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Comparative Performance of Microscopy and Nested PCR for the Detection of *Cryptosporidium* Species in Patients Living with HIV/AIDS in Abidjan (Côte d'Ivoire)

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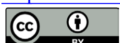

Open Access

Abstract

Cryptosporidium spp. infection is one of the causes of diarrhea in people living with HIV/AIDS. The objective of this study is to compare the sensitivity of microscopy and molecular biology to determine the prevalence of *Cryptosporidium* spp. in Patients Living With HIV (PLWH). This is a descriptive cross-sectional study conducted in three care centers for people living with HIV/AIDS in Abidjan. It took place from November 2018 to March 2020. Sociodemographic data were obtained via a questionnaire. Stool and blood samples were collected and analyzed for microscopy and Nested PCR detection of *Cryptosporidium* spp. Blood samples were analyzed for CD4+ count. A total of 363 stool samples were collected from the three sites. Individuals aged 40 - 50 years (36.52%) were most likely to participate in the study. HIV Type 1 accounted for 86.22% of the study population. The samples collected consisted of 47.65% diarrheal stool. Microscopic examination of the stool yielded a prevalence of 3.86% for *Cryptosporidium* spp. while the prevalence was 3.96% with molecular identification. No statistically significant difference was observed between these two prevalences ($\chi^2 = 0.26$; $p = 0.609$). CD4+ count was the factor associated with *Cryptosporidium* spp. infection for both microscopy (OR = 0.887, p = 0.001) and PCR (OR = 0.896, p = 0.001). This study demonstrated that Nested PCR improves the detection of *Cryptosporidium* spp. in patient diagnosis.

Keywords

Cryptosporidium, PCR, Microscopy, PLHIV, Côte D'Ivoire

1. Introduction

Infection caused by *Cryptosporidium* spp. is one of the most important causes of diarrhea and death associated with diarrhea primarily in infants and immunocompromised individuals [1]. The first human cases of this infection were reported in the 1970s in immunocompromised children and adults [2]. The pathogen responsible for this infection belongs to the genus *Cryptosporidium* which is a protozoan, obligate intracellular parasite that infects the epithelial cells of the digestive and respiratory tracts of a wide variety of hosts. It is recognized as one of the most important diarrheal pathogens affecting human populations worldwide, particularly in Sub-Saharan Africa [2]. There are 38 recognized species of *Cryptosporidium* spp. that differ in host specificity and public health importance [3]. Of these, *C. hominis* and *C. parvum* are the primary sources of cryptosporidiosis in humans [2] [3]. *C. hominis* is generally associated with human infection while *C. parvum* is as much related to human infection as it is to the infection of animals, especially young ruminants [2]. More than 20 species have been identified at the molecular level as causing zoonotic cryptosporidiosis in humans [4]. The life cycle of *Cryptosporidium* alternates between asexual and sexual reproduction in a single host. Sexual recombination results in oocyst production and is therefore essential for parasite transmission, but recombination may also play a role in ongoing host infection [5] [6] [7]. The diagnosis of these parasitic diseases relies primarily on microscopic examination of stool for many laboratories [8] [9]. This diagnosis involves several steps including a fresh direct examination to identify vegetative forms followed by a staining step from the stool concentration pellet to detect parasites in cases of low infestation [10]. However, the process of microscopic identification of intestinal parasites requires a lot of time up to several hours, and careful microscopic observation, which often leads to misdiagnosis [11]. All these steps make it difficult to control cryptosporidiosis. The sensitivity of the infection in humans depends on factors such as the immune status of the subject, which is characteristic of people living with HIV/AIDS [12]. Noting the importance of this parasitosis in PLHIV, the objective of this work was to compare the sensitivity of microscopy with that of molecular biology (Nested PCR) for the determination of the prevalence of *Cryptosporidium* spp. in PLHIV in Abidjan.

2. Methodology

2.1. Framework of the Study

The study was conducted from November 2018 to March 2020 in three care centers in the Abidjan district, namely the Urban Community Health Training

Center (FSUCOM) of Anonkoua-Kouté, the Research and Training Care Center (CePReF) of Yopougon Attié, and the Pneumo-Physiology Department (PPH) of the University Hospital Center (CHU) of Cocody. This was a cross-sectional, prospective, and descriptive study that involved adult patients managed in each of the 3 study sites who gave informed consent. The three study sites were chosen to be representative of the Abidjan district. Microscopic and molecular analyses of this study were performed at the Parasitology Unit of the Institut Pasteur of Côte d'Ivoire. The immunological analyses were performed in each of the management centers of our study.

2.2. Recruitment of Patients Included in the Study

Before participation in the study, the objectives of the study and the procedures to be followed were explained in simpler and more accessible terms to the patients. Therefore, each patient enrolled in this project gave informed consent by signing a consent form in French according to the patient's preference. Sociodemographic data (age, gender, place of residence and occupation), clinical data (medical history, HIV status, weight loss, vomiting, abdominal pain and fever) were collected. Some biological data (HIV/AIDS status, CD4/mm^3) were also collected from all patients included in the study, using a questionnaire administered by the treating physician.

For patients who reported diarrhea, additional questions were asked to determine the cause of the diarrhea. The physical examination was performed by the attending physician at each management center. At the time of enrollment, each patient was informed about the parasitological analysis of the stool and the assessment of the CD4+ T-cell count to be performed on each purple tube blood sample. A 125-mL sterile stool jar was distributed to each volunteer for collection of their stool samples.

- **Inclusion criteria**

Participants of the study included HIV-infected adults over 18 years of age old, with or without diarrhea. All the participants were monitored in a health care center in Abidjan and their approval written informed consent was obtained.

- **Exclusion criteria**

Persons excluded from the study were those under 18 years of age, patients that were not followed in a treatment center, and those who refused to give their written informed consent.

2.3. Sample Collection

Patient stools collected at each site were sent to the Parasitology Unit of the Institut Pasteur de Côte d'Ivoire (IPCI) and analyzed within two hours of release to identify vegetative forms of intestinal parasites. The collected stool samples were separated into three samples: the first sample was preserved in potassium dichromate ($K_2CR_2O_7$), the second sample was preserved in sodium chloride (NaCl) 9‰, while the third sample was used for microscopic examinations.

2.4. Microscopic Examinations

Once the stool arrives at the IPCI Parasitology Unit laboratory, it is first examined macroscopically by triturating it with a clean rod to note its consistency (appearance) and the presence of blood or mucus. Once the macroscopic examination is completed, 1 g of stool is taken from different places (3 to 4 places depending on the stool appearance) with a thin clean rod. This sample is diluted in a drop of physiological water (9‰) placed on a slide and covered with a coverslip. This preparation is observed under a light microscope with a 10× and 40× objective respectively (this preparation must not be thick and must be read completely). To better identify some parasites, a drop of Lugol's was finally placed on the edges of the cover slip and the observation was done at the 40× objective. Finally, the staining by the Zielh Neelsen method was performed in order to highlight the parasite oocysts under the light microscope, first at the ×40 objective and then at the ×100 magnification.

2.4.1. Modified Ritchie Method (PBS-Ether Concentration)

After the fresh direct examination step, the modified Ritchie method was used in order to concentrate the parasite elements that were not detected by direct examination. To do this, approximately 2 g of fresh stool is mixed in 10 mL of PBS solution in a clean container using a clean rod. The mixture is filtered through a small, clean, fine strainer. Eight mL of the filtrate was collected in a 15 mL centrifuge tube (Falcon® tube) to which 4 mL of ether was added using the scale on the 15 mL Falcon® tube. The Falcon® 15 mL tube is capped and shaken to obtain a homogeneous emulsion. This tube is centrifuged at 2000 rpm for 3 minutes to break the emulsion. At the end of this step, the tube shows different phases from top to bottom: an ether phase (fat), a lipophilic residue layer, an aqueous phase (PBS solution) and the pellet. The tube is then emptied abruptly [13]. Using a Pasteur pipette, two to four drops of the centrifugation pellet are spread between slides and coverslips and examined under the microscope for further cysts, eggs and parasites. A part of these pellets obtained is then used for the modified Zielh-Neelsen staining.

2.4.2. Microscopic Detection by the Modified Zielh-Neelsen Stain

The pellets obtained by the modified Ritchie method are spread on slides to make thin smears. The smear slides are then air-dried and fixed with methanol for 5 minutes and immersed in Zielh's phenol fuchsin for 1 hour. After rinsing with water, a decoloration with 2% sulfuric acid solution is performed for 20 seconds. This decoloration is followed by a new water rinse, and then the slides are immersed in a 5% malachite green solution for 5 minutes for counterstaining. After rinsing with water and drying at room temperature, the slides are examined under a light microscope at ×40 objective and then at ×100 magnification with immersion oil. This simple staining technique has been used to highlight oocysts (resistance form) of coccidia which are sometimes difficult to detect by direct observation.

2.5. Molecular Examinations

2.5.1. Extraction of DNA from *Cryptosporidium* spp.

Cryptosporidium spp. DNA was extracted from the pellet obtained by the fresh stool concentration technique (stool enrichment technique). 200 uL of each sample was added to a 2 mL cryotube to undergo heat shock (freeze-thaw step) in order to break down the *Cryptosporidium* spp. oocyst wall. After the freeze-thaw step, as much of each heat shocked pellet as possible was transferred to the Promega Extraction Kit columns. This kit was used to extract cryptosporidia DNA following the manufacturer's protocol. At the end of the extraction, 300 uL of DNA eluate was obtained.

2.5.2. Nested PCR of COWP Gene

During the study, a portion of the gene coding for the *Cryptosporidium* spp. wall protein was targeted. This hypervariable and highly polymorphic region was subjected to Nested PCR [14]. For this purpose, two pairs of primers targeting the COWP gene were used: The first primer pair Oocry 3 (5'-AGA TTA ACA GAA TGC CCA CCA GGT A-3') and Oocry 4 (5'-CCA TGA TGA TGT CCT GGA TTT TGT A-3') was followed by the second pair of primers Oocry1 (5'-CCT GGA TAT CTC GAC AAT-3') and Oocry 2 (5'-GCG AAC TAA TCG ATC TCT CT-3'). The reaction medium for the first PCR was composed of 5 µL of DNA extract, 1 µL of each Oocry 3/Oocry 4 primer concentrated to 10 µM of each, 5 µL of SolisBioDyne HOT FIREPolBlend Master Mix Ready to Load and 13 µL of sterile water to reach a total volume of 25 µL. The second PCR reaction medium is the same except for 2 µL of the first PCR amplicon and 16 µL of sterile water to be added to make a total volume of 25 µL. The amplification programs of the first and second PCR are identical, except for the number of cycles of the second PCR which is increased from 30 to 40: denaturation of the DNA for 10 min at 95˚C followed by 30 cycles of 95˚C for 30 s, 52˚C for 1 min and 72˚C for 1 min and finally 7 min of elongation. The program for the second PCR was the same except that the number of cycles was increased from 30 to 40. The amplified fragments were revealed on a 1.5% agarose gel by electrophoresis and observed using Biorad GelDoc Imager.

The COWP gene targeted by the two primer pairs through Nested PCR, revealed fragments of 640 base pairs corresponding to the genus *Cryptosporidium.*

2.6. Consent and Ethics Approval

This study was approved by the National Ethics Committee for Life Sciences and Health (CNESVS) of Côte d'Ivoire. All patients were informed of the objectives of the study protocol and an informed consent form was presented to them to obtain their agreement before the collection of stool and blood samples.

2.7. Statistical Analysis

R software (version R x64 3.6.0 and R i386 3.6.0) was used for the analyses. Prevalence of infection is the proportion of positive samples out of all tested sam-

ples. Prevalence of infection was compared with sex and age group using the chi-square test or Ficher's exact test (when at least one cell was less than 5).

The sensitivity of a diagnostic technique is the ability of the method to correctly identify the presence of a parasitic infection.

The specificity of a diagnostic technique is a measure of the performance of the test when used on negative individuals.

$$\text{Sensibility}(\%) = \frac{\text{Number of true positive cases}}{\text{Number of true positive cases} + \text{Number of false negative cases}} \times 100$$

$$\text{Specificity}(\%) = \frac{\text{Number of true negative cases}}{\text{Number of true negative cases} + \text{Number of false positive cases}} \times 100$$

Cohen's kappa coefficient (ƙ) assesses the degree of agreement between diagnostic techniques and uses the 2 × 2 contingency table. The statistical coefficient (ƙ) was used to determine the strength of agreement based on the following criteria:

—<0 = no agreement;

—0 - 0.20 = poor agreement;

—0.21 - 0.40 = average agreement;

—0.41 - 0.60 = moderate agreement;

—0.61 - 0.80 = considerable agreement;

—0.81 - 1 = near perfect agreement.

P-values less than 0.05% and 95% confidence intervals were considered statistically significant associations.

Logistic regression was used to determine factors associated with *Cryptosporidium* spp. using R software (versions Ri3864.0.0.0). For this purpose, the association between *Cryptosporidium* spp. infections was defined as the independent variable.

The dependent variables were analyzed using univariate analysis and the strength of each association was measured by an Odds Ratio (OR). All variables with a minimum p-value criterion (p ≤ 0.2) were specified and included in the multivariate analysis at a 95% Confidence Interval (CI). A p-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Socio-Demographic Description of the Study Population

The parasitological survey was conducted on 363 HIV-infected patients of whom 114 (31.40%) were men [95% CI 37 - 47] and 249 (68.59%) were women [95% CI 52 - 62].

The mean age of the participants was 44.87 years with extremes ranging from 18 years to 72 years (Table 1). The age groups of 40 - 50 and 51 - 61 years were the most represented with respectively 37.74% and 27.82% of the studied population. Patients of 40 - 50 years (36.52%) old were the most numerous to participate in the study. However, the lowest participation was observed in adults over

Table 1. Some socio-demographic characteristics of the study population.

Variables	Study population	
Sex	**N**	**%**
Male	114	31.404
Female	249	68.595
Age range		
18 - 28	27	7.438
29 - 39	78	21.76
40 - 50	137	37.741
51 - 61	101	27.823
More than 61	18	4.95
ND	1	0.27
Profession		
Pupil	3	0.8264
Student	7	1.928
Liberal function	244	67.217
Public servant	29	7.98
Unemployed	80	22.038
HIV type		
HIV 1	313	86.225
HIV 1 & 2	8	2.2038
HIV 2	42	11.570
ARV treatment		
Yes	337	92.83
No	21	5.785
ND	5	1.377
Total	**363**	**100**

Note: ND: Not defined.

61 years old (2.20%).

Several types of HIV were identified in this study in which HIV Type 1 represented 86.22% of the study population.

The liberal profession (67.21%) was predominant among all the professions in the study population. Professional occupation (67.21%) was predominant among all occupations in the study population (Table 1). The majority (92.83%) of the PLHIV in the study were on ARV treatment (Table 1).

3.2. Prevalence of *Cryptosporidium* spp. Infection by Microscopy

Direct examination of samples by Zielh Nelseen staining revealed 14 samples positive for *Cryptosporidium* spp. (Figure 1) out of 363 samples, representing

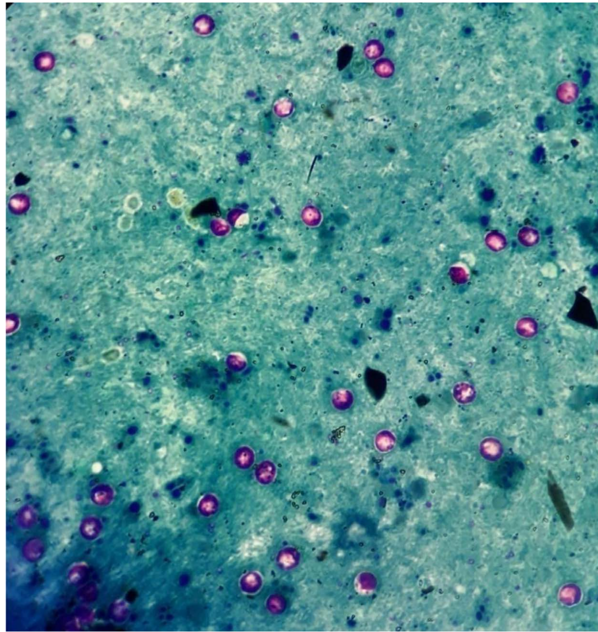

Figure 1. *Cryptosporidium* spp. observed with a light microscope (G × 100).

3.86%. Only the genus of the parasite has been identified.

A total of 14 of 363 stool samples examined microscopically contained *Cryptosporidium* spp. oocysts, for a prevalence rate of 3.86%. Males (4.38%) were more parasitized than females (3.61%) (**Figure 2**). No statistically significant difference was observed (p-value = 0.962). The 29 - 39 age group had the highest prevalence rate (8.97%) (**Figure 3**).

3.3. Prevalence of *Cryptosporidium* spp. Infection by Molecular Biology (PCR)

The COWP gene targeted by the two primer pairs by Nested PCR revealed fragments of 640 base pairs corresponding to the genus *Cryptosporidium*. Analysis of the Nested PCR (molecular biology) results identified 18 samples positive for *Cryptosporidium* spp. (**Figure 4**) out of 363 samples analyzed, a proportion of 4.95%. This analysis technique was used to identify 18 egg-infested samples of the genus *Cryptosporidium.*

Figure 5 shows that the prevalence of Cryptosporidiosis infection in females (5.22%) is higher than in males (4.38%). However, this difference is not significant (χ^2 = 1.3415, p = 0.247). In terms of age, the 29 - 39 age group had the highest prevalence rate (10.25%) (**Figure 6**). However, statistical analysis showed that there was significant variation between age groups (χ^2 = 43.0411, p = 0.00481).

3.4. Performance of Nested PCR and Microscopy in the Detection of *Cryptosporidium* spp.

The observed sensitivity rate showed that PCR is more sensitive than microscopy,

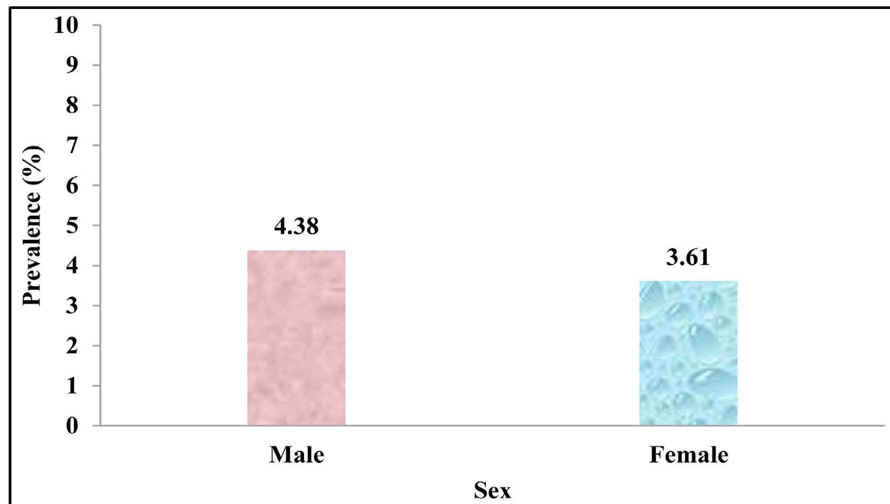

Figure 2. Prevalence of *Cryptosporidium* spp. by microscopy in relation to sex.

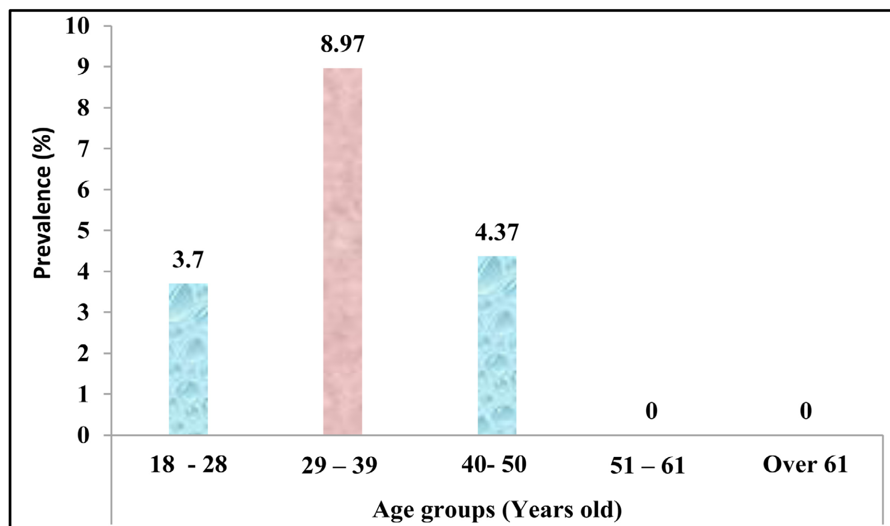

Figure 3. Prevalence of *Cryptosporidium* spp. by microscopy according to age group.

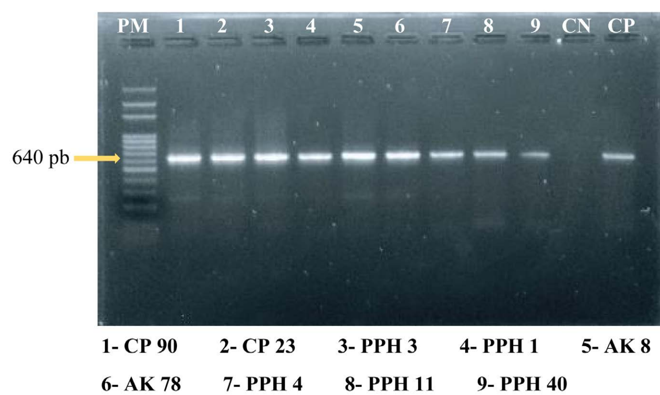

Figure 4. Nested PCR product (640 bp) after PCR amplification of the gene encoding the wall protein of *Cryptosporidium* spp. The PM lane denotes the molecular weight, the lanes numbered 1 to 9 contain positive *Cryptosporidium* spp. samples, and the CN lane represents the negative control while the CP lane shows the positive control.

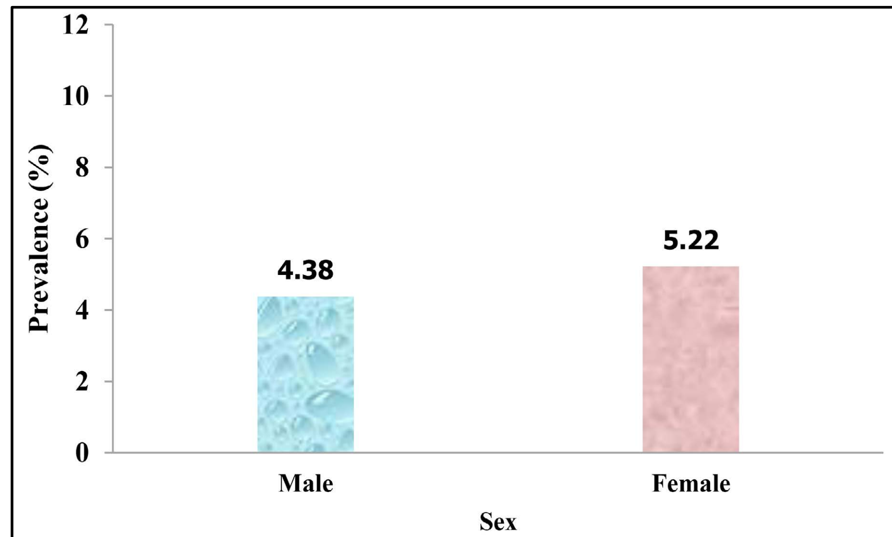

Figure 5. Prevalence of *Cryptosporidium* spp. by Nested PCR according to sex.

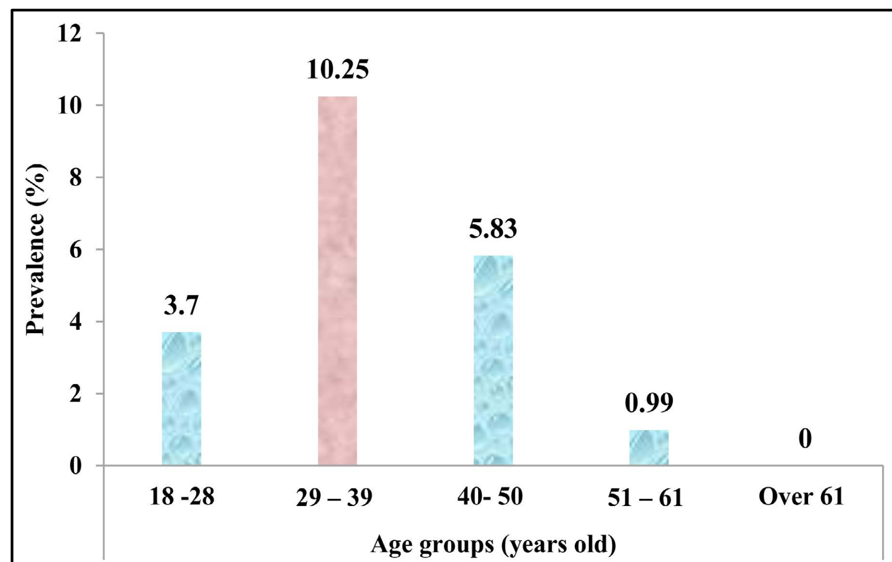

Figure 6. Prevalence of *Cryptosporidium* spp. by Nested PCR according to age group.

but no statistically significant difference was observed (p-value = 0.24). A Cohen's kappa of 0.17 was obtained between the diagnostic techniques, reflecting poor agreement between these two diagnostic techniques for the identification of *Cryptosporidium* spp. (Table 2).

3.5. Factors Associated with *Cryptosporidium* Relative to the Sex, Occupation, Type of HIV, ARV Treatment, Previous Diarrheal and CD4 Count by Test Type

Concerning the main variables identified, namely gender, age, occupation, only CD4 count was the factor associated with *Cryptosporidium* spp. infection for both microscopy (OR = 0.887, p = 0.001) and PCR (OR = 0.896, p = 0.001) (Table 3).

Table 2. Comparative sensitivity of microscopy techniques and Nested PCR for the detection of *Cryptosporidium* spp. and agreement between Nested PCR and microscopy techniques for the detection of *Cryptosporidium* spp.

Method	Numberof sample examined	Numberof positive detected	Sensitivity (%)	Specificity (%)	Strain discrimination
Nested PCR	363	18	100	100	Specificity: 0.24
Microscopy	363	14	77.77	97.21	Sensitivity: 0.95
	Microscopy			**Kappa Cohen**	
PCR	**Positive**	**Négative**			
Positive	14	4			
Négative	0	0		**k = 0.17**	
Total	14	4			

Table 3. Factors associated with *Cryptosporidium* spp. according to the type of test.

	Cryptosporidium spp. n (%)									
Explanatory variable	**Microscopie**					**PCR**				
		Univariate analyses		Multivariate analyses			Univariate analyses		Multivariate analyses	
Sex	n (%)	OR	P	OR	P	n (%)	OR	P	OR	P
Male	5 (1.38)	1.22	0.72	NA	NA	13 (3.58)	_	0.55	NA	NA
Female	9 (2.48)					5 (1.37)				
Occupation										
Public servant	3 (7.31)					5 (1.37)				
No public servant	11 (3.52)	_	0.99	NA	NA	13 (3.58)	_	1	NA	NA
Learner	0 (0)					0 (0)				
Type of HIV										
HIV 1	13 (3.58)					15 (4.13)				
HIV 2	1 (0.28)	0.46	0.43	NA	NA	3 (0.82)	1.95	0.48	NA	NA
HIV 1 & 2	0 (0)					0 (0)				
ARV treatment										
Yes	10 (2.75)	_	0.55	NA	NA	14 (3.85)	NA	NA	NA	NA
No	4 (1.10)					4 (1.10)				
Previous diarrheal										
Yes	11 (3.52)	1.87	0.40	NA	NA	1 (0.41)	0.246	0.30	NA	NA
No	3 (7.31)					2 (1.68)				
CD4 count										
<200	12 (3.30)					13 (3.58)				
200 - 500	1 (0.28)	0.04	0.0001*	0.887	0.001**	3 (0.82)	0.041	0.0001	0.89	0.001**
≥500	1 (0.28)					2 (0.55)				

Note: NA: Not Applicable; P: p-value; OR: Odds Ratio.

4. Discussion

The study revealed a prevalence rate of 3.86% of *Cryptosporidium* spp. for microscopy versus 4.95% for molecular biology (Nestd PCR). However, this difference was not statistically significant (p-value ≥ 0.005). These results are similar to those of Chalmers and collaborators [15], who obtained a lower prevalence of *Cryptosporidium* spp. by microscopy compared to molecular biology. In the present study, based on microscopy observation, males (4.38%) were more infested than females (3.61%). In contrast, Nested PCR analysis indicated that females (5.22%) were more infested than males (4.38%). No significant difference was observed between sex either for microscopy (p-value = 0.962) or for Nested PCR (p-value = 0.247). The results of the analysis of the relationship between sex and parasitemia of our work are similar to these of Kassi and collaborators [16] obtained among people living with HIV at the CHU de Treichville in 2017 in Abidjan. Similar results were also reported by Wumba and collaborators [17] in the Democratic Republic of Congo among PLHIV, where they observed the absence of a significant association between gender and the identified parasite (p-value = 0.46). The results of this study showed greater sensitivity and specificity of the Nested PCR method compared to microscopy, but no statistically significant difference was observed in terms of sensitivity (p-value = 0.24) or specificity (p-value = 0.95). Similar studies on the comparison of cryptosporidiosis diagnostic tests conducted in the United Kingdom [15] and Ethiopia [18] also showed the lower sensitivity of microscopy procedures used to diagnose *Cryptospordium* spp.

According to the results obtained, assays through PCR were very sensitive and specific techniques for the detection of *Cryptosporidium* spp. genes. These results are similar to those of Bath and collaborators [11], where they compared Nested PCR and microscopy for the detection of cryptosporidiosis in veal calves.

A Cohen's kappa of approximately 0.17 was obtained between the diagnostic tests. This reflects poor agreement between these two diagnostic tests for identification of *Cryptosporidium* spp. This value obtained could be explained by the fact that Nested PCR analysis is a more sensitive technique for the identification of *Cryptosporidium* spp. eggs than microscopic analysis. This finding was also highlighted by Chalmers and collaborators in the UK [15] and Hailu and collaborators [18] in Ethiopia.

The study showed a correlation between CD4 count and the presence of *Cryptosporidium* spp. ($\chi^2 = 29.968$; p-value = 0.0001; r = −0.2438). This could be explained by the fact that a decrease in CD4 count in the organism predisposes to the occurrence of opportunistic diseases such as *Cryptosporidium* in people living with HIV. These results are similar to those of Bissong and collaborators [19] who showed a correlation between opportunistic parasites and CD4 count, specifically for *Cryptosporidium parvum* in people living with HIV/AIDS at the Bamenda Regional Hospital in Cameroon.

5. Conclusion

This study showed a correlation between the presence of *Cryptosporidium* spp. and CD4 count in both diagnostic techniques (Microscopic analysis (OR = 0.887; p-value = 0.001) and Nested PCR (OR = 0.896; p-value = 0.001)). Nested PCR has proven to be more sensitive than microscopic techniques. Better management of PLWH relies on a good diagnosis of opportunistic diseases such as Cryptosporidiosis. It is imperative to use PCR methods to better diagnose Cryptosporidiosis in order to better manage people living with HIV/AIDS.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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