PHYNOTYPIC AND MOLECULAR DETECTION OF CRYPTOSPORIDIUM ISOLATES FROM DIARRHOETIC PATIENTS ATTENDING FEDERAL MEDICAL CENTRE YENAGOA

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ABSTRACT
Background: *Cryptosporidium* is a genus of apicomplex an parasitic alveolates, one of the most common causes of infectious diarrhoea in humans.
Aim:to determine the prevalence of *Cryptosporidium* species from diarrhoeic stool samples collected from human immunodeficiency virus (HIV/AIDS) children and the elderly attending the Federal Medical Centre Yenagoa.
Methodology: A total of two hundred and six (206) human faecal samples were collected, of which one hundred and six (106) from males and one hundred (100) from females, out of the 206, 119 were from HIV/AIDS. Samples analyzed for the presence of *Cryptosporidium* species using Modified Ziehl Neelsen staining (MZN) technique and Polymerase Chain Reaction (PCR).
Results: Out of the 206 specimens examined by Modified Ziehl Neelsen Stain 20(9.7%) were positive for *Cryptosporidium* species; from these 20 positive samples, 12(5.8%) were from HIV positive patients, 9(4.4%) were detected in children of the age-group 1-10 years, 4(1.9%) in 11-20 years, and 7(3.5%) in 20 years and above.
Out of 158 watery diarrhoeic and 48 semi-formed non-diarrhoeic samples, 16(7.8%) and 4(1.9%) were positive for Cryptosporidium species respectively. Three (3) out of the 20 positive samples by Modified Ziehl Neelsen stain subjected to molecular analysis using PCR technique were positive to *Cryptosporidium* species. Out of these 3 positive samples, 2(0.9%) were from children of age-group 1-10 years, and 1(0.48%) from adults above 20 years. Out of these same 3-positive to molecular analysis 2(0.97%) were females and 1(0.49%) was male.
Conclusion: Females had higher rate of Cryptosporidiosis than their male counterparts.
Keywords: Cryptosporidiosis, ZN technique, Diarrhoeic Stool, HIV patients, PCR

INTRODUCTION
Cryptosporidiosis is a disease caused by a protozoaparasite named Cryptosporidium, in Apicomplexa phylum. This disease has affinity for the distal small intestine and respiratory tract in both individuals with a normal and abnormal functioning immune system such as people living with HIV/AIDS leading to diarrhea accompanied with an unexplained cough (Sponseller *et al.,*2014). Diarrhoea is considered as one of the universal health challenges which could result into mortality and morbidity especially among the immunocompromised patients. The global record shows over 50 million deaths in all ages due to diarrhea and ranks third among diseases responsible for human mortality according to World Health Organization (Ayinmode, 2014; WHO 1991). Consumption of contaminated food or water, excessive alcohol can result into acute diarrhea in individuals; such attacks could disappear within a day or two even if it is not treated.

A serious intestinal disorder could be as a result of chronic diarrhea; while food poisoning and microorganisms such as *Staphylococcus, Clostridium, Salmonella, E. coli* O157*, Campylobacter, Cryptosporidium*, and Norwalk virus can cause acute diarrhea. Infective diarrhoea could be acquired from the droplets of adenoviruses or echoviruses infections. (Banwat *et al.*,2003; CDC, 2009). Ali *et al.* (2014) stated that since 19th century there has been an existence of protozoan causing diseases in human, though initially they were known to be attached to rodents, insects, birds and human primates. Previously the most common causative agents of diarrhea among protozoa include *Giardia lamblia, Entamoeba histolytica, Balantidium coli* and *Coccidian* agents like *Isospora belli, Cyclospora cayetenensis, Microsporidia* and *Cryptosporidium parvum* (Ali *et al.,* 2014). In Africa continent, about 70% of Human Immunodeficiency Virus (HIV) infected patients are affected with chronic diarrhea and immune suppressed individuals can come down with severe symptoms that could be fatal; faecally contaminated water can speed its spread through the fecal-oral route (CDC. 2016; Wang *et al*., 2018). *Cryptosporidium* species are parasites that are mostly isolated from the faecal specimens of patients living with HIV presenting with diarrhea experiencing dehydration. Fluid rehydration, electrolytes adjustment is effective management (Ahmadpour *et al*., 2020).Cryptosporidium species was identified in 1976 as one of the most common waterborne diseases and its cyst (oocysts) can be transmitted in the environment when ingested, it is established in the small intestine resulting into intestinal epithelial tissues disease. These protozoa have one-week incubation period and present with clinical signs of profuse, offensive, watery diarrhoea, coupled with abdominal pain, vomiting and fever. The prevention and control of this parasite has become a public health concern. Prevention methods include the installation of more advanced filtration systems, constant testing of water supply, and educating populations about the health implications. Clasen *et al.* (2007) stated that cryptosporidiosis could be difficult to treat.

Cryptosporidiosis was categorized among the Neglected Tropical Diseases (NTD) in 2004 by World Health Organization (WHO) especially in developing countries due to poverty and lack of medical equipment for diagnosis and adequate treatment. Since this declaration Medical Laboratory Scientists in Nigeria have not been checking for this parasite from diarrheic stool in the laboratory. Nwabuisi (2001) in his study stated that few studies have been carried out on this Cryptosporidium species, a parasite common among the immune compromised individuals due to lack of effective treatment and control which eventually leads to their vulnerability. Identification and analysis of these parasites in children and Human Immunodeficiency Virus patients residing in South-Western and Mid-Western States by molecular technology using Polymerase Chain Reaction (PCR) Technique could be of great advantageous awareness according to some authors; Akinbo *et al. (*2010); Molloy *et al.* 2011; Ayinmode *et al.* 2011 and 2014, Maikai *et al. (*2012); Akinbo *et al. (*2013); Ayinmode *et al.(*2014). Studies conducted by many authors Bialeka *et al*., 2012; Paul *et al*., 2017 and Bouzid *et al*., 2013 stated that PCR has the ability to detect the genotypes of infection diseases and recommended PCR as a useful method in the diagnosis of Cryptosporidium infections due to its precision and higher sensitivity than the staining method. However, no information was available on the species circulating in humans in South-South Nigeria. This study was carried out to evaluate the occurrence, diversity and transmission dynamics of *Cryptosporidium* species in patients diagnosed of diarrhoea or gastroenteritis and immmuno-compromised patients accessing services at the Federal Medical Centre Yenagoa, Bayelsa State. The study aimed at detection and confirmation of *Cryptosporidium* species by modified Ziehl Nelseen Stain (MZN) and Polymerase Chain Reaction (PCR).

MATERIALS AND METHODS

Study Area

This study was conducted at the Federal Medical Centre (FMCY), Yenagoa; FMCY is situated in the heart of Yenagoa the state capital, with over 2000 staff and 425 bed-compliment; the Molecular Research Laboratory at Niger- Delta University, Amassoma all in Bayelsa state, Southern part of Nigeria, Bayelsa has a total population of about 1,704,515 and covers 21,100 Square Kilometers with capital at Yenagoa. Yenagoa has an area of 706 km² and a population of 266,008 at the 2006 census. Bayelsa State is geographically located within Latitude 4°15′ North, 5°23′ South and longitude 5°22′ West and 6°45′ East. It shares boundaries with Delta State on the North, Rivers State on the East and the Atlantic Ocean on the West and South (Bayelsa State Government, 2022).

Ethical Considerations

Ethical clearance was obtained from the Federal Medical Centre Yenagoa, ethical and scientific committee; and data from study patients was treated with utmost confidentiality.

The study involved all age groups. For those under the age of eighteen, unconscious and mentally sick, their parents, guidance gave consent.

Study Population

Two hundred and six (206) Patients examined include: HIV positive; Immuno – competent; Children below 10 years all presenting with diarrhea.

Sample Size Determination/Source

Two hundred and six patients who visited the Federal Medical Centre Yenagoa during the period of this study, presenting with diarrhoea were recruited and sample size was determined using the formula for calculating sample size below.

$n = \frac{z^2 \times pq}{d^2}$

Where n = Number of samples

z = Standard normal deviation at 95% confidence interval = 1.96

p = Proportion or otherwise previous prevalence of Cryptosporidiosis in a study carried out in, Nigerian to be studied in the study area.

q = 1 - P

d = the desired level of Precision (Precision limits) (0.05) (Daniel, 1999).

$$\text{Therefore } n = \frac{(1.96)2 \times 0.16 \times (1 - 0.16)}{(0.05)^2} = \frac{3.8416 \times 0.16 \times 0.84}{(0.05)^2}$$

$$= \frac{0.614656 \times 0.84}{(0.05)^2} = \frac{0.51631104}{0.0025}$$

$$= 206.524 = 206 \text{ samples}$$

Sample Collection and Processing

Two hundred and six (206) Faecal Samples were collected between February – December 2019 and January -February 2021 into sterile wide mouth plastic containers and examined immediately.

Parasitological Techniques

Macroscopy

All stool samples collected were examined macroscopically for colour, consistency (formed, Semi-formed, unformed or watery); presence of blood, mucus and adult parasites.

Formol Ethyl-Acetate Sedimentation Technique

The samples collected were analyzed by homogenizing a 0.5 g portion of faecal specimen in 3 ml of 10% formol-saline in a test-tube. The faecal suspensions were filtered through a 2 mm sieve. Four millilitres of ethyl-acetate wereadded to the faecal suspension, shaken vigorously for 1 min and then centrifuged at 2600 g for 10 min. The faecal debris at the interface was loosened with an applicator stick and the supernatant decanted.

The tube was tapped gently to loosen and re-suspended the faecal deposit at the bottom. The deposit was put on a clean grease-free glass slide, covered with a cover slip and examined wet using x10 and x40 objective lens of the microscope. The deposit was allowed to dry and then stained by modified Ziehl Neelsen staining technique (WHO, 2004; Cheesbrough, 2005); this was viewed under the microscope using x100 objective lens for the presence of Oocysts *of Cryptosporidium (*Cheesbrough, 2005*).*

Molecular Analysis

All diarrheic faecal samples collected from children, immuno-competent and HIV-infected persons were screened for c*ryptosporidium* using polymerase chain reaction (PCR) technique targeting the 18S SSU rRNA and *gp60* genes.

Extraction of total genomic DNA from Faecal Specimens Using Fastdna® Zymo-Spin™ Kit (Chemical Method) ProtocoL

For optimal performance, beta-mercaptoethanol (user supplied) was added to the Genomic Lysis Buffered0.5% (v/v) i.e., 0.5ml per 100 ml.

About 150 mg of fecal sample was added to a ZR BashingBead™ Lysis Tube (0.1 &0.5 mm). 750 µl BashingBead™ Buffer added to the tube; secured in a bead beater fitted with a 2 ml tube holder assembly and processed at maximum speed for ≥ 5 minutes. The ZR

Bashing Bead Lysis Tube (0.1 & 0.5 mm) was centrifuged in a microcentrifuge at ≥ 10,000 x g for 1 minute. Up to 400 µl of the supernatant was transferred to a Zymo-Spin™ III-F Filter in a Collection Tube and Centrifuged at 8,000 x g for 1 minute. Then, 6.800 µl of the mixture was transferred to a Zymo-Spin™ IICR Column in a Collection. Tube and centrifuged at 10,000 x g for 1 minute. The flow Tube was discarded and the procedure repeated. About 0.2ml DNA Pre-Wash Buffer was added to the Zymo-Spin™ IICR Column in a new Collection Tube and centrifuged at 10,000 x g for 1 minute. 0,5 ml g-DNA Wash Buffer added to the Zymo-Spin™ IICR Column and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin™ IICR Column was transferred to a clean 1.5 ml microcentrifuge tube and added 100 µl (50 µl minimum) DNA Elution Buffer directly to the column matrix; centrifuged at 10,000 x g for 30 seconds to elute the DNA; placed a Zymo-Spin™ III-HRC Filter in a clean Collection Tube and added 600 µl Prep Solution; this was centrifuged at 8,000 x g for 3 minutes. The eluted DNA was transferred to a prepared Zymo-Spin™ III-HRC Filter in a clean 1.5 ml microcentrifuge tube and centrifuge at exactly 16,000 x g for 3 minutes. The filtered DNA is now suitable for PCR

DNA Quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer.

The equipment was initialized with 2ul of sterile distilled water and blanked using normal saline. Two microlitre of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button.

Cryptosporidium Gene Amplification

The Cryptosporidium gene of the isolates were amplified using Nested PCR Method. Isolates were amplified using CRY18S-OF 5'-GGTGACTCATAATAACTTTACGG-3' and CRY18S-OR: 5'-ACGCTATTGGAGCTGGAATTAC-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 30 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.5uM and 2uL of extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 59ºC for 30 seconds; extension, 68ºC for 30 seconds for 30 cycles and final extension, 68ºC for 5 minutes as the primary amplification.

The secondary amplification using CRY18S-1F 5'-TAAACGCTAGGGTATTGGCC-3' andCRY18S-1R: 5'-CAGACTTGCCCTCCAATTGATA-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 30 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.5uM and 1uL of the amplicon of the primary amplification as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 59ºC for 30 seconds; extension, 68ºC for 30 seconds for 30 cycles and final extension, 68ºC for 5 minutes.

Agarose Gel Electrophoresis

The product resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a UV trans-illuminator photo imager (Edman and Kwon-Chung, 1990).

Data Analysis

Data was analyzed using software statistical package for social sciences (SPSS) version 21.0 the results were expressed as percentage. Difference in percentage differences were assessed by chi square (X^2). Tests with a probability value of $P< 0.05$ were considered statistically significant (Hays, 1973).

RESULTS

Distribution of Participants by Age and Gender for Prevalence of *Cryptosporidium* Species Using Modified Ziehl Neelsen Stain. Two hundred and six (206{106 males, 100 females) specimens analyzed by modified Ziehl Neelsen stain, 14(14%) out of 100 females and 6(5.7%) out of 106 males were positive to *Cryptosporidium* species. The age range of participants from 1-10; 11-20 and above 20 years was shown in table 1. The highest prevalence of *Cryptosporidiosis* was recorded in the age group 1 – 10 years while the lowest prevalence was in the age group > 20 years. A total of 20(9.7%) were positive for *Cryptosporidium* while 186(90.3%) was negative, out of the 20 positive cases of cryptosporidiosis, 9(4.4%) were detected in children in the age-group 1-10 years. However, cases of cryptosporidiosis detected in age-groups 11-20 years were 4(1.9%). Patients whose age were > 20 years had infection rate of 7(3.5%) (Table 1).

Distribution of *Cryptosporidium* Species by Age and Gender Using Polymerase Chain Reaction Technique is described in this table. Out of twenty positive(20 {14 females, 6 males}) specimens to *Cryptosporidium* by Ziehl Neelsen staining method subjected to analysis using PCR technique, 3(1.46%) were positive; two 2(0.97%) females and 1(0.49%) male were positive for *Cryptosporidium* species.Both techniques are operative depending on the technical expertise of the operators. Out of the 3 positive samples of cryptosporidiosis, 2(0.97%) were detected amongst patients of age-group 1-10 years, and 1(0.48%) in >20 years (Table 2).

Frequency Distribution of *Cryptosporidium* species in HIV participants using Modified Ziehl Nelseen stains and polymerase chain reaction technique is described here in table 3.Out of 206 participants,119(78,37.9% females; (19.9%) males were HIV positive patients. Out of the 119 HIV positive samples, 12 were positive to cryptosporidium (4.35%) were females while (1.45%) were male, while 107 were negative to cryptosporidium. The number of patients between the age of (1-10) years are 80, (11-20) years are 60 while the age group >20 years are 66. The age range of participants from 1-10 years; 4(1.9%) was positive by MZN while 2(0.97%) was positive by PCR. 11-20 years; 3(1.5%) was positive by MZN and for 20 years and above; 5(2.4%) was positive by MZN while 1(0.48%) was positive by PCR (Table 3).

Table 1: Distribution of Participants by Age and Gender for Prevalence of *Cryptosporidium* Species Using Modified Ziehl Neelsen Stain

Age	Sex		No of Participants	*Cryptosporidium* species (%)	
(Years)	Male	Female	MZN	Positive	Negative
1 -10	42(20.4)	38(18.4)	80(38.8)	9(4.4%)	71
11- 20	28(13.6)	32(15.5)	60(29.1)	4(1.9%)	56
>20	36(17.5)	30(14.6)	66(32.0)	7 (3.5%)	59
Total	106(51.5)	100(48.5)	206	20(9.7%)	186(90.3%)

Table 2: Distribution of Cryptosporidium Species by Age and Gender Using Polymerase Chain Reaction Technique

Age	Sex		No of Participants	C*ryptosporidium* species (%)	
(Years)	Male	Female	PCR	Positive	Negative
1 -10	2(10.0)	7(35.0)	9(4.4%)	2(0.97%)	7
11- 20	2(10.0)	2(10.0)	4(1.9%)	0	4
>20	2(10.0)	5(25.0)	7 (3.5%)	1(0.48%)	6
Total	6(30%)	14(70%)	20(9.7%)	3(1.46%)	17(8.3)

Table 3: Frequency Distribution of C*ryptosporidium* species in HIV participants by Modified Ziehl Neelsen stains and Polymerase Chain Reaction techniques

Age	Sex		No of Participants		C*ryptosporidium* species (%)	
(Years)	Male	Female	ve	-ve	MZN	PCR
1 -10	18(8.7)	29(14.1)	47(22.8)	33(16.0)	4(1.9)	2(0.97%)
11- 20	9(4.4)	17(8.3)	26(12.7)	34(16.5)	3(1.5)	0
>20	19(9.2)	27(13.2)	46(22 .3)	20(9.7)	5 (2.4)	1(0.48)
Total	46(22.3)	73(35.4)	119(57.7)	87(42.2)	12(5.8)	3(1.46)

Distribution of *Cryptosporidium* Species by Age and Gender Using Polymerase Chain Reaction Technique is explained in this table**.** This table shows that of the 20 (14 females; 6males) specimens that were positive to *Cryptosporidium* by Ziehl Neelsen staining method subjected to analysis using PCR technique, 3(1.46%) positive to cryptosporidiosis include 2(0.97%) females and 1(0.49%) male; of the 3 positive samples of cryptosporidiosis, 2(0.97%) were detected amongst patients of age-group 1-10 years, and 1(0.48%) in >20 year (Table 4).

Table 4: Distribution of *Cryptosporidium* species by age and gender for apparently healthy patients presenting with Diarrhoea using Modified Ziehl Nelseen stains and polymerase chain reaction technique

Age	Sex		No of Participants	*Cryptosporidium* species	
(Years)	Male	Female		MZN	PCR
1 -10	24	9	33(16.0)	5 (2.4)	0
11- 20	19	15	34(16.5)	1 (0.48)	0
>20	17	3	20(9.7)	2 (0.97)	0
Total	60(29.1)	27(13.1)	87(42.2)	8(3.9)	

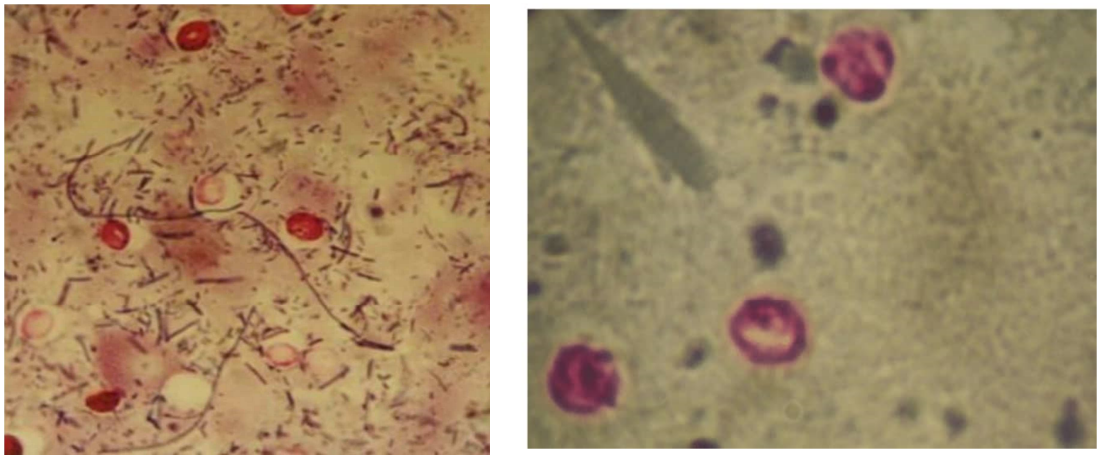

Fig. 2&3: Pictorial Representation of Modified Ziehl Neelsen Staining Showing *Cryptosporidium* species

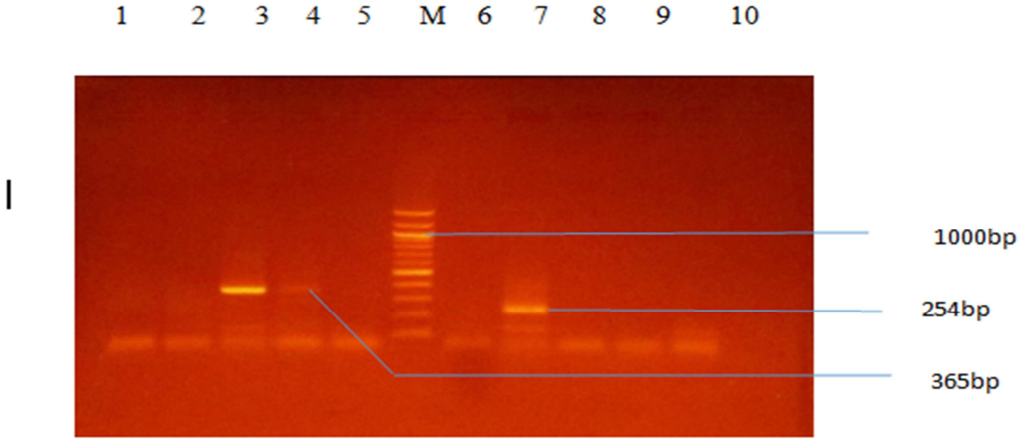

Plate 1: Showing Agarose gel electrophoresis of *Cryptosporidium* gene diversity of some selected *Cryptosporidium* isolates from human faeces. Lane 3,4 represents *Cryptosporidium* gene band (365bp) and 7 represents *Cryptosporidium* gene band (254bp).
Lane M represents the 100bp Molecular ladder of1500bp.

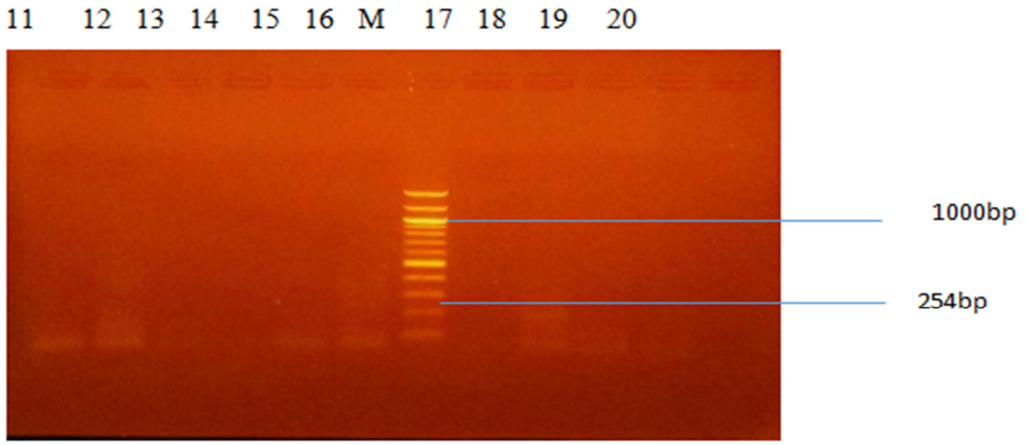

Plate 2: Agarose gel electrophoresis of *Cryptosporidium* gene diversity of some selected. *Cryptosporidium* isolates from human faeces.Lane11–12 represents negative amplification of different isolates of the *Cryptosporidium* parvum.The254bpon the Molecular ladder represents the supposed base pair of one of the diversities of *Cryptosporidium* gene. Lane M represents the100b

DISCUSSION

This study showed that the prevalence of *Cryptosporidium* infection in Diarrhoeic stools collected from Federal Medical Centre Yenagoa was 9.7% by Modified Ziehl Neelsen stain due to compromised immune system vulnerability among the immuno-compromised persons examined. This finding agrees with the work of Xiao, (2014) who reported high occurrence of *Cryptosporidium* infections(12.2%)in immuno-compromised patients. In addition, the findings are in agreement, though higher prevalence of 22.2%, with the work done by Akinbo *et al.,* (2013), amongst mid-western Nigerians and stated that high occurrence of *Cryptosporidium* infections is common amongst people that are HIV-positive due to their compromised immunity. Molecular confirmation of the presence of Cryptosporidium by PCR technique revealed lower rate of 1.46% in comparison with traditional (modified Ziehl Neelsen staining technique) (9.7%) at $p<0.05$; this is in agreement with Morgan *et al.* (2014) who reported 36% and 39% respectively. Molecular and traditional findings in this study could be attributed to effectiveness in the skill acquisition in terms of focusing, reporting and interpretation of result.

Of one hundred and six(206)study subjects, one hundred and nineteen {119, 41(19.9% male; 78(37.9% female}were collected from HIV patients; the findings revealed 60% and 100% positivity by MZN and PCR respectively. This is consistent with previous reports that *Cryptosporidium* causes diseases mostly in the immune-compromised individuals by

Ayinmode *et al. (*2011). Observations also showed that patients in the age group 1-10 years had a higher *Cryptosporidium* infection than 11 – 20 years and above 20. The higher prevalence range of *Cryptosporidium* in the age group 1-10 year is in agreement with previous reports of Ayinmode *et al*. (2011) who reported similar results in their study and stated that *Cryptosporidium* causes diseases mostly in the immune-compromised individuals such as children and the elderly. Similarly, the lower prevalence among the age groups of 11 – 20 years and > 20 years agrees with the report of Upton, (2008), that these age groups are more immune competent, hence diarrhoea caused by *Cryptosporidium* species could be self-limiting. The high prevalence of the parasite in children as seen in this study could be attributed to low immunity, poor hygiene and the misuse of antibiotic chemotherapy without prescription and in unrestricted doses which is widely obtainable in Nigeria. The analysis of the prevalence of *Cryptosporidium* infections among age groups using Chi-square (X^2) showed a statistically significant ($p < 0.05$) association between age and rates of infection. This data also revealed a significant difference ($p<0.05$) in the prevalence of *Cryptosporidium* infections between females their male counterparts with females preponderance over males; Ayinmode *et al*. (2011) gave a similar report and stated that this could be as a result of wider opening of the females genital part which is vulnerable to infection in comparison with males.

The finding is in agreement with the report of Das *et al.,* (2016), who in a similar study reported a higher (2.0%) and 4.5% by Sonia *et al*., (2019) sensitivity of PCR in the work done in Qatar among the immigrant workers. Results of Bialeka *et al*., (2012) also revealed that sensitivity of immunologic methods is sufficient for the *Cryptosporidium* screening in feces samples and showed that PCR did not lead to any significant increase in sensitivity. However, Zeigler *et al*. (2017) announced the increase in sensitivity by PCR in *Cryptosporidium* diagnosis.

CONCLUSION

The prevalence rate of *Cryptosporidium* Species in diarrhoeic stools in this study showed to be higher in immune-compromised individuals than in immune-competent, Females demonstrated to have higher prevalence rate of Cryptosporidiosis infection than their male counterparts.

This study revealed that molecular method employing PCR is more specific and sensitive than the staining (Modified Ziehl Neelsen) techniques. Thus, there is need to carryout PCR on stain positive individuals before administrating therapy as PCR is more specific to Cryptosporidiosis.

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