International Journal of Advanced Research, Volume 6, Issue 1, 2023
Article DOI: https://doi.org/10.37284/ijar.6.1.1059

International Journal of Advanced Research
ijar.eanso.org
Volume 6, Issue 1, 2023
Print ISSN: 2707-7802 | Online ISSN: 2707-7810
Title DOI: https://doi.org/10.37284/2707-7810

EAST AFRICAN NATURE & SCIENCE ORGANIZATION

Original Article

Molecular Studies of Polyparasitism among Women in Oju and Obi LGAs of Benue State

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Article DOI: https://doi.org/10.37284/ijar.6.1.1059

Publication Date:

23 January 2023

Keywords:

Polyparasitism, Molecular, Infection, Women, Oju, Obi.

ABSTRACT

The molecular studies of polyparasitism among women in Obi and Oju LGAs of Benue State was investigated. Stool, blood, and urine samples were collected from women in the study location and processed molecularly using PCR technology. Zymo research quick-DNA universal kit (spin-column), and QIAamp DNA stool mini kit were used in the extraction of genomic DNA from stool, urine, and blood samples. The extraction and purification process was based on spin column chromatography. PCR amplification of the genes (*Pf*18S, *Sh*73, *Sh*77, *Eh*1, *Tv18S* and *Cox* 1), was carried out. The conditions and volume of the reaction was determined by the master mix used. A check gel was performed on 2% TAE agarose gel to confirm the amplified bands with 100 base pair DNA Ladder (TRANSGEN BIOTECH, CAT NO. BM 311). This ready-to-use product was premixed with 1× DNA loading buffer (0.02% bromo-phenol blue and 5% glycerol). The bands were visualized under UV light. The purity of the DNA extracted from the isolates as reflected in the OD260:OD280 ratios are within the 1.8 and 2.0 acceptable values. The parasites encountered were *Plasmodium falciparum*, *Schistosoma haematobium*, *Trichomonas vaginalis*, *Ascaris lumbricoides* and *Entameoba histolytica*. From the genus specific primers from PCR, 158bp fragment was obtained which correlates with *Ascaris lumbricoides* (Cox 1), 112bp and 178bp were also observed correlating to the presence of *Trichomonas vaginalis* and *Entamoeba histolytica* respectively. Also, 201bp fragment were observed correlating with *Plasmodium falciparum*, and 262bp and 350bp were determined which correlates with the presence of *Schistosoma haematobium*. It was concluded that the women of Obi and Oju suffer from polyparasitism as the amplification of genes confirms the presence of *Plasmodium falciparum*, *Schistosoma haematobium*, *Trichomonas vaginalis*, *Ascaris lumbricoides* and *Entameoba histolytica*. There is need for enlightenment campaigns against parasitic infections and polyparasitism in Oju and Obi and efforts of the government is needed to ensure provision of efficient molecular facilities for accurate diagnosis of infection.

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APA CITATION
Uweh, P. O., Omudu, E. A. & Atu, B. O. (2023). Molecular Studies of Polyparasitism among Women in Oju and Obi LGAs of Benue State. *International Journal of Advanced Research, 6*(1), 9-22. https://doi.org/10.37284/ijar.6.1.1059

CHICAGO CITATION
Uweh, Philomena Okwa, Edward Agbo Omudu and Bernard Ortwer Atu. 2023. "Molecular Studies of Polyparasitism among Women in Oju and Obi LGAs of Benue State." *International Journal of Advanced Research* 6 (1), 9-22. https://doi.org/10.37284/ijar.6.1.1059.

HARVARD CITATION
Uweh, P. O., Omudu, E. A. & Atu, B. O. (2023) "Molecular Studies of Polyparasitism among Women in Oju and Obi LGAs of Benue State.", *International Journal of Advanced Research*, 6(1), pp. 9-22. doi: 10.37284/ijar.6.1.1059.

IEEE CITATION
P. O. Uweh, E. A. Omudu & B. O. Atu "Molecular Studies of Polyparasitism among Women in Oju and Obi LGAs of Benue State.", *IJAR*, vol. 6, no. 1, pp. 9-22, Jan. 2023.

MLA CITATION
Uweh, Philomena Okwa, Edward Agbo Omudu & Bernard Ortwer Atu. "Molecular Studies of Polyparasitism among Women in Oju and Obi LGAs of Benue State.". *International Journal of Advanced Research*, Vol. 6, no. 1, Jan. 2023, pp. 9-22, doi:10.37284/ijar.6.1.1059.

INTRODUCTION

Intestinal parasitic infections remain one of the most pressing public health problems where one third of the world population is estimated to be infected. The most affected people are children, women, and the poor people living in the tropics and subtropics where there is poor personal hygiene and environmental sanitation, overcrowding, and limited access to clean water (De Silva *et al.,* 2003; Prajapati *et al.,* 2012). A concurrent infection with more than one parasitic species, a term referred to as polyparasitism is also commonly observed among the same population (Pullan & Brooker, 2008).

Studies across multiple epidemiological settings have shown that polyparasitism is the norm rather than the exception and occurs at different frequencies than would be expected under assumptions of independence (Fleming *et al.,* 2006; Howard *et al.,* 2002; Tchuem *et al.,* 2003). Interactions between parasites in humans can be synergistic or antagonistic. For example, studies have demonstrated a positive association between intensity and concurrent infection of helminth species, suggesting that individuals harbouring multiple helminth species also harbour the most intense infections (Fleming *et al.,* 2006; Howard *et al.,* 2002; Tchuem *et al.,* 2003). It is conceivable therefore that polyparasitism may have a greater impact on morbidity than single species infections since morbidity is typically related to infection intensity for most parasite species. Multiple species infections may also increase susceptibility to other infections (Mwangi *et al.,* 2006; Druilhe *et al.,* 2005).

In many laboratories of developing countries, microscopic examination of stools for detection of cysts, oocysts, and trophozoites remains the diagnostic method of choice, due to the low cost of reagents. However, optical microscopy requires considerable technical expertise, is time consuming and, in most cases, does not allow determination of the parasite at the species/genotype level. Molecular methods are more expensive and require specific equipment, but have higher specificity and sensitivity compared to microscopy, and are therefore increasingly used to detect and characterize gastrointestinal parasites. Furthermore, the large genetic variability observed among isolates of intestinal parasite species has led to the description of many genotypes and subtypes that have been found to differ in host range, zoonotic potential, and clinical significance therefore strongly impacting on our knowledge of the epidemiology of parasitic infection (David *et al.,* 2015).

In addition to the above, it has been observed that the sensitivity of microscopy for different parasites does not exceed 60% even if concentration methods and skilful technical assistance are available. Nowadays a number of PCR-based assays have been developed for the identification of

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gastrointestinal parasites directly from human faeces. These assays have been proven to be considerably more sensitive than microscopy, highly specific, and in contrast to microscopy are able to differentiate between the species and the sub-type level (Koffi *et al.*, 2014). Also, several workers have reported the presence of Pf188, sh73, sh77 and Eh1 genes in the identification of polyparasitism in stool, urine, and blood respectively (Faten *et al.* 2017; Mehru *et al.* 2021). This calls for investigation into the molecular diagnosis of polyparasitism in women in Nigeria, Benue State and Oju and Obi Local Government Areas in particular. This could then contribute to appropriate policies aimed at controlling polyparasitism.

MATERIAL AND METHODS

Study Area

The study was carried out in Oju and Obi local government areas in the southern part of Benue State. Oju is located between latitude $6^{o}6^{1}$N and $6^{o}9^{1}$E and Longitude $8^{o}10^{1}$E and $8^{o}25^{1}$E in the south eastern part of Benue State. Oju local government area covers an area of 1,283 km². It is bounded in the south by Cross River State, in the east by Konshisha local government area, in the north by Obi and Gwer East local government areas, in the west by Ado local government area, and in the south-west by Ebonyi State. The widely disputed result of the 2006 national population and housing census put the population of Obi local government area of Benue State at 98,707, with 49,143 males and 49,564 females (Department of Geography, Benue State University, Makurdi, 2015). Obi local government area covers an area of 423 km². It is bounded in the north-east by Gwer East local government area, in the south by Oju local government area, in the west by Ado local government area, and in the north by Otukpo local government area.

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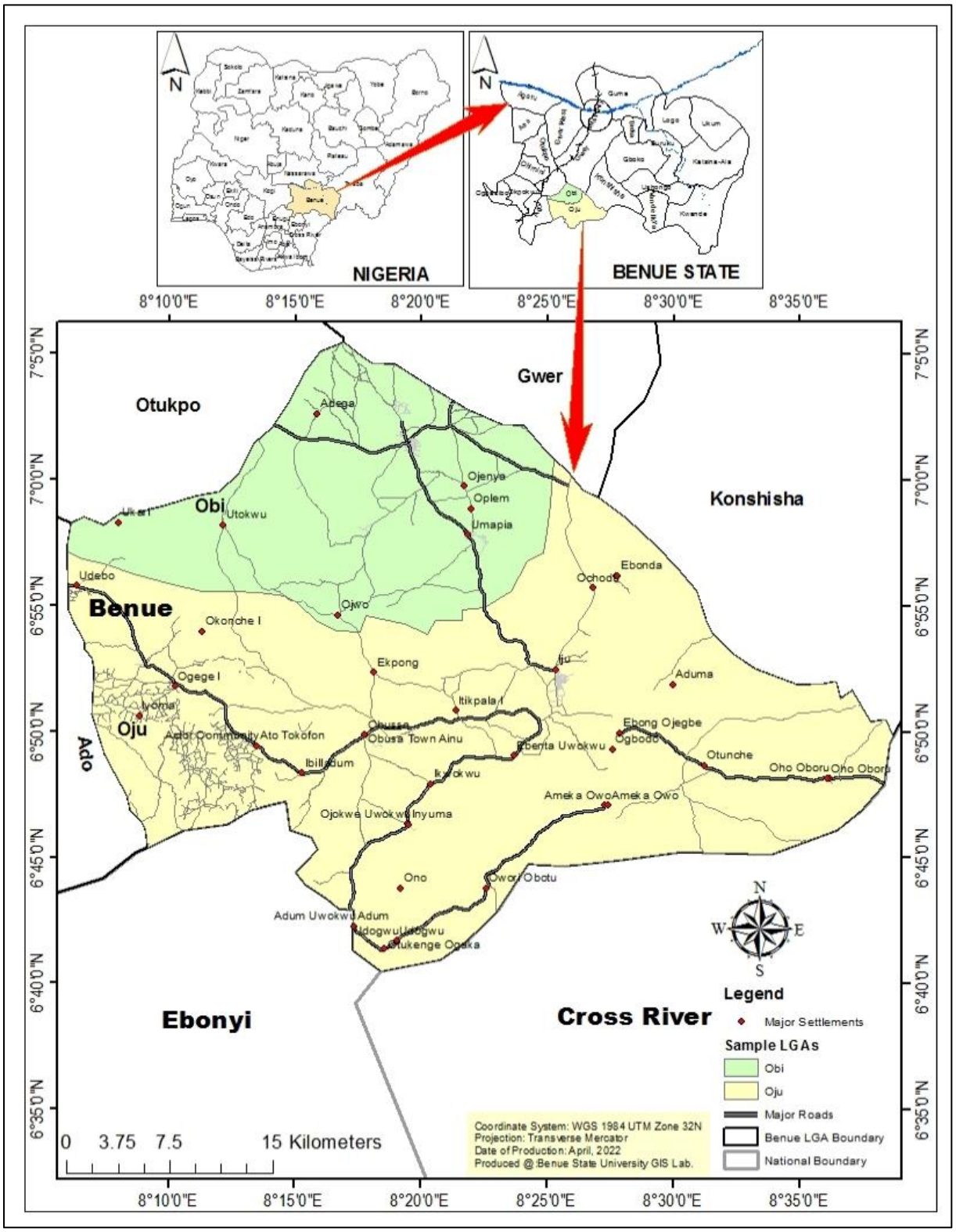
Figure 1: Map of Benue State showing Location of Oju and Obi Local Government Area



Source: Department of Geography, Benue State University, Makurdi

Article DOI: https://doi.org/10.37284/ijar.6.1.1059

Experimental Design

A Cross sectional study design was adopted for the study. All women in the areas visited were given equal opportunity to participate in the study. Women were briefed on the health implication of being infected with parasites, the dangers of parasitism, and the relevance of the study to each participant. Participation was made voluntary. Volunteers were made to fill a written consent form indicating their willingness to participate in the study.

Ethical Consideration

Ethical approval was obtained from the Benue State Ministry of Health (Ref Number: MOH/STA/204/VOL1/182) prior to the commencement of the study. This was in accordance with the requirements for conducting research on human subjects. Also, informed consent forms were distributed to the women and were verbally translated to the women in their local languages. Only women who consented to the study by signing the consent forms were recruited for the study.

Sample Collection

Specimen bottles were distributed to the consenting women who participated in the study. They return the containers with urine and stool in them. Blood was however collected intravenously by laboratory technicians from women using appropriate aseptic techniques. Blood samples were examined immediately, while stool and urine samples were taken to the laboratory for microscopic examination.

Demographic information, clinical manifestations of parasites as well as information pertaining to parasitism were collected from the study subjects using well-structured questionnaires so as to enable adequate correlation of results obtained with demographic variables.

Molecular Analysis of Samples

Representative samples were sent to FOWM Biotechnology Limited, Yaba, Lagos and Molecular Laboratory, Covenant University Centre for Research, Innovation and Development (CUCRID) for DNA extraction and amplification (Polymerase Chain Reaction).

Extraction of Genomic DNA

Plasmodium falciparum-infected blood smear positive samples from women in Obi and Oju LGA were collected on Whatman ® FTA Elute cards (GE Lifesciences, MA, USA). Zymo research quick-DNA universal kit (spin-column) was used in the extraction of genomic DNA. Sterilized paper puncher was used to punch out the blood stained on the paper while sterilized needle was employed in cutting the paper into four pieces and suspended in 1500 µl of phosphate buffered saline (isotonic buffer) and pelleted using centrifugation. The pellet was resuspended in 200 µl BioFluid (cell buffer). 20 µl proteinase K was equally added to the suspension. 200 µl of lysis buffer (contains RNase) was added to the sample; this helps with the lyses of the blood cells.

The contents of the tube were mixed thoroughly and incubated at 55 ^{o}C for 10 minutes. 1 volume of genomic binding buffer was then added to the digested sample and mixed thoroughly. This neutralized the sample and caused precipitation of the proteins and genomic DNA. The resulting suspension was spun and the clarified lysate containing the genomic DNA was transferred to Bioteke's spin column in a collection tube and centrifuged at 12,000 rpm for 1 minute. The collection tube was discarded with the flow through. The column was transferred into a new collection tube. 400 µl pre-wash buffer was added to the column in the new collection tube and centrifuged for 1 minute. The collection tube was emptied. 700 µl wash buffer was added to the tube and centrifuged for 1 minute. The flow through in the collected tube was emptied.

Finally, 200 µl of wash buffer was added and centrifuged for 1 minute at 12,000 rpm. The collection tube was discarded with the flow through. The purified product was eluted with the elution buffer in a new micro centrifuge tube and incubated for 5 minutes on an echotherm. A check gel was performed on 1% TAE agarose gel to resolve extracted genomic DNA bands with 100 bp Plus DNA Ladder (TRANSGEN BIOTECH, CAT NO. BM 311). The ladder comprised of twelve linear

Article DOI: https://doi.org/10.37284/ijar.6.1.1059

double-stranded DNA bands. This ready-to-use product was premixed with 1× DNA loading buffer (0.02% bromophenol blue and 5% glycerol). The bands were visualized under ultraviolet (UV) light.

Extraction of DNA from Stool and Urine

DNA was extracted with the QIAamp DNA stool mini kit (Qiagen Benelux, Venlo, The Netherlands) from 1 gram of faeces that was dissolved into 5 mL ASL buffer (Qiagen). 200 ml urine sediment was processed for DNA extraction with the QIAamp DNA mini kit (Qiagen) after centrifugation of 10 mL of urine and three wash/centrifugation steps. These steps were carefully carried out as described by Cnops and Van, (2010) and Cnop *et al*. (2012).

Genomic DNA was extracted directly from all samples using a QIAmp_ DNA Mini Kit (QIAGEN, CA, USA) in accordance with the manufacturer's instructions. 200 mg of stool sample was mixed with 1.4 ml ASL buffer in a microcentrifuge tube and vortexed until the sample was thoroughly homogenized. Following incubation at 70 ºC for 5 minutes, samples were centrifuged at 25,200 rpm. InhibitEx tablets were subsequently added to the samples, followed by vortexing and centrifugation at 25,200 rpm. Next, supernatants were transferred to new tubes, and then proteinase K was added. The tubes were re-incubated at 70 ºC for 10 min. All samples were then mixed with ethanol and transferred to spin columns. After washing, DNA was eluted in 100 μl elution buffer and immediately employed for PCR analysis.

Determination of the Purity of DNA (Nanodrop Spectrophotometry)

The purity of the DNA was determined using the Nanodrop spectrophotometer. About 0.4 µl was added to the spectrophotometer and run. The purity of a DNA was reflected in the OD260: OD 280 ratios and must be between 1.8 and 2.00 (Cnop *et al.*, 2012).

Agarose Gel Electrophoresis

1.0 g of agarose was dissolved in 100 ml of TAE buffer in a 250 ml beaker and heated to boil until the agarose dissolved completely. 4 µl ethidium bromide was added to the dissolved agarose solution as dye and mixed. The gel was allowed to cool to about 50 ºC and poured into the electrophoresis tray with the casting comb at the one end of the tray. The casting comb was removed after the agarose has completely solidified.

Amplification of Resistance Genes

Amplification of the genes (*Pf*18S, *Sh*73, *Sh*77, *Eh*1, *Tv18S* and *Cox* 1) using the polymerase chain reaction was carried out. The conditions and volume of the reaction was determined by the master mix used. Solis BioDyne ready to load master mix (5x) was used for the reaction. PCR was performed in a 20 µl reaction under the following conditions: initial denaturation at 95 °C for 5 min, followed by denaturation at 95 °C for 30 sec, annealing between 54-66 °C for 1 min (the annealing temperature is determined by the primer used), extension at 72 °C for 2 min, and a final extension at 72 °C for 5 min. Total number of cycles was 35 cycles. A check gel was performed on 2% TAE agarose gel to confirm the amplified bands with 100 bp Plus DNA Ladder (TRANSGEN BIOTECH, CAT NO. BM 311). The bands were visualized under ultraviolet (UV) light.

RESULTS

The purity and concentration of the DNA extracted from parasites in stool of women in Obi and Oju LGA is as presented in *Table 1*. The purity of the DNA is reflected in the OD260:OD280 ratio and must be between 1.8 and 2.0. Values less than 1.8 indicates protein contamination while above 2.0 indicates RNA contamination. The absorbance of the extracted DNA ranged between 1.80 and 2.00. The result showed that the extracted DNAs are neither contaminated by RNA nor proteins.

Table 2 shows the purity and concentration of DNA extracted from parasites in urine of women in Obi and Oju LGA. The absorbance of the extracted DNA ranged between 1.85 and 2.00. This showed that the extracted DNAs are neither contaminated by RNA nor proteins. The extracted DNAs are pure with the exception of OJU 73 (1.79). The purity and concentration of DNA extracted from blood of women in Obi and Oju LGA is as presented in *Table 3*. The absorbance of the extracted DNA ranged between 1.82 and 2.00. This showed that the extracted DNAs are neither contaminated by RNA nor proteins.

Article DOI: https://doi.org/10.37284/ijar.6.1.1059

Table 1: Purity and Concentration of Genomic DNA extracted from Stool

Sample ID	Nucleic acid concentration (ng/µl)	Absorbance (260:280)
OBI 31	367.3	1.92
OBI 42	735.6	1.92
OBI 17	657.6	1.89
OJU 53	745.4	1.81
OJU 15	698.5	1.98
OBI 45	745.4	1.93
OJU 70	678.9	1.90
OJU 73	457.8	1.80
OBI 28	456.9	1.99
OBI 51	767.4	1.80
OJU 32	568.8	1.83
OJU 38	758.8	1.87
OBI 22	673.6	1.99
OBI 27	854.0	1.80
OBI 92	606.4	1.82
OJU 200	704.1	1.94
OJU 196	678.6	2.00
OJU 199	545.8	1.91

Table 2: Purity and Concentration of Genomic DNA extracted from Urine

Sample ID	Nucleic acid concentration (ng/µl)	Absorbance (260:280)
OBI 31	654.3	1.89
OBI 42	764.2	1.93
OBI 17	561.9	1.88
OJU 53	697.6	1.89
OJU 15	578.6	1.91
OBI 45	647.0	1.94
OJU 70	893.4	1.87
OJU 73	632.6	1.79
OBI 28	346.8	1.92
OBI 51	566.5	1.85
OJU 32	619.4	1.93
OJU 38	643.8	1.97
OBI 22	587.6	1.99
OBI 27	834.7	1.96
OBI 92	678.5	1.89
OJU 200	778.3	2.00
OJU 196	845.5	1.97
OJU 199	483.8	1.96

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Table 3: Purity and Concentration of Genomic DNA extracted from Blood

Sample ID	Nucleic acid concentration (ng/µl)	Absorbance (260:280)
OBI 31	329.1	1.84
OBI 42	833.5	1.89
OBI 17	621.7	1.98
OJU 53	587.3	1.89
OJU 15	671.6	1.92
OBI 45	661.8	1.90
OJU 70	701.3	1.85
OJU 73	425.6	1.82
OBI 28	393.7	2.00
OBI 51	733.9	1.92
OJU 32	517.8	1.83
OJU 58	658.8	1.87
OBI 22	573.6	1.88
OBI 27	801.7	1.86
OBI 92	606.4	1.93
OJU 200	704.1	1.94
OJU 196	739.6	1.95
OJU 199	583.2	1.91

The presence of plasmodium (*Pf 18S*) in blood samples of women in Obi and Oju was determined (Gel *Plate 1*). The bands as shown in wells 1 and 14 indicated that samples OBI 58 and OJU 200 had plasmodium. Gel *Plate 2* showed the presence of *S. haematobium* (*Sh73*) in sample from OBI 31. The band is as shown in well 10. The presence of *Trichomonas vaginalis* was determined in urine samples of women in Obi and Oju LGA. This is as shown in gel *Plate 3*. The bands as seen in wells 1-8 indicated that samples from OBI 31, OBI 17, OJU 73, OJU 53, OBI 27, OJU 70, OBI 92, OJU 200 had *Trichomonas vaginalis.*

Gel *Plate 4* showed the presence of *Ascaris lumbricoides* from stool samples of women in Obi and Oju LGA (OBI 53, OBI 28, OBI 45, OBI 92, OBI 199, OBI 31, OBI 17, OJU 196, OJU15, OBI 58). The bands as seen in wells 1-8 indicated the presence of *A. lumbricoides* in samples taken from the women. The presence of *E. histolytica* was determined. This is as shown in Gel *Plate 5*. The bands as shown in wells 9 and 10 (OBI 92, OJU 200) indicated the presence of *E. histolytica.* Gel plate 6 does not show the presence of any parasite. The absence of bands showed that no parasite was seen.

Gel *Plates 1-5 V* confirms the presence of *Plasmodium falciparum, Schistosoma haematobium, Trichomonas vaginalis*, *Ascaris lumbricoides* and *Entameoba histolytica.* Gel *Plate 6* showed samples that do not contain parasites. This buttresses the sensitivity of polymerase chain reaction. Samples (OBI 17, OBI 27, OBI 28, OBI 31, OBI 45, OBI 53, OBI 58, OBI 92, OBI 199, OJU 15, OJU 53, OJU 70, OJU 73, OJU 196, OJU 200) confirmed the presence of parasites in women of Obi and Oju LGA. Samples (OBI 17, OBI 31, OBI 192 and OJU 200) confirmed poly-parasitism in women of Obi and Oju LGA.

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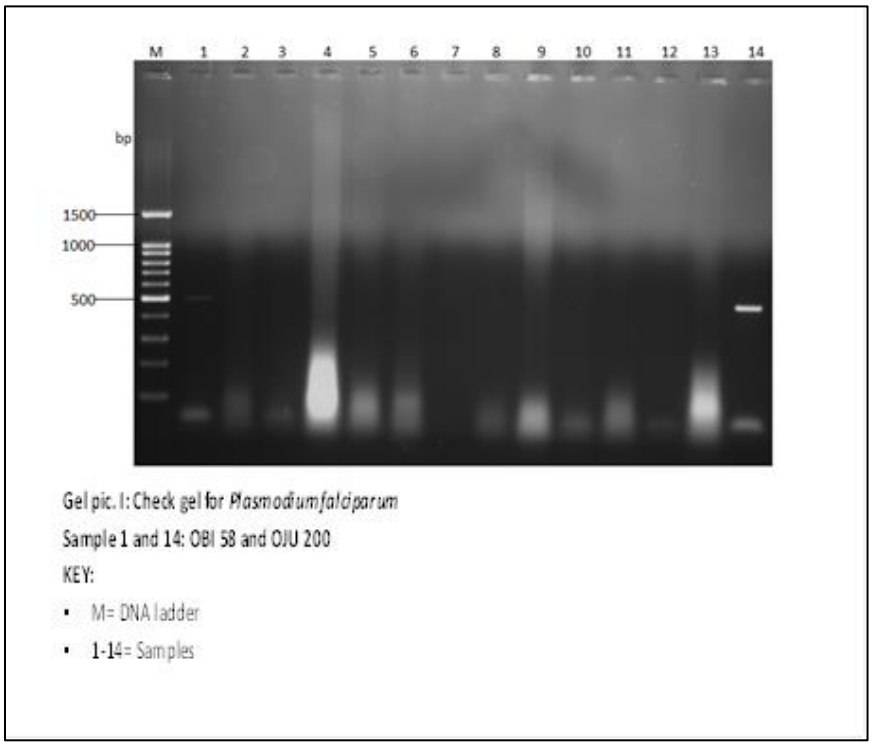
Plate 1: Gel Documentation of Detected *Plasmodium falciparum*



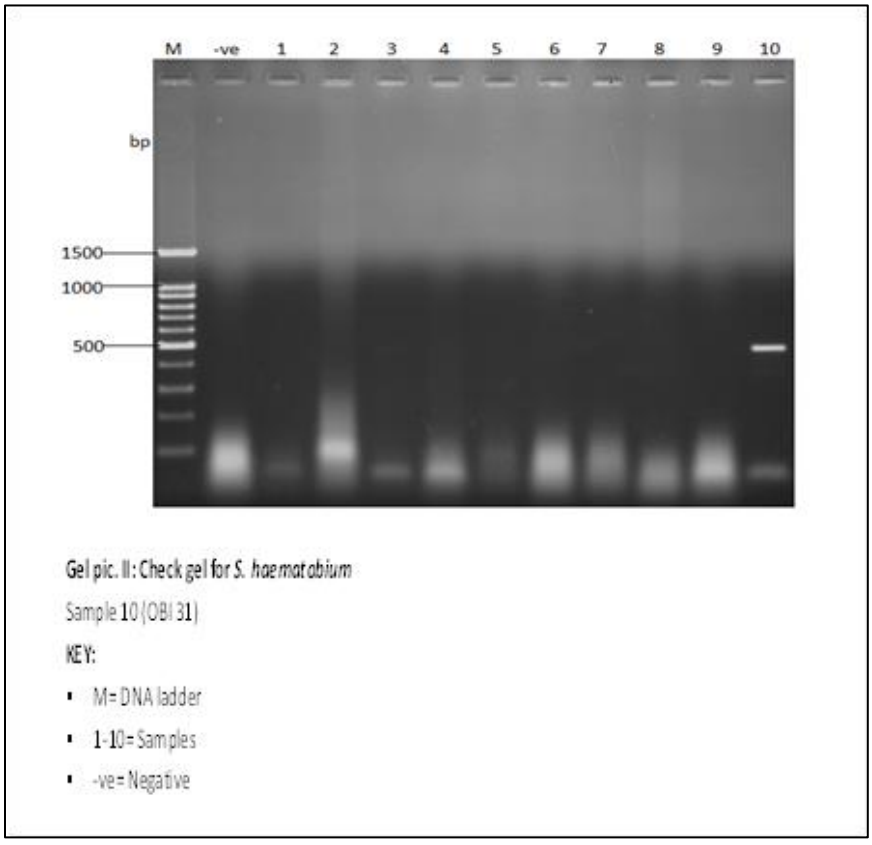
Gel pic. I: Check gel for *Plasmodiumfalciparum*

Sample 1 and 14: OBI 58 and OJU 200

KEY:

- M= DNA ladder
- 1-14= Samples

Plate 2: Gel Documentation of Detected *Schistosoma haematobium*



Gel pic. II: Check gel for *S. haematobium*

Sample 10 (OBI 31)

KEY:

- M= DNA ladder
- 1-10= Samples
- -ve= Negative

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Plate 3: Gel Documentation of Detected *Trichomonas vaginalis*

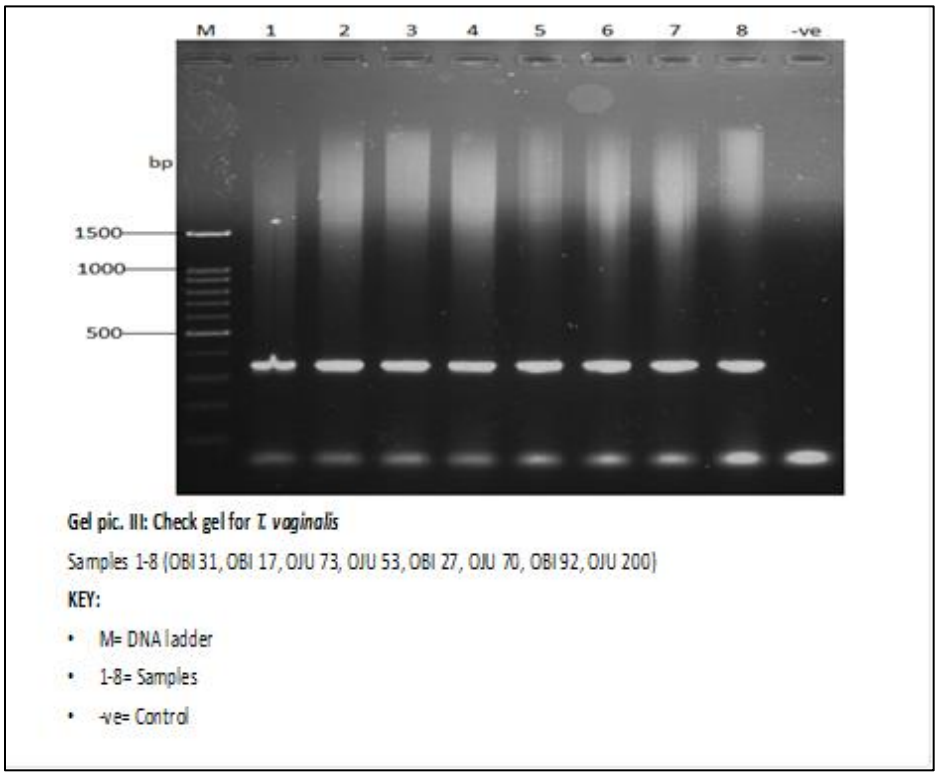
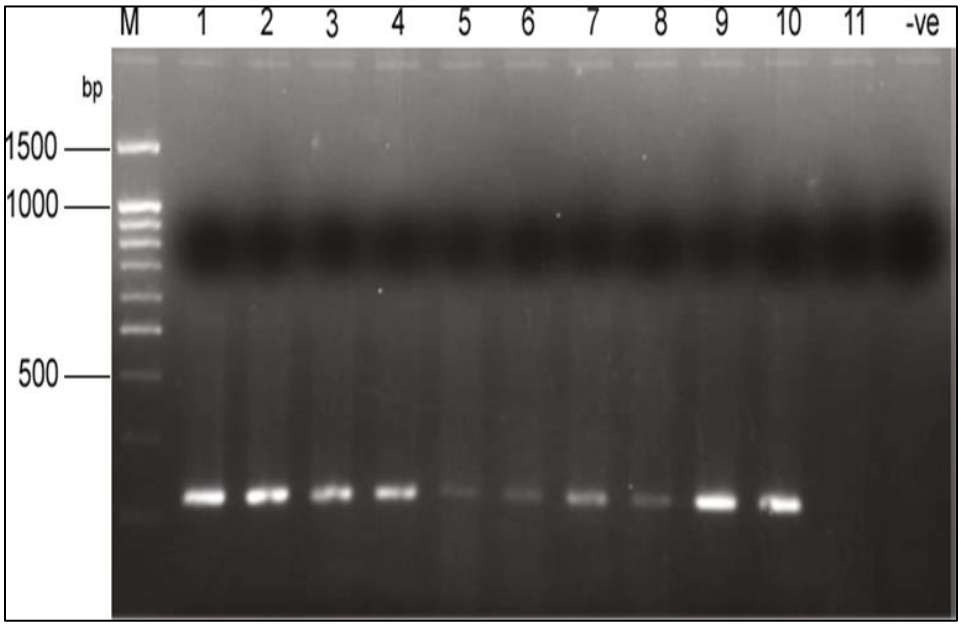


Plate 4: Gel Documentation of Detected *Ascaris lumbricoides*

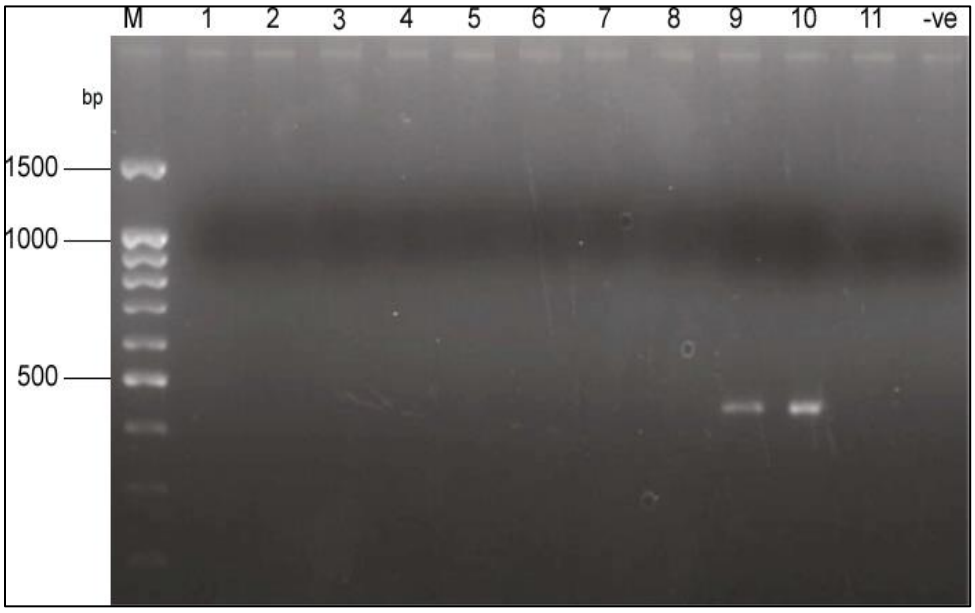


KEY: M = DNA ladder; 1-11= Samples; -ve= Control

Samples 1-10 (OBI 53, OBI 28, OBI 45, OBI 92, OBI 199, OBI 31, OBI 17, OJU 196, OJU15, OBI 58)

Article DOI: https://doi.org/10.37284/ijar.6.1.1059

Plate 5: Gel Documentation of Detected Entamoeba histolytica

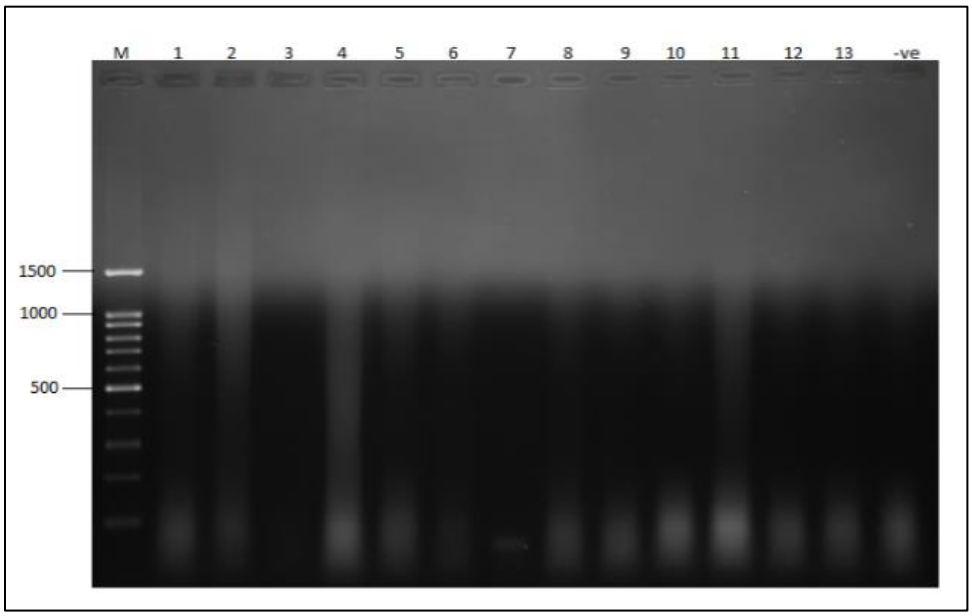


KEY: M = DNA ladder, 1-11= Samples, -ve= Control

Gel *Plate 5:* Check gel for *Entameoba histolytica*

Samples 9 and 10 (OBI 92, OJU 200)

Plate 6: Gel Documentation of Detected E. histolytica



KEY: M= DNA ladder; 1-13= Samples (OBI 20, OBI 25, OBI 30, OBI 37, OBI 150, OBI 165, OJU 32, OJU 72, OJU 78, OJU 92, OJU 160, OJU 170, OJU 195); -ve= Control

Gel *Plate 6*: Check gel for *E. histolytica*

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From the genus specific primers from PCR, 158bp fragment was obtained which correlates with *Ascaris lumbricoides* (Cox 1), 112bp and 178bp were also observed correlating to the presence of *Trichomonas vaginalis* and *Entamoeba histolytica* respectively. Also, 201bp fragment were observed correlating with *Plasmodium falciparum*, and 262bp and 350bp were determined which correlates with the presence of *Schistosoma haematobium* as shown in *Table 4*.

Table 4: PCR primers sequences for the intestinal parasites isolated from Women in Oju and Obi, Benue State

Species	Primer Sequence	Amplicon size	Annealing Temperature
A. lumbricoides (Cox1)	F: CCAGCTGACGCACTCGCTTGG R: ATGGTTGAGGTCTCCGTATGT	158bp fragment	59 ^{o}C
P. falciparum (Pf18S)	F: CTT AAC CTG CTA ATT AGC G, R: ATT CCT CGT TCA AGA TTA ATA ATT	201bp fragment	58 ^{o}C
T. vaginalis (Tv18S)	F: TGAATCAACACGGGGAAAC R: ACCCTCTAAGGCTCGCAGT	112bp fragment	62 ^{o}C
S. heamatobium (Sh 73)	F: CCTTGGTCACGTGATTTTC R: CTCCACCTTATGCCGGTC	262bp fragment	55 ^{o}C
S. heamatobium (Sh 77)	F: CCTGGGTGTGCACGTCTTGTT R: CTAAATGCCTAGCAACGGGGC	350bp fragment	58 ^{o}C
E. histolytica (Eh1)	F: TCT AAT TTC GAT TAG AAC TCT R: TCC CTA CCTATT AGA CAT AGC	174bp fragment	58 ^{o}C

DISCUSSION

Parasites encountered among women in Oju and Obi LGAs include: *Plasmodium* species, *Schistosoma haematobium, Ascaris lumbricoides, Entamoeba histolytica, Taenia species, Trichuris trichuria* and Hookworm. These parasites encountered in this study are similar to those reported by Omudu *et al.* (2012) where *Plasmodium falciparum* was also reported to be of higher dominance than the other parasites. The parasites reported were also similar to those reported by Alaku *et al.* (2019) in Nassarawa State, Nigeria. It is also similar to the report of Akor *et al.* (2019) where three of the parasites reported in this study were found among the study population. The presence of parasites such as *Plasmodium* spp. can be attributed to the presence of adequate breeding grounds for mosquitoes as well as the low adoption of preventive methods such as utilization of insecticide treated nets by the women and use of insecticides.

Amplicaton of genes through polymerase chain reaction indicated the presence of *Pf18S, Sh 73, Sh 77, Eh 1, Tv18S,* and *Cox 1*. These genes are known to code for polyparasitism in stool, urine, and blood respectively and are utilized in their identification (Faten *et al.,* 2017; Mehru *et al.* 2021). Their presence in the PCR analysis of stool, blood, and urine of women in Oju and Obi confirmed the presence of polyparasites and the specific polyparasites present. This is consistent with the findings of Cnops *et al*., (2013); Pechangou *et al*., (2015); Faten *et al*., (2017); Lucrecia *et al*., (2017); Lloyd *et al*., (2018), and Grabias *et al*., (2019) who reported that PCR analysis reveals the presence of polyparasitism, the DNA markers, and consequently, the polyparasites present.

Molecular analysis of parasites by PCR was seen to be more specific in identifying parasites such as *Trichomonas vaginalis* which not found by microscopic techniques. This is a pointer to the high specificity of PCR techniques in the detection of parasites and for studies pertaining to polyparasitism. This finding agrees with previous reports where molecular characterization of polyparasites by molecular techniques was recommended in place of microscopic and serological techniques (Pechangou *et al*., 2015; Lucrecia *et al*., 2017; Lloyd *et al*., 2018; Grabias *et al*., 2019).

Article DOI: https://doi.org/10.37284/ijar.6.1.1059

CONCLUSION

The parasites dominant in Oju and Obi Local Government Areas of Benue State include: *Plasmodium species, Schistosoma haematobium, Ascaris lumbricoides*, and *Entamoeba histolytica*. The genes Pf18S, Sh 73, Sh 77, Eh 1, Tv18S and Cox 1 that codes for *Plasmodium falciparum, Schistosoma haematobium, Entameoba histolytica,* and *Ascaris lumbricoides* were present in the women examined thus confirming the presence of polyparasitism in the study area. It was further revealed that PCR analysis of subjects is more specific than microscopic techniques and parasites which could not be detected by microscopy or serology can be evidently identified using PCR molecular technique. This is an implication for the need for improvement in diagnostic methods for parasitic infections in Nigeria if the fight against parasitic infection is to be actualized. Also, the presence of these parasites suggests the existence of a significant public health hazard in the study area and calls for action by the individuals, government, and non-governmental organizations to help curb the rate of polyparasitism among women in Oju and Obi Local Government Area of Benue State.

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