COMPARISON OF MODIFIED ZIEHL–NEELSEN, AURAMINE-PHENOL AND IMMUNOFLUORESCENCE ANTIBODY TEST FOR THE DETECTION OF CRYPTOSPORIDIUM SPECIES AT JIMMA UNIVERSITY MEDICAL CENTER, SOUTH WEST ETHIOPIA.

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A THESIS PAPER SUBMITTED TO THE SCHOOL OF MEDICAL LABORATORY SCIENCES, FACULTY OF HEALTH SCIENCES, INSTITUTE OF HEALTH, JIMMA UNIVERSITY, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTERS OF SCIENCE IN MEDICAL PARASITOLOGY.

August, 2019
JIMMA, ETHIOPIA

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Abstract

Background: *Cryptosporidium species infections cause severe diarrhea especially in children and immuno-compromised people worldwide. However, laboratory detection and identification of the Oocyst stage of these parasites seems overlooked with the routine saline wet mount stool examination.*

Objective: *The objective of this study was to compare Modified Zeihl-Neelson (MZN), Auramine Phenol (AP) and Immunofluorescent Antibody test (IFAT) for the detection of Cryptosporidium species infections in under five years old children with diarrhea at Jimma University Medical Center (JUMC).*

Method: *A cross-sectional study design was conducted from January 01, 2019 to March 30, 2019 enrolling a total of 221 children. Stool specimen was collected and examined by the direct saline wet mount, formol-ether oocyst concentration, MZN, AP and IFAT staining with a smear made both from direct and concentrated sediment. Comparison of diagnostic performances was evaluated in calculating for sensitivity, specificity, NPV and PPV, agreement compared with kappa values and level of significance at p-value of < 0.05 with the 95% confidence interval.*

Result: *The overall prevalence of intestinal parasites was 91(41.2%). Intestinal coccidian parasites detected by MZN after formol-ether concentration was 34 (15.84%), of which Cryptosporidium spp. accounts for 23 (10.4%), C. cayatenensis 8 (3.6%), and C. belli 3 (1.4%). Whereas, 47(21.26%) was detected in AP staining after formal-ether Oocyst concentration technique. Of this, Cryptosporidium spp. accounts 39 (17.64%), C. cayatenensis 6 (2.7%), & C. belli 2 (0.9%). Cryptosporidium species detected by IFAT was 54 (24.4%). Generally, the sensitivity of MZN & AP against the Gold standard test was (42.6%) & (72.2%), respectively so that AP revealed a better NPV (92.0%) as compared to the MZN (84.34%).*

Conclusion and recommendation: *the prevalence of Cryptosporidium species are still underestimated due to the low sensitivity of diagnostic methods used. Therefore, it is preferable to use the accessible, rapid, sensitive and specific method. According to findings in here, it is recommended to use Auramine phenol staining technique for the detection of intestinal Cryptosporidiosis in routine diagnostic in health facilities.*

Key words: *Cryptosporidium species, Cyclospora cayatenensis, Cystoisospora belli, sensitivity, specificity, NPV, PPV, MZN, AP, IFAT Jimma, Ethiopia.*

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List of acronyms

JUMC	Jimma University medical center
AP	Auramine-phenol
PCR	Polymerase chain reaction
MZN	Modified Ziehl-Neelsen
IFAT	Immunofluorescence antibody test
PPV	Positive predictive value
NPV	Negative predictive value

CHAPTER ONE

INTRODUCTION

1.1. Background

The World Health Organization (WHO) ranks diarrheal disease as the second highest cause of morbidity and mortality in children in the developing world. In those countries the impact of protozoan pathogens represents a major cause of gastrointestinal illnesses and is becoming of growing impact(1). Of which intestinal coccidian parasitic infections cause severe diarrhea especially in under five children and in immune-compromised people worldwide. The intracellular intestinal protozoan parasites such as *Cryptosporidium* species, *Cyclospora cayatenensis,* and *Cystoisospora belli* are among the major causes of diarrhea in developing countries(2). These parasitic infections are facultative pathogenic organism, which uses human beings not only as a host but also for multiplication. Serious pathological changes are not common in immune-competent infected individuals. Although the infection is self-limiting in immune-competent hosts, which readily clear the parasites, but it may cause persistent diarrhea and severe mal-absorption in immune deficient hosts(3).

Among intestinal coccidian parasites, *Cryptosporidium* infection is acquired through the ingestion of contaminated food or water with sporulated oocysts, person-to-person spread and contact with infected animals. Then, motile sporozoites, attach to intestinal epithelial cells. The trophozoite undergoes an asexual replication (merogony), resulting in the production of eight merozoites (type I meronts). Merozoites, released into the intestinal lumen, infect new intestinal epithelial cells, and originate type II meronts, characterized by four merozoites. The merozoites can undergo a sexual cycle (gametogony) and develop into macrogametocytes. The microgametocyte produces numerous microgametes which are released into the intestinal lumen(4). A microgamete will fuse with a macrogamete and the resulting zygote undergoes sporogony. Fully sporulated thick and thin oocysts are shed into the intestinal lumen at the completion of sporogony. The infectious thick oocysts are excreted in the feces, thus completing the life cycle. An autoinfection in which excystation takes place within the same host may also be possible and is mediated by thin-walled oocysts(5).

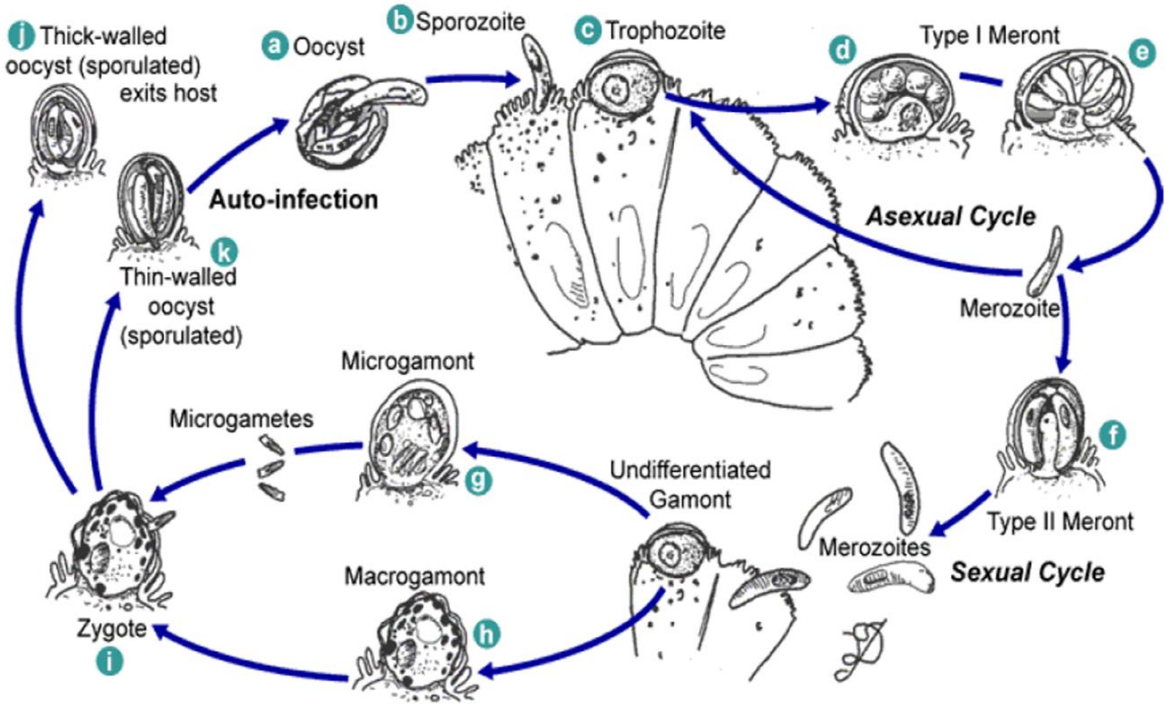

Figure 1: Life Cycle of Cryptosporidium.

Whereas, *Cyclospora cayatenensis* is an obligate intracellular coccidian parasite which is closely related to Eimeria species and humans are the only known hosts. It is responsible for significant morbidity and foodborne outbreaks in children and AIDS patients. Children, older adults, and the immune-compromised are more susceptible to the disease(6). The symptoms include diarrhea, which is sometimes explosive; anorexia, nausea, vomiting, abdominal bloating, cramps, weight loss, mal-absorption, fatigue, low-grade fever and body aches. *Cyclospora cayatenensis* cause diarrhea in both immune-competent, immune-compromised hosts and most cases of disease occur in young children (7). This protozoan parasite is a fecal-oral pathogen in which the oocyst from excreta must mature in the environment to become infectious. Sporulation occurs in the environment after days or weeks at temperatures between 22 °C and 32°C which results in the division of the sporont into two sporocysts each containing two elongated sporozoites. Food and water can serve as vehicles for transmission(3).

Then, matured oocysts excyst the sporozoites in the gastrointestinal tract to invade the epithelial cells of the upper small intestine (preferably the jejunum). Type I meronts give rise to 8-12 merozoites that then infect neighboring epithelial cells; this asexual reproduction is

often quite prolific. Type II meronts form later, and the subsequent type II merozoites also invade neighboring cells. Some of these meronts form macrogametes, while others undergo multiple fission events and form microgametocytes containing flagellated microgametes. Once fertilization occurs, an environmentally resistant wall is formed and the oocyst passes out of the host and into the environment(8).

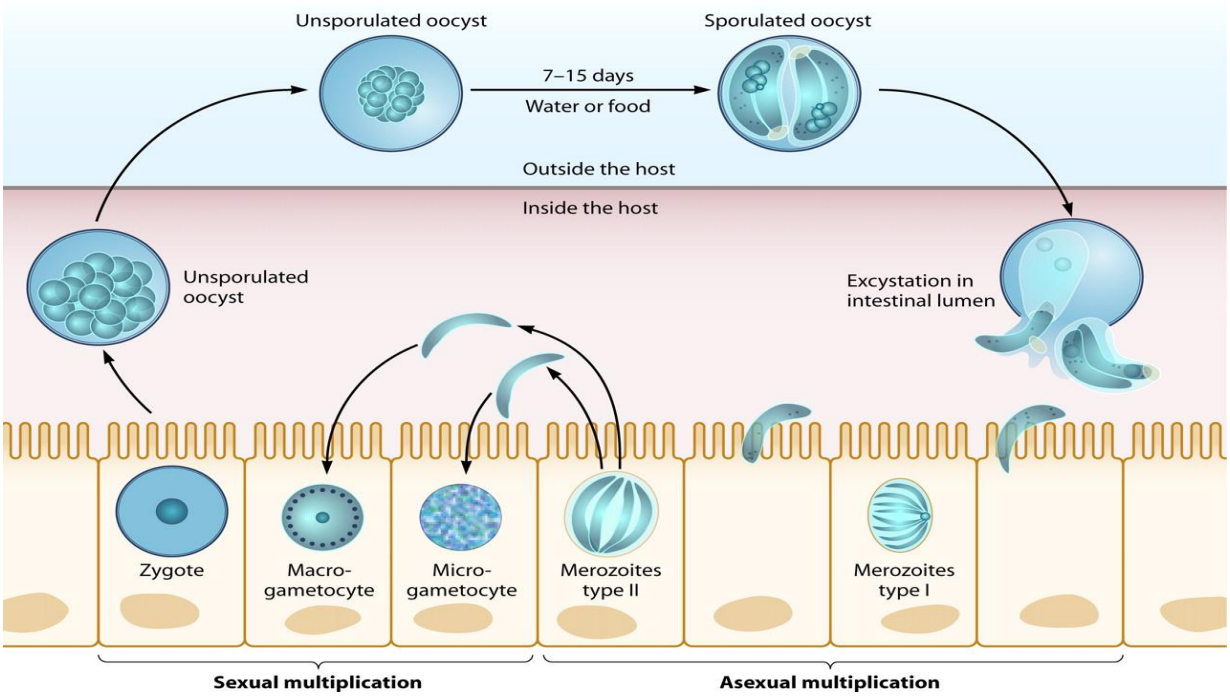

Figure 2: Life cycle of *Cyclospora cayatenensis*

The *Cystoisospora belli* is thought to be the only *Cystoisospora* species that infects humans. At the time of excretion in the stool, the oocyst is immature and usually contains just one sporoblast. During maturation, the sporoblast divides in to two and secrete a cyst wall, thus becoming sporocysts which then devide twice resulting four sporozoites (9). After ingestion of an infectious sporulated oocyst, it will excyst and release sporozoites into the small intestine, where they penetrate mucosal intestinal epithelial cells of the distal duodenum and enterocytes of the proximal jejunum and develop into trophozoites(10). Both sexual and asexual stages of development occur. Oocysts are produced, passed in the faeces and then mature outside of the body in 2-3 days depending on the environmental conditions. Symptoms include diarrhea, headache, fever, malaise, abdominal pain, vomiting, dehydration, and weight loss(11).

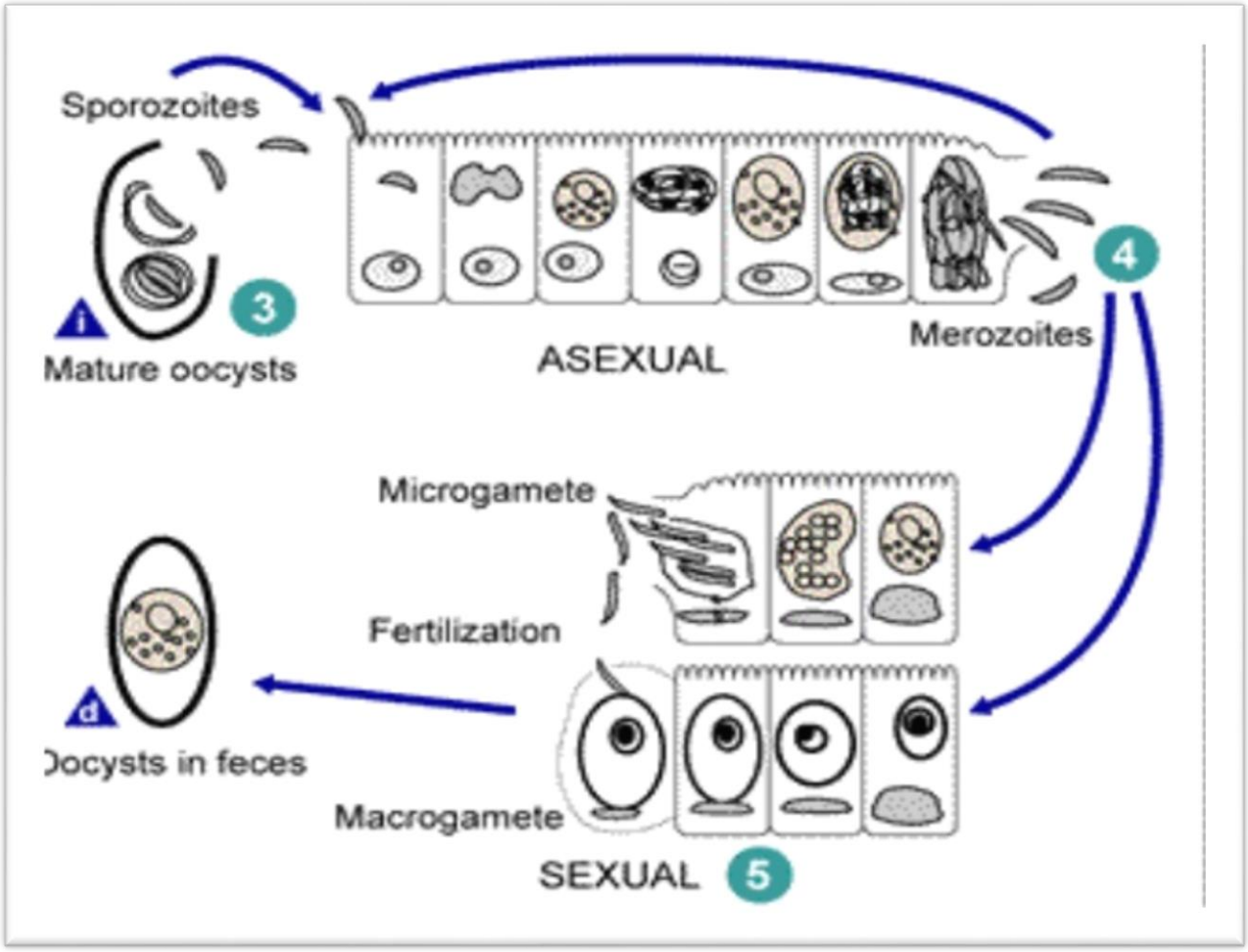

Figure 3: Life cycle of *Cystoisospora belli*

Although Cotrimoxazole is prescribed for the treatment of *Cyclospora cayatenensis* and *Cystoisospora belli,* still no effective treatments are available. Also specific treatment strategies for cryptosporidiosis have been followed for more than three decades, yet despite the evaluation of nearly a thousand chemotherapeutic agents, therapies able to clear the host of *Cryptosporidium* species are lacking. For prevention consistent hand hygiene, safe food and water practices are critical for preventing infection, particularly in patients with immune-compromised and children(12). Because of its high sensitivity compared to other assays, immunofluorescence is now considered the gold standard for detecting *Cryptosporidium* species in stool samples(13).

1.2. Statement of the problem

Intestinal coccidian protozoa are important etiological agents of diarrhea, particularly in children, yet the public health risk they pose is often neglected. *Cryptosporidium* species is among the leading causes of moderate to severe diarrhea in children of under 5 year(14). The burden of pediatrics diarrheal disease in sub-Saharan Africa exposed that *Cryptosporidium* species is second to rotavirus as a contributor to moderate-to-severe diarrheal disease during the first 5 years of life. It has been estimated that 2.9 million *Cryptosporidium* species attributable cases occur annually in children aged < 24 months in sub-Saharan Africa and infection is associated with a greater than two-fold increase in mortality in children aged 12 to 23 months(9). The epidemiology *of Cryptosporidium* species infection involves both direct transmission from animals to humans or from person to person, as well as indirect transmission through ingestion of water and food contaminated with infectious oocytes(5).

Persistent diarrhea caused by intestinal coccidian infection is the leading cause of death in children of under 5 years age in developing countries (15) in which out of 1.5 million deaths every year is because of diarrheal diseases (16). Persistent diarrhea caused by intestinal coccidian accounts 30-50 % of child mortality. In Africa, each child experiences five episodes of diarrhea per year and 800,000 children die each year from diarrhea and dehydration. According to Ethiopian Central statistics agency of 2012 report demographic and Health Survey piloted in 2005 and 2011 showed the prevalence of diarrhea in under five in the two weeks period to be 24% and 13%, respectively(17).

The *Cryptosporidium* species, *Cystoisospora belli* and *Cyclospora cayatenensis* commonly causes diarrhea in immune-compromised patients. Although bacterial etiologies of diarrhea in children under 5 years of age in Ethiopia have been reported(18), *Cryptosporidium* species prevalence was shown to range from 3.3% -12.2% in children. The weaker immune function in children attributes for higher prevalence and clinical impact in these groups(19). As there is no effective therapy available to treat *Cryptosporidium* species associated gastroenteritis in early childhood, infections may cause malnutrition, impaired physical and cognitive development, and death (20).

Detection of intestinal parasites by microscopic examination is a well-described laboratory technique that is widely used for examining stools by various procedures in different institutions of the world. The diagnostic challenges faced by the clinical laboratory diagnosis in the detection of intestinal coccidian parasites are suboptimal in physician requesting practices of the test; as they are not requesting organism-specific tests even during out breaks and inadequate access to patients information by laboratory personal(21). Additionally, routine saline wet mount examination of stool is not satisfactory for the detection of *Cryptosporidium* species, *Cystoisospora belli* and *Cyclospora cayatenensis* oocysts (22).

Gold standard test (with 100% accuracy) does not exist for detection of intestinal parasites identification of the etiological agent responsible for the disease, assess drug efficacy, monitor the effectiveness of control programs and obtain better understanding of the epidemiology of intestinal parasites (23). As a matter of fact, evaluation of the efficiencies of available diagnostic methods is important in the search for accurate diagnostic techniques to provide adequate patient care.

1.3. Significant of the study

The different diagnostic techniques may influence the detection of intestinal coccidian protozoan parasites among risk group population segments like children aged less than five years old. For the cases of *Cryptosporidium* species, *Cystoisospora belli* and *Cyclospora cayatenensis* infections in particular, it is important to provide a timely and accurate diagnosis as they are totally overlooked by the current health care laboratory diagnosis system.

Therefore, the current study has compared and evaluated different diagnostic methods for the detection and identification of intestinal coccidian to fill the gap of the challenges in diagnosis. Additionally, literatures on the area are scarce as detection of these parasites is not practiced as childcare in health institutions in Ethiopia. Therefore, the outcome of this study will help:

1. For introducing better diagnostic test method which can aid for patient care and treatment.
2. Policy makers in designing appropriate diagnostic methods.
3. Researchers as a base line data in identifying thematic areas on the matter for further study.

CHAPTER TWO

LITERATURE REVIEW

According to the study done in Netherlands, a strong association of diarrhea-causing protozoa with age was noted with *Cryptosporidium* species being detected in (21.8%) of 110 children aged <5 years (24). In Italy, the research article reported on global distribution, public health and Clinical Impact of the Protozoan Pathogen stated that water-associated outbreaks of parasitic protozoan diseases worldwide reported was 50.8% for *Cryptosporidium* species, 1.8% *Cyclospora cayatenensis* and 0.9% *Cystoisospora belli* (25).

In South Africa, Stool samples were collected and investigated for the diagnosis of *Cryptosporidium* species with the MZN technique. As a result, the overall prevalence of *Cryptosporidium* species was 5.6% (26). Similarly in Ghana Acra, like in many developing countries diarrheal diseases remain a major cause of morbidity and mortality among children. In a hospital-based study among children hospitalized for acute diarrhea recorded (22.2%) prevalence of *Cryptosporidium* species to be more common than diarrhea caused by other enteric parasites. *Cryptosporidium* species infection was relatively common among age groups 13-24 months (27).

The study done on Prevalence of intestinal opportunistic parasites infections in the University hospital of Burkina Faso, majority of the parasitic infections was waterborne (64.3 %) consisting of high prevalence of *Cryptosporidium* species (26.5 %) followed by *Cyclospora cayatenensis* (0.7 %) and *Cystoisospora belli* (0.7 %)(30). Whereas in Kenya, the research reported on the overall prevalence of intestinal parasitic infection at least with one parasite positive were (25.6%)(28). Another research reported in Egypt in detection of *Cryptosporidium* species infection among Children with diarrhea stated that from a total of 177 children presenting with diarrhea, *Cryptosporidium* species was detected in 27 samples (15.3%)(29).

The study done in southern Ethiopia showed the overall prevalence of *Cryptosporidium species* to be (13.2%) by MZN techniques(33). Another study done in Ethiopia, in Hawassa the overall prevalence of intestinal parasitic infection in under five years old children with diarrhea was 26.6% (19). In Yirgalem Hospital, among intestinal Protozoan Parasites in diarrheal Children a single stool sample was collected and examined by formol-ether concentration, and MZN. The

finding showed that (53.84%) prevalence of *Cryptosporidium* species infection which was higher in the age groups of ≤4 years old(34). Similarly in Bahir Dar, the prevalence of intestinal parasitic infection reported as 65.5% which were infected by one or more intestinal parasites(32). In a study conducted on 222 children of under five years of age who had diarrhea and on 74 children who had no diarrhea in selected Hospitals in Addis Ababa, a single stool specimens were collected and screened for intestinal parasitic infections. Among the emerging Opportunistic parasites detected in diarrhea children were *Cryptosporidium* species (8.1%), & *Cystoisospora belli* (2.3%) (31).

Whereas, a method comparison study conducted in Canada show that the fluorescent AP stains method has proved use for staining oocysts of *Cryptosporidium* species and *Cystoisospora belli.* They were described as irregular staining while, *Cyclospora cayatenensis* stains very poorly, and the weak fluorescence renders this method unsuitable for this parasite(36). On the other hand, a research conducted in United Kingdom showed the sensitivity of immunofluorescences antibody test was (97.4%) in comparison of diagnostic sensitivity and specificity of diagnostic assays used in stool samples for *Cryptosporidium* species (35).

Also another study done in India, Laboratory diagnosis of intestinal Cryptosporidiosis conventionally relies on demonstration of oocysts in stool samples by MZN staining. However, microscopic examination of MZN stained smears is time-consuming, tedious and has low sensitivity of 37-90%(37). Also another study conducted in India for evaluation of Immunological methods based on either antigen detection or antibody detection. These methods have reported to yield good sensitivity and specificity in the range of 93%–100%. While antigen detection tests are useful for diagnosis of acute infection, antibody detection tests are useful in sero-epidemiological surveys for *Cryptosporidium* species(38).

Even if Immunofluorescent and AP staining techniques had a better performance, most of studies from Ethiopia focused on the less sensitive MZN technique for these particular parasites. As far as literature search was done on the area in Ethiopia, a gap was identified for a need for new tool most appropriate for the detection and identification of these parasites especially the most common one i.e. cryptosporidium species with diagnostic method with improved performances.

CHAPTER THREE

OBJECTIVE

3.1. General objective

To compare the diagnostic test performance of MZN and AP with IFAT test methods for the detection of *Cryptosporidium* species at JUMC.

3.2. Specific objectives

1. To determine the overall prevalence of *Cryptosporidium* species, infections among children of less than five years old.
2. To compare the diagnostic test performance of (MZN,AP and IFAT) diagnostic test methods for the detection of *Cryptosporidium* species.

CHAPTER FOUR

METHODS AND MATERIALS

4.1. Study area

The study was conducted at Jimma university medical center Jimma town, South West Ethiopia. The town is located 346 K.ms away from the capital of Ethiopia, Addis Ababa and has total surface area of 4,623 hectares. The total projected population of the town from 2012 central statistical agency (CSA) census report is 207,573. The town has a temperature that ranges from 20-30 ^{0}c and the average annual rainfall of 800-2500mm^{3} and the town has an altitude of 1750-2000m above sea level(39).

Regarding health, facilities in Jimma town there are governmental and non-governmental health institutions. One governmental Medical center, one General Hospital, One primary private Hospital, four health centers and 51 private clinics, 19 drug stores and 33 pharmacies are found in the town.

4.2. Study period

The study was conducted in JUMC from January01, 2019 to March 30, 2019.

4.3. Study design

A cross sectional study design was used for diagnostic test methods comparison during the study period.

4.4. Study variables

The study variables were: *Cryptosporidium* species infections, Sensitivity, Specificity, Positive predictive value, Negative Predictive value, Modified Zeihl-Neelson technique, Auramine Phenol staining technique, and immunofluorescent antibody test.

4.5. Populations

4.5.1. Source population

All children coming to JUMC for any clinical service during the study period.

4.5.2. Study population

All children of under five years old with diarrhea attending JUMC pediatrics clinic during the study period.

4.6. Eligibility criteria

4.6.1. Inclusion criteria

All diarrheic children of under five years old and whose parents/guardians are volunteer and signed informed consent.

4.6.2. Exclusion criteria

The study populations who were unable to give sufficient stool sample.

4.7. Sample size and sampling technique

4.7.1. Sample size

Considering sample size for method comparison studies could be done on 30 - 200 positive samples, the sample size was calculated using the general formula for a single population proportion to be on the safe side:

$$n = \frac{(Z\ \alpha/2)^2\ p\ (1-p)}{d^2}$$

Where n= the minimum sample size

$Z\ \alpha/2$ =1.96 (95% confidence level)

p= 0.5 d= margin of error (5%) Therefore, the value of "n'' will be calculated as follows:

$$n = \frac{(1.96)^2 \times 0.5(1-0.5)}{(0.05)^2}, \quad n=384$$

Since the total population of children who are attending JUMC pediatrics clinic is less than 10, 000, we corrected sample size by using correction formula; (N=422 from hospital data logbook).

*A 422 children less than 5 years were seeking help due to diarrhea at JUMC pediatrics ward from October 01 to December 01/2017.

= N x n/N+n, Where, N= 422, n=384 422 x384/422+384 = **201+10%= 221**

4.7.2 Sampling technique

Convenient purposive sampling technique was used until the predetermined sample size is achieved.

4.8. Stool specimen collection and processing

A single, 5 grams of fresh stool specimens was collected from study participants in clean, labeled stool cups and blindly investigated by different persons for test methods as follows:

Saline wet mount: is made by mixing a small quantity (about 2 mg) of stool in a drop of saline placed on a clean glass slide. The smear is then examined under microscope with 10x and 40x magnifications. Saline wet mount is used for the detection of trophozoites and cysts of protozoa, and eggs and larvae of helminthes.

Formol- ether Oocyst concentration technique: About 7 ml of 10 % formol water was added to approximately 1 g of feces and mixed using an applicator stick. The stool sample then sieved with cotton gauze and transferred to 15 ml centrifuge tube Falcon. After adding 3 ml of diethyl ether to the mixture and hand shaking, the content was centrifuged at 1000 rpm for 2 min. The supernatant was poured and a drop of sediment was transferred to slide. Finally, smear was prepared on a glass slide for MZN and AP staining procedures.

The MZN staining technique: The principle of MZN technique is that it allows for detection of so-called "acid-fast organisms" such as coccidian intestinal protozoa. Once these organisms are stained with a specific dye, they are difficult to decolorize, and retain a red color even when treated with acid and alcohol (while all other structures present in the sample will decolorize). Methylene blue is used as counter stain, hence only the acid-fast organisms will appear as red-colored and can thus easily be detected in a stool sample with the blue background.

Stool sample was smeared on glass slide from direct and after concentrated sediment then, allowed to air dry and fixed in methanol for 3 minutes. It was then stained by carbol-fuchsin 1% for 15-20 minutes and rinsed thoroughly with a tap water. Acid alcohol of 0.5% was used as a decolorizer 15-20 seconds and 0.1% methylene blue for 30-60 seconds as a counter stain, then rinsed thoroughly and air dried. Finally examined under microscope using 40x and 100x oil immersion objectives for the presence of cyst/oocyst.

The AP staining: The fluorochrome dye, Auramine-phenol staining, for the detection of oocysts is often only performed when the accurate staining characteristics of auramine-O, which stains nucleic acids found in the acid-fast cell wall of organisms which resist decolorization by acid-alcohol. The counterstain, potassium permanganate, reduces tissue and

its debris non-fluorescent, thus reducing the possibility of artifacts. The oocyst visualized under ultraviolet light appears bright yellow green.

A smear was prepared from direct and concentrated sediment, air dry (smears should be medium to thick), fix in methanol for 3min. Then, flood the slide with Auramine-phenol solution and leave the solution on the slide for 15min. After rinsing with tap water, decolorize the slide with 0.5% acid alcohol and leave the alcohol on the slide for 2 min. Rinse with tap water and counterstain the slide with 0.1% potassium permanganate and leave the counterstain fluid on the slide for 2 min. again rinse with tap water, drain and air dry. Finally, examine with 20x and 40x objective the PrimoStar iLED fluorescence microscope (blue light) for the presence of oocyst/cyst.

The IFAT staining: is an antigen detection method using antibodies labeled with fluorescent reporters. Cryptosporidium oocysts can be detected using monoclonal antibody against oocyst wall antigen. These monoclonal antibodies basically recognize the epitopes on the surface of oocysts. Place between 5-20µl of stool sample on the graded well, spread the sample material with applicator stick or a sterile inoculation loop. Let the sample dry, either in room temperature (approximately 15-30 minutes) or in incubator at 37^{O}c, and fix with a drop of absolute methanol. After all the methanol has evaporated, add 5-15 µl of Aqua-Glu antibodies. Incubate the sample in humid chamber (box with damp tissue and lid) place in the room temperature for 40 minutes or longer. Apply 1 drop of counterstain per well and Incubate for 1 minute at room temperature. Rinse the slide free of counterstain by adding 1 drop of water and Place a drop of mounting medium or water before covering with coverslip for examination. Finally, smears were examined at 20x and 40x magnification for detection of protozoan oocysts/cyst. The detailed procedures for the methods are customized from crypto-POC study SOP which is annexed in the document.

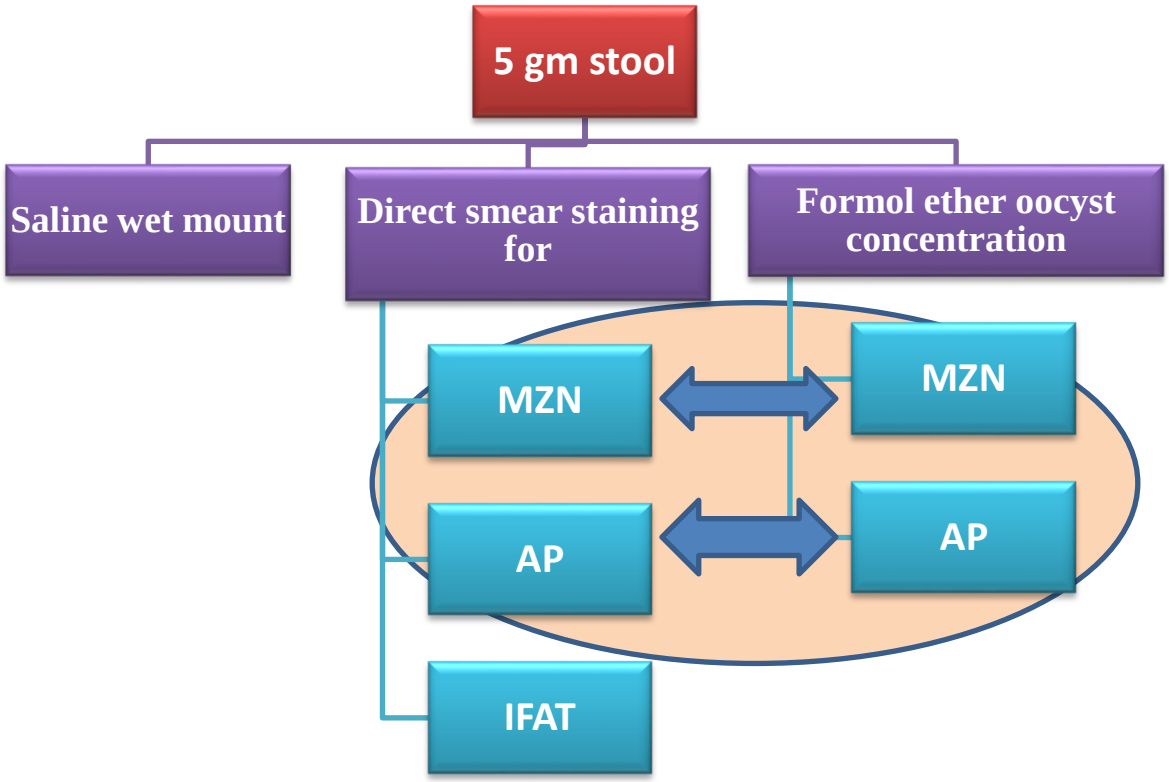

Figure 4: **Laboratory Sample processing work flow chart**

4.9. Data analysis

Data were entered to Epidata 4.4 manager software and analyzed using SPSS version 20. Considering immunofluorescent antibody test as a gold standard for *Cryptosporidium* species, the diagnostic test Sensitivity, specificity, positive predictive value, Negative predictive value of MZN, AP and IFAT were analyzed and compared. Agreement of the two methods in detecting intestinal coccidian parasites was determined by Kappa test interpreted as, from 0.01 to 0.20 as slight agreement, from 0.21 to 0.40 as fair agreement, 0.41–0.60 as moderate agreement, 0.61–0.80 as substantial agreement and 0.81–0.99 as perfect agreement(40).

4.10. Material and reagents

- Carbol-fuchsin,
- Auramine-Phenol
- Microscopy slide
- 0.1%Methyleneblue.
- 3%acidalcohol
- 1%Acidalcohol
- Coverslip
- 0.1% Potassium permanganate
- Florescence Microscopy

- 0.85%normalsalin
- IFAT reagents
- Ether
- Formol 10%
- Centrifuge
- Stool cup

4.11. Data quality assurance

Short training was given on the sample collection and laboratory diagnosis of intestinal coccidian parasites for the data collectors on the objective of the study. Standard Operating procedure was followed during specimen collection and processing. Manufacturers' instruction and test protocols were strictly followed when running the wet mount, formal-ether concentration, and MZN, AP, and IFAT methods along with running the known positive controls. The equipment used was checked for proper functioning. The entire positive slides and (10%) of the negative slides were double checked by another blinded technologist and when result disagreement found between them decision was given by the principal investigator.

4.12. Ethical considerations

Ethical approval of the project proposal was obtained from Jimma University ethical review board committee. Official permission was received from the hospital administration. All respondents were asked for their permission and written informed consent was obtained before sample collection. Confidentiality of individual patients' information was maintained during sample collection, analysis and interpretation. Children who become positive for intestinal parasitic infections were linked to the health professional for possible care and treatments**.**

4.13. Plan for dissemination

Final result report was submitted to the School of Medical Laboratory Sciences, Jimma University and the findings will be presented to the academics of the University. The report will also be submitted to the Jimma university Medical center administration. Finally, manuscript will be submitted to scientific reputable journals for publication.

CHAPTER FIVE

RESULTS

1. Socio demographic characteristics

A total of 221 under five years old children with diarrhea were enrolled in this study. Of which, 116 (52.5%) of them were males and the rest 105 (47.5%) were females. The age of participants ranges from 4 to 59 months with the mean age of 35.3 ± 13.6 months. Majority of participants were in the age group 36-47 months (32.1%) followed by 48-59 months (30.3%). Also, most of them were urban residents 134 (60.6%) while 87 (39.4%) were from rural area (Table 1).

Table 1: Socio-demographic characteristics of a study participants in the study of detection of intestinal coccidian parasites in diarrheic under five year's old children at JUMC from January 01, to March 30, 2019.

Category		Frequency	Percent
Age in Months	<6months	1	0.5%
	6-11 months	11	5.0%
	12-23 months	26	11.8%
	24-35 months	45	20.4%
	36-47 months	71	32.1%
	48-59 months	67	30.3%
Sex	Males	116	52.5%
	Females	105	47.5%
Residence	Urban	134	60.6%
	Rural	87	39.4%

2. Prevalence of parasites

The overall prevalence of intestinal parasitic infections was 41.2 % (91/221). A total of intestinal parasites were identified such as *G. lamblia, E. histolytica/dispar, Ascaris. lumbricoides, H.nana, H.worm, S. stercoralis*, and intestinal coccidian parasites, *Cryptosporidium* species, *C. cayatenensis, Cystoisospora belli* among diarrheic children which

are detected with at least one of the diagnostic test method. The prevalence of intestinal parasitic infection detected by direct wet mount saline method was 19(8.59%). The leading intestinal parasites detected with this method were trophozoite of *Giardia lambilia* 6 (2.7%), ova of *H.nana* 5 (2.3%) and *Ascaris lumbricoid* 4 (1.8%). Whereas any of intestinal coccidian protozoan parasites were not detected with the direct wet mount saline method (Fig. 2)

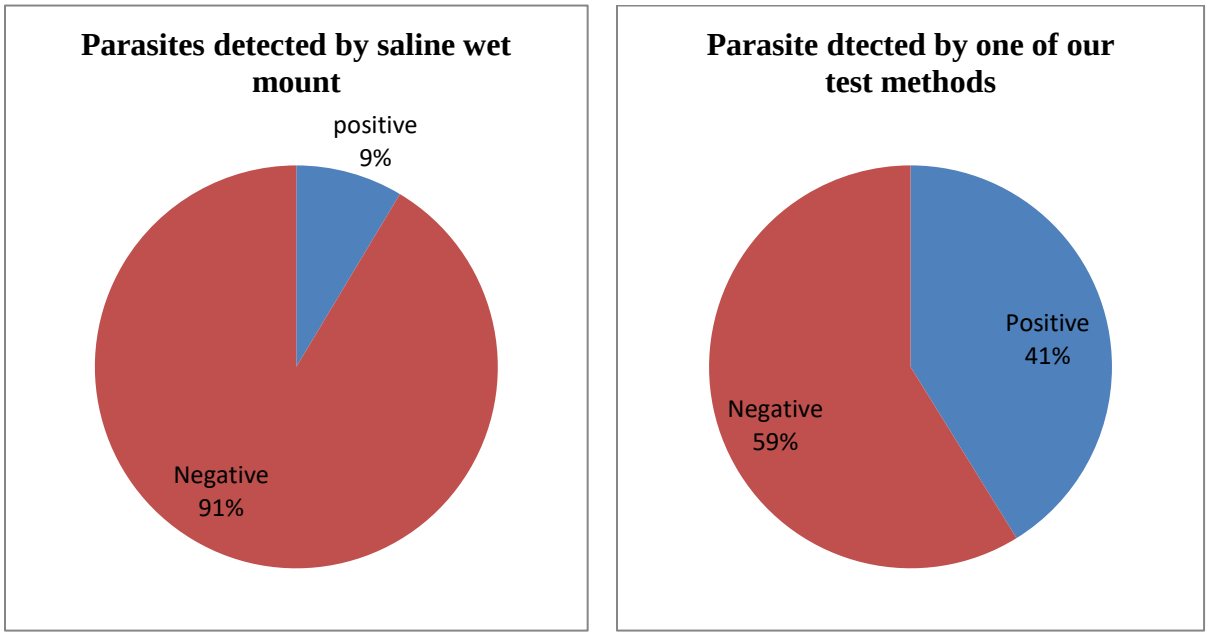

Figure 5: Overall intestinal parasites detected by saline wet mount test method and at least by one of test methods.

The overall prevalence of *Cryptosporidium* species detected by IFAT test method was 54 (24.4%). Whereas the overall of *Cyclospora cayatenensis* 8 (3.6%), and *Cystoisospora belli* 3 (1.4%) were detected by MZN test method. However, the intestinal coccidian parasites detected by MZN from formal-ether oocyst concentration technique sediment was 34 (15.84%), of which *Cryptosporidium spp*. Accounts for 23 (10.4%), *Cyclospora cayatenensis* 8 (3.6%), and *Cystoisospora belli* 3 (1.4%) (Fig.3). similarly, in AP staining test method after formal-ether Oocyst concentration technique was 47 (21.26%) (Fig. 3).

Whereas, in the absence of *Cyclospora cayatenensis* and *Cystoisospora belli*, these parasites were not detected by IFAT test method because of Aqua-Glu fluorescein-labeled monoclonal antibody reagent which was a direct immunofluorescence specific for simultaneous detection of Giardia cyst and *Cryptosporidium* species oocysts in stool sample. Hence, the prevalence of *Giardia lambilia* cyst was 27 (12.21%) of which 14 (6.33%) was detected as co-infection with *Cryptosporidium* species and *Giardia cyst* only in 13 (5.88%) (Fig.3).

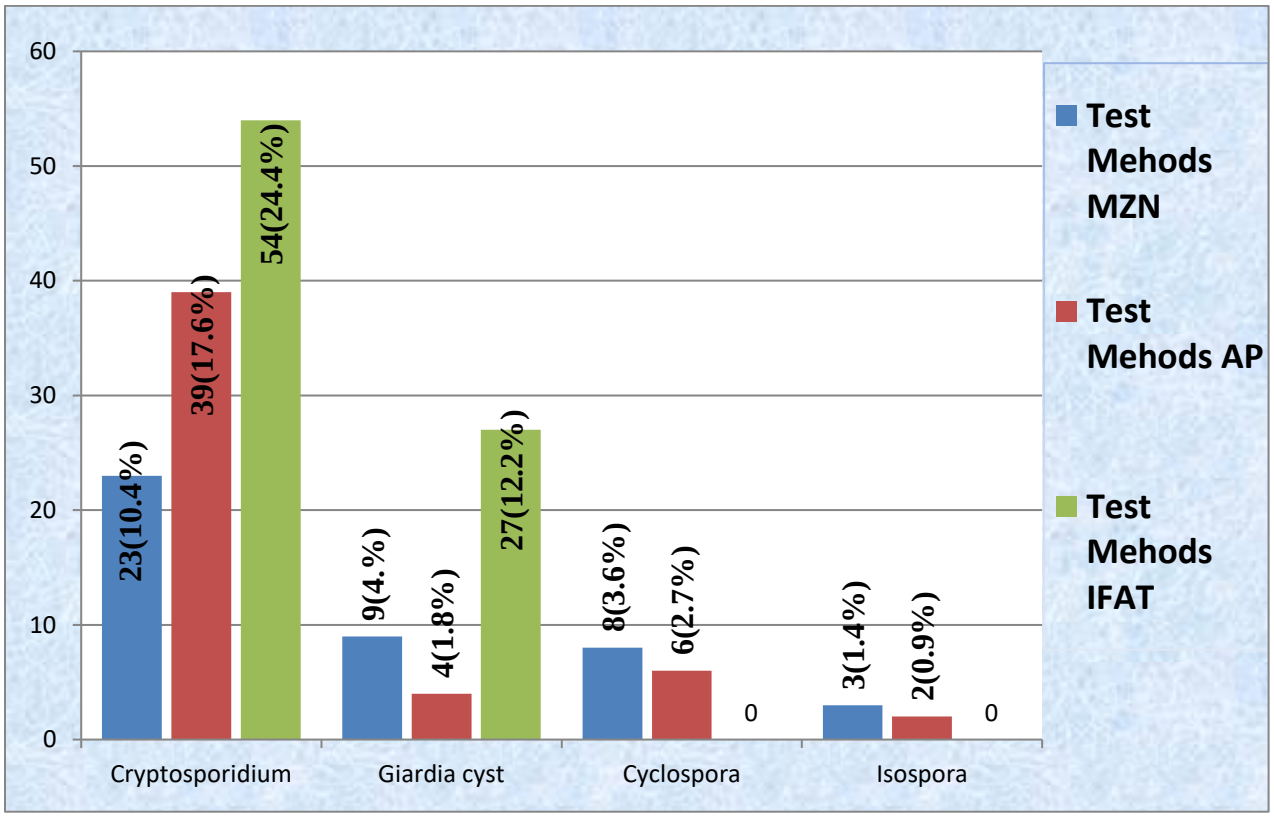

Figure 6: **Detection of intestinal coccidian parasites by MZN, AP, and IFAT test methods**

Modified Ziehl-Neelsen staining technique, AP staining technique and IFAT were compared for the detection of *Cryptosporidium* species in this study. Additionally, MZN and AP technique was compared for their detection improvement of the direct stool smear and after concentrating by formol-ether oocyst concentration technique. As a result, 7 (3.2%) improvement of the detection of parasite was observed with MZN from 16 (7.2%) to 23 (10.4%) and AP from 32 (14.5%) to 39 (17.7%) in direct smear and after concentration, respectively (Fig:3).

However, IFAT test method showed detection of 54 (24.4%) for *cryptosporidium* species. As it is the gold standard test, the agreement of diagnostic test methods for the detection of *Cryptosporidium* species was compared against it. Thus, AP staining technique showed better and statistically significant (p-value<0.05) agreement in detecting *Cryptosporidium* species 32 (14.5%) in direct and 39 (17.7%) after concentration technique compared with IFAT test method. Although, statistically significant (p-value<0.05) agreement was observed between MZN and IFAT in detecting 16 (7.2%) in direct and 23 (10.4%) after concentration technique, AP staining showed approximately twice detection capacity than MZN (Table 2).

Table 2: Comparison of MZN, AP and IFAT diagnostic test methods in detecting *Cryptosporidium* Species in diarrheic under five years old children at JUMC from January 01, to March 30,2019.

Types of diagnostic methods	**Result**			**P-value**
	Direct smear Positive (%)	**Concentration smear Positive (%)**	**Improved detection %**	
MZN	16(7.2%)	23(10.4%)	7(3.2%)	0.001*
AP	32(14.5%)	39(17.7%)	7(3.2%)	0.001*
IFAT	54(24.4%)*	-	-	-

**p-value is 0.001 statistical significance tested by Mc nemar test method*

** IFAT test performed only from direct smear method.*

3. Performance of diagnostic techniques

In this study the sensitivity and negative predictive value for the MZN direct smear test method was 29.6% and 80.41% respectively, and with the kappa value 0.36 which indicate that MZN direct smear test method has shown fair agreement with the Gold standard test method, IFAT. Whereas, the sensitivity and NPV for MZN test method after formol-ether concentration was 42.6% and 84.34% respectively, with kappa value 0.52, which implies that MZN test method after formol-ether concentration has shown moderate agreement when compared with the gold standard test method (Table 3).

AP staining direct smear method sensitivity and NPV indicate 59.3% and 88.34% respectively. Also the test method agreement measurement kappa value shows a value of 0.68, this indicate

that AP staining direct smear method has substantial agreement with the gold standard method. Whereas, the sensitivity and NPV for the AP staining after formol-ether concentration method was 72.2% and 92% respectively with the kappa value of 0.80 which has substantial agreement with gold standard test method (Table 3).

Table 3: The performance of diagnostic technique of *Cryptosporidium* species against the Gold standard method in diarrheic under five year's old children at JUMC from January 01, to March 30, 2019.

Diagnostic techniques		**IFAT as Gold standard for *Cryptosporidium* species**				
		sensitivity	Specificity	PPV	NPV	Kappa value
MZN	Direct smear	29.6%	100%	100%	80.41%	0.36
	Concentration	42.6%	100%	100%	84.34%	0.52*
AP	Direct smear	59.3%	100%	100%	88.35%	0.68
	Concentration	72.2%	100%	100%	92.0%	0.80*

**p-value 0.001 is statistical significance for direct and concentration smear test method by Mc nemar test method.*

**Kappa value-0.01-0.20 slight agreement, 0.21-0.40 fair agreement, 0.41-0.60 moderate agreement, 0.61-0.80 substantial agreement, and 0.81-0.99 perfect agreement*

However, MZN and AP diagnostic methods performance comparison was not done with IFAT test for *C. cayatenensis* and *C. belli* due to the fact they could not be detected with this method. But the figure showed 8(3.6%) and 6(2.7%) detection of *C. cayatenensis* and 3(1.4%) and 2(0.9%) detection of *C. belli* with MZN and AP techniques, respectively.

Table 4: Comparison of MZN and AP test methods for the detection of *C. cayatenensis* and *C. belli* in diarrheic under five year's old children at JUMC from January 01, to March 30, 2019.

Detected Parasites	**MZN**	**AP**	**IFAT**
Cyclospora cayatenensis	8 (3.6%)	6 (2.7%)	-
Cystoisospora belli	3 (1.4%)	2 (0.9%)	-

CHAPTER SIX

DISCUSSION

In this study the overall prevalence of intestinal parasitic infections detected with at least one of the diagnostic test method was 91(41.2%). This finding is higher than the prevalence reported from Hawassa (26.6%)(18), and it is lower than the result of study done in Bahir Dar (65.5%)(32) in Ethiopia. When compared with a study conducted in Kenya with the overall prevalence of intestinal parasitic infection among children was (26.5%)(28), which is lower than our study finding. These variations could be due to difference in diagnostic methods, hygienic practice, age group of the children, environmental factors, seasonal variation and socio-economic status of the children parents/caregivers.

The overall detection rate of *Cryptosporidium* species infections detected by IFAT test method was 54 (24.4%). Because of its superior detection capacity and recommended to use Immunofluorescence antibody test as a gold standard reference method, we have compared the sensitivity, specificity, NPV and PPV of MZN and AP against it. Although, Immunofluorescence antibody test outperformed than MZN and AP methods, its true values for sensitivity and specificity were not calculated in this study. Whereas, any of intestinal coccidian protozoan parasites were not detected with direct saline wet mount microscopy.

For the detection of *Cryptosporidium* species, the study result revealed that MZN test method shown low performance with the sensitivity value of (42.6 %) and in AP with (72.2 %) showing improvement. This result agrees favorably with the similar study conducted in United Kingdom,(36). Also this study was in agreement with the study done for the detection rate of *Cryptosporidium* species with the sensitivity MZN (42.6 %) in India, MZN staining has low sensitivity of 37-90%(35).

However, MZN has low sensitivity to detect *Cryptosporidium* species; while it was widely performed as a diagnostic method in most studies. The study finding showed that, MZN exhibited very low capacity for the detection of *Cryptosporidium* species and as compared to the AP and IFAT techniques. This suggested that the use of MZN method alone for *Cryptosporidium* species infections identification is insufficient and may lead to false negative results. However, AP outweighs in its sensitivity to MZN to be used in health center and

hospitals as it is available for other diagnostic purposes in Ethiopia such as in national laboratory, regional laboratories, Hospitals and health center laboratories.

In this study, the detection rate of *Cyclospora cayatenensis* and *Cystoisospora belli* by MZN and AP was (3.6%), (1.4%) and (2.7%), (0.9%) respectively. This finding is higher in MZN test method than AP staining. This was similar with previous study conducted in Canada, the detection ability of AP staining for these parasites are poor(41).

Generally, this finding indicates an improved performance for detecting *Cryptosporidium* species with AP technique. Concentrating the stool prior staining methods also show detection performance difference.

In this study AP staining after formol-concentration method improved more detection rate for *Cryptosporidium* species when compared for its sensitivity, NPV, and Kappa value with the MZN test methods. But, both diagnostic tests performance showed 100% specificity and PPV. In addition, this methods show statistically significant difference after the formol ether concentration technique with a *p v*alue <0.05 when tested by Mc Nemar test method.

Whereas, *Cyclospora cayatenensis* and *Cystoisospora belli* were unable to be detected by IFAT test method, because of the IFAT reagent is specific for the detection of Giardia cyst and *Cryptosporidium* species. Then, we couldn't compare with the gold standard method. However, MZN showed an improved detection was compared with AP test method. Hence, MZN was considered and recommended as confirmatory for these two particular parasites as AP staining test method.

CHAPTER SEVEN

CONCLUSION AND RECOMMENDATION

7.1 Conclusion

The prevalence of *Cryptosporidium* species are still underestimated in our country due to the low sensitivity of diagnostic method used in health facilities laboratory which mainly relies on microscopy saline wet mount. However, the outcome results of this study showed that AP is a simple fluorescent staining, highly sensitive and specific. Therefore, the AP staining technique could be promising in diagnosis of intestinal Cryptosporidiosis.

7.2 Recommendation

Based on the above conclusion the following points are recommended:

- High infection rate of *Cryptosporidium* species were seen then, prevention and control methods (health education) important for the community.
- It is preferable to use the accessible, rapid, sensitive and specific method that is Auramine phenol staining technique for the detection of intestinal coccidian parasites especially for intestinal cryptosporidiosis.

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ANNEX -I Laboratory result report format

Jimma university

Institute of health and faculty of health sciences

School of medical laboratory sciences

Laboratory result report format for the detection of intestinal coccidian protozoan parasites among under five years old children with diarrhea at JUMC.

Sample ID_____ Date of data collection_____

Age in months _____ Sex 1. Male 2. Female

Residence 1. Urban 2. Rural

1. Direct saline & iodine preparation

A. protozoan 1. Trophozoite of_____

2. Cyst of _____

B. Helminthes 1. Ova of_____

2. Larva of_____

3. No O/p seen_____

2. Modified Ziehl-Neelsen technique 1. Cyst of_____

2. *Cryptosporidium* oocysts Present ☐ Absent ☐

3. *Cystoisospora belli* oocysts Present ☐ Absent ☐

4. *Cyclospora cayatenensis* oocysts resent ☐ Absent ☐

3. Auramine-phenol (AP) staining technique 1. Cyst of_____

2. *Cryptosporidium* oocysts Present ☐ Absent ☐

3. *Cystoisospora belli* oocysts Present ☐ Absent ☐

4. *Cyclospora cayatenensis* oocysts Present ☐ Absent ☐

4. Immunofluorecent Antibody test (IFAT) technique

1. *Giardia lambilia* cyst Positive ☐ Negative ☐

2. *Cryptosporidium* oocysts Positive ☐ Negative ☐

Laboratory technologists Name _____

Sign._____

Date_____

Annex II- Standard operating procedures (SOP)

1. Direct saline wet mount examination

A portion of stool will examined by direct saline wet mount preparation (0.85 % sodium chloride solution) to observe motile larva, trophozoites and ova of intestinal parasites under light microscope at 10× and 40× magnifications.

2. Formol- ether Oocyst concentration technique

About 7 ml of 10 % formalin will added to approximately 1 g of feces and mixed using an applicator stick. The stool sample will sieved with cotton gauze and transferred to 15 ml centrifuge tube Falcon. After adding 3 ml of diethyl ether to the mixture and hand shaking, the content will centrifuged at 1000 rpm for 2 min. The supernatant will pour and a drop of sediment will transferred to slide. Finally, the entire zone under the cover slip was systematically examined using 10X and 40X objective lenses to observe ova, cyst and larvae of different intestinal parasites.

3. Modified Ziehl-Neelsen method

Use of the modified Ziehl-Neelsen stain for faecal smears has already been established for coccidian protozoa, in particular, oocysts of *Cryptosporidium* species, but it is also useful to confirm the presence of oocysts of *Cystoisospora belli* and *Cyclospora cayetanensis*.

1. Faecal smears are made either directly from the stool sample or from the concentration deposit.
2. Allow to air dry.
3. Fix in methanol for 3 minutes.
4. Stain with strong carbol fuchsin 3% for 15-20 minutes.
5. Rinse thoroughly in tap water.
6. Decolorize in acid alcohol 3% (1% HCl in methanol) for 15-20 seconds.
7. Rinse thoroughly in tap water.
8. Counterstain with 0.1% methylene blue for 30-60 seconds.
9. Rinse thoroughly and air dry.
10. Examine using x40 and x100 objectives.

4. Auramine-phenol (AP) staining method

The fluorochrome dye, Auramine phenol staining, for the detection of oocysts is often only performed when the accurate staining characteristics of auramine-O, which stains nucleic acids found in the acid-fast cell wall of organisms which resist decolorization by acid-alcohol. The counterstain, potassium permanganate, reduces tissue and its debris non-fluorescent, thus reducing the possibility of artifacts. The oocyst visualized under ultraviolet light appears bright yellow green.

1. Prepare a smear and air dry (smear should be medium to thick)

2. Fix in absolute methanol for 1 min, then air dry before proceeding with staining

3. Flood the slide with Auramine-phenol solution (0.1% auramine-O) (ready-made commercial stain, or prepared according to the MOH protocol) leave the solution on the slide for 15 minutes. Do not heat.

4. Rinse with tap water (from a beaker, not directly from the tap). Drain excess water from the slide.

5. Flood the slide with 0.5% acid ethanol and leave the destaining solution on the slide for 2 minutes.

6. Rinse with tap water (from a beaker, not directly from the tap) Drain excess water from the slide.

7. Flood the slide with 0.1 % Potassium permanganate and leave the counterstain fluid on the slide for 2 minutes. The timing of this step is critical.

8. Rinse with tap water (from a beaker, not directly from the tap) Drain excess water from the slide, and air dry. Do not blot because some blotting materials may fluorescence.

9. Examine with X 20 objective and X 10 eyepiece lens, and the primostar iLED fluorescence microscope (blue light). The whole sample area should be examined for the presence of fluorescent oocysts. Suspicious objects can be re- examined with a 40x objective or with oil-immersion and the 100x objective.

5. Immunofluorescent antibody test (IFAT) microscopy

Antigen detection methods using antibodies labeled with fluorescent reporters. *Cryptosporidium* oocysts can be using monoclonal antibody against oocyst wall antigen. These monoclonal antibodies basically recognize the epitopes on the surface of oocysts.

1. Use the graded 3-welled microscope slide, and write the sample identifier (number, name or code) on the slide.
2. Place between 5-20µl of sample materials (faeces concentrated by salt or formol- ether concentration) on the well. The smear must be thin. If necessary, spread the sample material with applicator stick or a sterile inoculation loop, being careful not to contact the surface of the slide.
3. Let the sample dry, either in room temperature (approximately 15-30 minutes) or in incubator at 37^{O}c.
4. Fix the with a drop (ca 45 µl) of absolute methanol
5. After all the methanol has evaporated, add 5-15 µl(depending on the amount of sample material) of Aqua-Glu antibodies. If necessary, spread the sample material with applicator stick or a sterile inoculation loop, being careful not to contact the surface of the slide.
6. Incubate the sample in humid chamber (box with damp tissue and lid) place in the room temperature for 40 minutes or longer.
7. Tap off surplus mab.(for example by tilting the slide, long edge down, and absorb excess fluid with soft paper placed at the edge of the slide well).
8. Gently add 1 drop of water to cover well (do not squirt directly on to well). Leave water drop on for 1 minute. Tap off.
9. Apply 1 drop of counterstain per well and Incubate for 1 minute at room temperature.
10. Rinse the slide free of counterstain by adding 1 drop of water (do not quirt directly on to well). Tap off. And Place a drop of mounting medium or water before covering with coverslip. And Examine sample on primostar iLED immunofluorescent microscope. Scan the whole well. Count the total number of cyst or Oocyst and the total number of observed fields of view using the x20 objective.

Annex- III Calculations for sensitivity and specificity

AP staining for *Cryptosporidium* spp. VS IFAT for *Cryptosporidium* Cross tabulation

Test method		IFAT Gold standard		
		Positive	Negative	Total
AP staining Concentration	Positive	39(72.2%)[a]	0[b]	39(17.6%)
	Negative	15(8.2%)[c]	167(91.8%)[d]	182(82.4%)
	Total	54(24.4%)	167(75.6%)	221(100%)
AP staining Direct smear	Positive	32(59.3%) [a]	0 [b]	32(14.5%)
	Negative	22(11.6%) [c]	167(84.4%) [d]	189(85.5%)
	Total	54(24.4%)	167(75.6%)	221(100%)

For AP staining Concentration:

Sensitivity=TP/(TP+FN) = 39/(39+15)=72.2% Specificity=TN/(TN+FP)=167/(167+0)=100%

NPV=TN/(TN+FN)= 167/(167+15)=92.% PPV=TP/(TP+FP)= 39/(39+0)=100%

For AP staining direct smear

Sensitivity=TP/(TP+FN) = 32/(32+22)=59.3% Specificity=TN/(TN+FP)=167/(167+0)=100%

NPV=TN/(TN+FN)= 167/(167+22)=84.4.% PPV=TP/(TP+FP)= 32/(32+0)=100%

MZN staining for *Cryptosporidium* spp. VS IFAT for *Cryptosporidium* Cross tabulation

Test method		IFAT Gold standard		
		Positive	Negative	Total
MZN staining Concentration	Positive	23(42.6%)[a]	0[b]	23(10.4%)
	Negative	31(15.7%)[c]	167(84.3%)[d]	198(89.6%)
	Total	54(24.4%)	167(75.6%)	221(100%)
MZN staining Direct smear	Positive	16(29.6%) [a]	0 [b]	16(7.3%)
	Negative	38(17.9%) [c]	167(81.1%) [d]	205(92.7%)
	Total	54(24.4%)	167 (75.6%)	221(100%)

For MZN staining Concentration

Sensitivity=TP/(TP+FN) = 23/(23+31)=42.6% Specificity=TN/(TN+FP)=167/(167+0)=100%

NPV=TN/(TN+FN)= 167/(167+31)=84.3% PPV=TP/(TP+FP)= 23/(23+0)=100%

For MZN staining direct smear

Sensitivity=TP/(TP+FN) = 16/(16+38)=29.6% Specificity=TN/(TN+FP)=167/(167+0)=100%

NPV=TN/(TN+FN)= 167/(167+38)=81.1% PPV=TP/(TP+FP)= 16/(16+0)=100%.

Annex-IV Consent form

I have been informed about a study. For this study, I have been requested to give my children stool sample. I have read /has been read to me all the information stated in the introductory part and I have had an opportunity to ask any ambiguous question I got satisfactory answer for all of my concerns. I have fully understood and gave my consent to give the stool specimen. It is therefore, with full understanding of the situation that I gave my informed consent and cooperate at my will in the course of the conduct of the study.

Name (Parents/guardian participant) ----------Signature ----------Date ----------

Name (investigator) ----------Signature ----------Date ---------

If illiterate: name of the independent witness, name

signature_____,_____/_____/_____(date,month,year)

Declaration Sheet

I, the undersigned, declare that this thesis is my own work. I have followed all ethical and technical principles of research in the preparation, data collection, data analysis and compilation of this thesis. Any scholarly matter that is included in the thesis has been given recognition through citation.

This thesis is submitted in partial fulfillment of the requirements for MSc. degree at Jimma University. I confidently declare that this thesis has not been submitted to any other institution anywhere for the award of any academic degree, diploma or certificate.

Principal investigator

Bahiru Terefe Legesse signature: _____ date_____

Advisors:

Mr. Yonas Alemu (MSc.) signature:_____ date_____

Mr.Mio Ayana (PhD fellow) signature:_____ date_____

Dr.Alemseged Abdissa (PhD) signature:_____ date_____

Thesis Examiner:

1. Dr. Melkamu Birhane (External Examiner) signature:_____ date_____
2. Mr. Tariku Belay (Internal Examiner) signature:_____ date_____