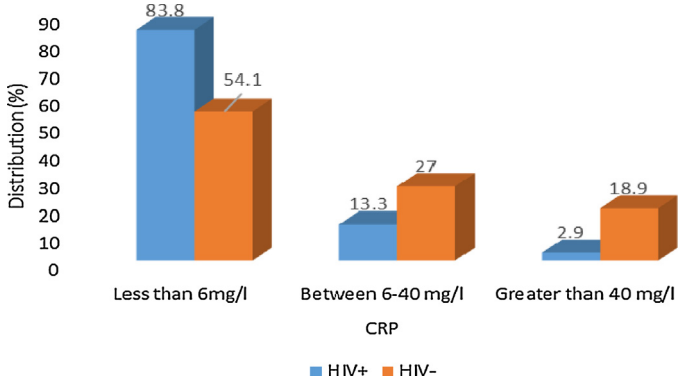

Figure 3 Distribution of CRP in HIV− and HIV+ patients: 37 (HIV−) and 68 (HIV+).

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6 mg/L) was observed in the majority of the seropositive (83.8%) and in 54.1% of the HIV− patients (all presenting a bacterial infection). However, a blood rate higher than 6 mg/L was observed in 45.9% of the HIV− patients, compared to 16.2% of the HIV+ patients. In addition, a very high CRP blood rate (greater than 40 mg/L) was observed in 18.9% of the HIV− patients, compared to only 2.9% of HIV+ individuals.

Table S6 summarizes laboratory parameters with bacterial resistance to less or more than three families of antibiotics according to HIV status. It could be deduced that lymphocytes and granulocytes had higher average values, and the CD4/CD8 ratio was higher, while CRP had lower average values in HIV+ patients than in HIV− patients, when the enteric bacteria were not multi-drug resistant (resistance to more than three families of antibiotics).

Discussion

Bacterial resistance to antibiotics is the aptitude of bacteria to grow in the presence of an antibiotic concentration higher than that inhibiting the majority of the same species [11]. Multi-resistance is defined as the resistance of a bacterium to more than three families of antibiotics [12]. In this work, we studied the organization of the defense system of HIV+ and HIV− patients in connection with the resistance of the bacteria responsible for enteric infections in the Mbouda AD LUCEM hospital.

The high incidence of HIV-infected adults (31−50 years old) found in our study of 55.88% was comparable to that found by Ouedraogo et al. [13] in Ouagadougou and Saliou et al. [14] in the G-spot hospital. This period corresponds to that of high sexual activity and therefore greater exposure to the risk of sexually transmitted infections.

The present results revealed that the distributions of infections with *E. coli* were highest in both HIV+ and HIV− patients, which could be explained by *E. coli* being the principal bacterium that normally colonizes the intestine [15]. The high prevalence of *Klebsiella* sp. infections in HIV+ patients (29.4%) compared to HIV− (2.7%) patients could be due to the disordered state of the defense mechanisms of HIV-infected persons. These results were in conformity with those obtained by Pupulin and collaborators in 2009, who reported that 49% of the diarrhea in HIV− patients were caused by *Escherichia coli* and 13% by *Klebsiella* sp. [16].

This study thus revealed that bacterial agents were significantly associated with increased resistance to antibiotics, such as CRO, GEN, CHL, and DOX for *Klebsiella* sp., GEN and CIP for *Enterobacter* sp., GEN for *Citrobacter* sp., CHL and DOX for *Salmonella* sp. and AMO, AMC, and CHL for *Serratia* sp., in HIV+ patients, compared to HIV− patients. These findings were in accordance with previous studies [17,18]. The resistance of *Klebsiella* sp. could be due to over-expression of the efflux pumps of the *Resistance Nodulation Cell Division* (RND) type [19−21]. The *Enterobacter* sp. resistance to GEN (25%) and CIP (37.5%) found in our study among HIV-seropositive patients was close to previously reported data [22]. The resistance of *Salmonella* sp. to CHL (75%) and DOX (75%) could be due to the use of antibiotics that induced the production by the bacteria of enzymes destroying the antimicrobials [23]. We also observed a resistance *Serratia* sp. to CIP (50%), CRO (50%) and CHL (50%) in the HIV− group. This resistance of *Serratia* sp. to CRO could be the result of an induction by these bacteria of the synthesis of cephalosporinases [24]. The increase in bacterial resistance to antibiotics tested among HIV+ patients compared to HIV− patients could be related to the frequent exposure of HIV+ patients to antibiotics within the framework of their follow ups.

The HIV+ patients had more pronounced lymphopenia than the HIV− patients as observed in this study. The lymphopenia found in 36.8% of HIV+ patients was twice as high as the value published by Oumar et al. [25] in Mali and Loua et al. [26] in Guinea Conakry. These anomalies were probably caused by infection with HIV and the drugs used within the framework of the follow-up of these patients.

On the level of CD4 and CD8 lymphocytes, we observed a reduction in the blood rates (blood rate of CD4 lymphocytes less than 500 cellules/μL: 66.2%) among HIV+ patients. These results were higher than those obtained by Dokekias et al. [26] in Congo Brazzaville, Loua et al. [27] in Guinea Conakry and Mouhari-Touré et al. [28] in Togo. In the present study, we observed that the CD4/CD8 ratio was generally less than 1 among the HIV-seropositive patients. These values are very closed to the results of Loua et al. [26] in 2011 (92.6% versus 88%) in the hematological profiles of patients infected with HIV in Guinea Conakry. These reductions in CD4 lymphocytes and CD4/CD8 ratio could be related to infection with HIV. Indeed, this virus infects the CD4 lymphocytes, which is the head of the orchestra of the immune reactions, causing a disordered state of the host's defense system.

Concerning CRP, the results presented here showed that 45.9% of the HIV− patients had blood rates higher than 6 mg/L, compared to 16% of HIV+ individuals. This weak blood rate among HIV+ patients could be attributed to the destruction of

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the CD4 lymphocytes and other cells, which are producers of pro-inflammatory cytokines, such as interleukin 6, which induces CRP synthesis at the level of the hepatocytes [29—31].

Enteric bacteria were more multi-drug resistant in the HIV+ (79.4%) patients than in the HIV— patients (29.7%). This increase in multi-resistance among HIV+ patients could be due either to prophylactic antibiotherapy used for the prevention of opportunistic infections when the CD4/CD8 ratio decreases during HIV infection or the potential antimicrobial activity of some antiretroviral drugs.

Low average values of CD4/CD8 ratio and CRP and high average values of lymphocytes and granulocytes in HIV+ patients compared to HIV— patients were observed in this study when the enteric bacteria were drug resistant. Acute infection with HIV causes a production of cells of the immune system, such as lymphocytes and granulocytes, and with time, these cells are destroyed, probably explaining the appearance of bacterial drug-resistance.

The data of this study might not be representative of the entire HIV-positive and HIV-negative populations of Mbouda; however, they could be of interest to physicians and other health professionals in Cameroon, given that they were collected in a reference health center involved in the management of HIV/AIDS and associated diseases. Because only one health center and a limited number of patients were involved in the present work, further studies will be performed to increase the reliability of these findings.

Conclusions

The present study revealed that the prevalence of enteric bacteria, as well as resistant phenotypes, was higher in HIV-infected persons. The anomalies observed in HIV+ patients were probably caused by immunodeficiency due to HIV infection. Hence, the resistance profiles of bacteria should be considered in HIV-infected patients to improve their health care. However, this work also indicated the need for adequate biological follow-up for the best monitoring of persons in Cameroon infected with HIV.

Funding

No funding sources.

Competing interests

None declared.

Ethical approval

Ethical clearance for this study was obtained from the Ethics Review and Consultancy Committee of the Cameroon Bioethics Initiative (CAMBIN) under reference number CBI/295/ERCC/CAMBIN on 16 September 2014.

Authors' contributions

WTJM and VK conceived of the study. WTJM performed the sampling and data collection. WTJM and VK participated in the analysis of the samples, data management and statistics. WTJM and VK drafted the manuscript. All of the authors read the manuscript and approved the final version prior to submission.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jiph.2016.04.009.

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