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Efficiency Evaluation of Immunochromatographic Strips Test in Comparison With Conventional and Molecular Techniques in Diagnosis of Some Acute Diarrheal Causes in pre School Patients in Babylon Province/ Iraq

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﴿بسم الله الرحمن الرحيم﴾

يا أَيُّهَا الَّذِينَ آمَنُوا إِذا قِيلَ لَكُمْ تَفَسَّحُوا فِي الْمَجالِسِ فَافْسَحُوا يَفْسَحِ اللَّهُ لَكُمْ وَ إِذا قِيلَ انْشُزُوا فَانْشُزُوا يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنْكُمْ وَ الَّذِينَ أُوتُوا الْعِلْمَ دَرَجاتٍ وَ اللَّهُ بِما تَعْمَلُونَ خَبِيرٌ

﴿صدق الله العظيم﴾

(سورة المجادلة، اية ١١)

DEDICATION

I dedicate this thesis

To … the compassionate my father

The kind heart my mother

To the dearest one (My sister Marium)

.

Zahraa...

2021

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Supervisors' certification

We certify that this thesis entitled " **Efficiency Evaluation of Immunochromatographic strips Test in Comparison With Conventional and Molecular Techniques in Diagnosis of Some Acute Diarrheal Causes in pre School patients in Babylon Province / Iraq.**" has been prepared and written under my supervision at the College of Applied Medical Sciences, University of Kerbala as a partial fulfillment of the requirements for the degree of Master in Clinical Laboratories.

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In view of the available recommendations, I forward this thesis for debate by the Examining Committee.

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Chairman of the Department of Clinical Laboratories.

Date:

Summary

The present study includes the identification of parasites, bacteria and viruses responsible for diarrheal infection in children under six years old suffering from acute diarrhea during the period from begning December 2020 till the end of May 2021,they were attending to Babylon teaching Hospital for Maternity and Children in the babylon city.

A total of 320 stool samples from both genders have been collected from the hospitalized patients to identifiy the causative agent of acute diarrhea, the primary detection of parasitic infection was carried out using microscopic and immunochromatography test (triage Rapid test) as well as Multiplex-PCR which used to detect three parasites infection by used (*EHCP8-S1*,*GLCP6-S1*and *CRY18s-S1*) genes. The Conventional PCR that used to identify bacteria were carried out to detect (*pho*) gene family for *E.coli* and *16srRNA* gene for *Salmonella spp*. On the other hand , among the viruses that causes diarrhea the results found *Rotavirus* infection was carried out using immunochromatography test

(Rapid test) with Reverse Transcriptase-PCR assay used to detect virus infection by using structural gene (*vp4 gene*).

The results showed 86 (26.87%) samples of parasites from 320 children stool sample which were divided into three type of parasites as 25 (29.6%) *E.histolytica*, 48 (55.82%) *G.lamblia*, and 13 (15.12%) *C.parvum* by using direct microscopic examination while, in immunocromatography assay the result found 8 (13.34%) , 41 (68.33%) and 11 (18.33%) for these parasites ,respectivelly . PCR assay showed 11 (16.41%), 45 (67.16%) and 11 (16.41%) for *E.histolytica*,*G.lamblia* and *C.parvum* respectively, in order to evaulate

immunocromatography assay. Among *Rotavirus* detection the virus was found 120 (37.5%) positive samples from 320 stool samples by using immunochromatography assay, while PCR assay it was found 84 (26.25%) positive *Rota virus* samples. Also the result found the sensitivity of *Rotavirus* (100%) and specificity (84.75%) , among bacterial isolates the results showed that there were 107 (33.43%) positive samples from 320 diarrhea children by using culturing and biochemical test. The results of bacterial infection by using PCR assay showed only 50 (15.62%) positive samples.it was found significant differences between parasitic and (viral,bacterial) infection.as well as high prevelance in males as 61.62%, 67.5% and 57.94% more than females as 38.37%, 32.5% and 42.05% in the detection of parasite,virus and bacteria respectivelly.

The result found that a significant differences for bacteria,parasite and virus, the rural area was found as 60.46%, 54.16% and 71.02% more prevelence in the parasite, viruse and bacteria infection more than urban area as 39.53%, 45.83% and 28.97% for parasites, viruses and bacteria, respectivelly.

The present study showed the distribution of children age with infectious agent,it was found significant differences in the virus and parasite. The highest number of *Rotavirus* and bacterial infection occurred in children have aged group less than two years as (49.16%), and (36.44%) respectively, it was slightly higher compare with other groups, while the more prevelance of parasites was recorded as (51.16%) in the 4-6 years.

The present study showed a significant increasing in viral infection (*Rotavirus*) that cause diarrhea in children in the winter season compare with hot season, otherwise higher parasites infection in hot season more than winter season.

Finally, current study indicated that the immunocromatography test have sensitivity as (66.67%, 93.18% and 100%),for detection of intestinal parasites (*E.histolytica,G.lamblia* and *C.parvum*) respectively, while the specificity of result by immunocromatography test were 94.55%,82.61% and 100% for *E.histolytica ,G.lamblia* and *C.parvum*,respectively.

The immunocromatography test result identify two parasites *C.parvum* with *G.lamblia* as adouble rather than single infection.

Among bacterial causes diarrhea, It was found the high number of total positive isolates from cultured 107 stool samples, 61 isolates (57%) for *E.coli* while 19 isolate (17.75%) for *Salmonella spp*.

The highest infection rate of watery diarrhea in children in the viral followed by bacterial then parasitic infection, (Triage panel test kit) was highly sensitive and specific for *G.lamblia,C.parvum* and *E.histolytica* and this assay could be utilized by personal that do not have extensive training in manual parasitological methods. The specificity and sensitivity of the triage panel test, it is approximaty to the *Rotavirus* kit that used for routine examination, which confirms the possibility of its use in the routine examination for the diagnosis of parasites.

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List of Abbreviations

Abbreviation	Meaning
cAMP	**Cyclic Adenosin monophosphate**
cGMP	**Cyclic Quanosin momophosphate**
C.P	***Cryptosporidium parvum***
D.W	**Distilled Water**
ELISA	**Enzyme Linked Immunosorbent Assay**
EMB	**Eosin Methylene Blue Agar**
E.H	***Entameba Histolytica***
G.L	***Giardia Lamblia***
H_2O_2	**Hydrogen Peroxide**
IC	**Immunocromatography**
ID50	**Infection Dose**
NSP4	**Non Structural Protein 4**
ORS	**Oral Rehydration Solution**
RT-PCR	**Rverse Transcriptase-Polymerase Chain Reaction**
S	**Second**
S-S agar	**Salmonella Shigella Agar**
TBE	**Tris Borate EDTA**
TNF	**Tumor Necrosis Factor**
VP	**Virus Protein**
WHO	**World Health Organization**
XLD	**Xylose Lysin Deoxy Cholate Agar**

CHAPTER ONE
INTRODUCTION
&LITRETURES REVIEW

1.1 Introduction

Diarrhea is defined as the production of stools of abnormally loose consistency, usually associated with excessive frequency of defecation and with excessive stool output (Yamada *et al*., 2011).

Diarrhea can be clinically divided into acute watery diarrhea which lasts several hours or days, acute bloody diarrhoea, and persistent diarrhoea which lasts 14 days or longer. Severe diarrhoea leads to fluid loss, and may be life-threatening, particularly in young children who are malnourished or have impaired immunity (Alaa *et al*., 2014). It is one of the leading causes of morbidity and mortality among children under five years of age in the developing world (Makobe *et al*., 2012). The inflammatory diarrhea occurs when there is damage to the mucosal lining or brush border, which leads to a passive loss of protein-rich fluids, and decreased the ability was to absorb these lost fluids. It can be caused by bacterial infections , viral infections, and It can be caused by parasitic infections (Sweetser, 2012). Infections with *Giardia duodenalis* (syn. *Giardia lamblia* or *Giardia intestinalis*) *Cryptosporidium* spp. and *Entamoeba histolytica* are considered to be among the most common and important causes of parasite-related diarrhea in human populations *(*Puebla *et al*., 2020) . Adadey and Quaye,(2017) they said accounting for about 20% of fatal diarrhea was occur globally, they are caused by viruses; associated with bacteria and some protozoans .

Rotavirus are the most common cause of viral gastroenteritis in children under 5 years of age. In 2013, more than 200 000 estimated deaths due to rotavirus occurred among children <5 years old worldwide, representing 37% of diarrhea deaths in this age group (Tate *et al*., 2016). Among the bacterial pathogens, *Escherichia coli* is an important etiologic agent of childhood

diarrhea and represents a major public health problem in developing countries (Nweze, 2009 and Gomes *et al*., 2016). *Salmonella* spp. predominantly cause a self-limiting diarrhoeal illness in healthy individuals (Feasey *et al*., 2012). The causes of diarrhea can be directly transmitted either through the fecal–oral route, tap water, food contaminated with diarrhea, or indirectly transmitted by some vectors (Al-Abbas, 2018).

Epidemiologic studies show that factors determining the occurrence of diarrhea in children are complex and the relative contribution of each factor varies as a function of interaction between socioeconomic, environmental and behavioral variables (Dessalegn *et al*., 2011). Clinical investigation of diarrhoeal aetiology can be expensive and time consuming and the results seldom directly affect patient treatment (Fauci & Morens, 2012). The principle diagnostic modalities have historically included Macroscopically, microscopy, culture, and antigen-based tests by different immunological pathways (Riddle *et al*., 2016).

Microscopy for parasites although inexpensive it can be time consuming and labor intensive, and diagnosis usually depends on the microscopist's level of expertise and training (Swierczewski *et al*., 2012). Culture methods are laborious and time consuming, with results often not available for 48 to 72 h (Humphries & Linscott, 2015).

Taniuchi *et al*, (2011) showed that molecular methodologies markedly improved the detection of enteric pathogens in stool samples based on polymerase chain reaction (PCR), most molecular test systems show higher sensitivity than microscopic or even cultural approaches (Wiemer *et al*., 2011). Studies have suggested that stool antigen assays offer an alternative to the routine microscopic examination method. They can be performed by laboratory technicians without the need for specific expertise and they provide the added

sensitivity required to confirm infections in patients with low parasite numbers (Al-Harthi & Jamjoom, 2007 and Gaafar, 2011).

Rapid detection techniques of fecal antigen have the potential to provide rapid, easy and cost effective diagnosis of enteric pathogen,one of them is triage qualitative Enzyme link immune assay EIA which is able to simultaneously detect specific antigens of *E. histolytica*, *C. parvum,* and *G. lamblia* in stool,it was simple , rapid and performed in approximately 15 min with fresh, frozen, unfixed human fecal specimens, and its sensitivity and specificity were 100% and 91.5%, respectively (Leiva *et al*., 2006 and Swierczewski *et al*., 2012). The others are immunochromatographic assay (ICA) is an attractive diagnostic tool because the test can be run individually, and the results are generally available in less than 30 min, has recently been developed for one-step,rapid and simultaneous detection of *rotaviruse* in human stool samples (Kim *et al*., 2014).

Serological diagnostic test,it is a quick test for some causative agent for diarrhea children,and some studies recorded as false positive,therfore;the study was aimed as the following:-

1.1.1 Aim of study :

Evaluation the efficiency of Immunochromatographic strip test by PCR assay used to detect gastrointestinal acute diarrhea causes and comparison with other diagnosis methods

The project can be a achieved through the following steps:

1-Comparison between modern immunochromatographic strip test

with routine diagnosis methods.

2- Comparsion between PCR assay and Immunochromatographic strip test to evaluate the sensitivity and specificity of Immunochromatographic strip test.

3-Isolation and identification of some bacteria from children suffering diarrhea and comparsion with PCR assay.

1.2 Literatures review:

1.2.1 Diarrhea :

Diarrhea can be defined as passage of loose or watery stools often associated with increased frequency of bowel movement,it's mean the passage within 24 hours of three or more watery or loose stool (Frank-Briggs, 2012). Generally,have high morbidity and mortality among children under the age of 5 years (Irfan *et al*., 2017). It is caused nearly 688 million cases worldwide, 499,000 cases and deaths among children under the age of 5 years especially in developing countries (Vos *et al*., 2016).

Diarrhea causes loss of a large amount of body fluids, salts, electrolytes, and other nutrients. Dehydration is the main cause of death, and also causes anorexia of the patient, as well as reduced intestinal ability to absorb water and nutrients (Yu *et al*., 2015). The causes of diarrhea can be transmitted either directly through the fecal–oral route, water, food contaminated with diarrhea, or indirectly by some vectors (Agustina *et al*., 2013). Intervention is required to prevent diarrhea which includes providing safe drinking water, use of improved sanitations, and hand washing with soap. Diarrhea can also be treated with oral rehydration solution (ORS) or a solution of clean water, sugar, and salt (Alemayehu *et al*., 2020).

1.2.2 Classification of diarrhea :

Diarrhea can be classified based on duration, pathophylogical mechanisms,and aetiological factor,according to duration, diarrhea can be acute, persistent and chronic, acute diarrhoea is of sudden onset, usually lasting less than 14 days and may be associated with fever and vomiting, persistent diarrhea usually begins as acute diarrhoea and lasts for about 14 days but less than one month,while chronic diarrhea usually begins insidiously and lasts longer than 28 days; it may be recurring or continuous (Joseph *et al*., 2017). Infectious agents are the most common causes of acute watery diarrhea among children in developing countries while chronic diarrhoea results from non-infectious causes like inherited metabolic disorders, sensitivity to gluten (celiac disease) or neoplasm (WHO, 2005 and Joseph *et al*., 2017).

Based on pathophysiological mechanisms, diarrhoea can be secretory, osmotic, dysentery, exudative, inflammatory or due to impaired motility, secretory diarrhea means that there is an increase in the active secretion, or there is an inhibition of absorption,the most common cause of this type of diarrhea is a enterotoxin that stimulates the secretion of anions, especially chloride ions (Cl–) (Ammoury & Ghishan, 2012 and Moon *et al*., 2015).

Osmotic diarrhea occurs when too much water is drawn into the bowels, if a person drinks solutions with excessive sugar or excessive salt,these can draw water from the body into the bowel and cause osmotic diarrhea (Purwar, 2012 and Moon *et al*., 2015). Inflammatory diarrhea occurs when there is damage to the mucosal lining or brush border, which leads to a passive loss of protein-rich fluids and a decreased ability to absorb these lost fluids, it can be caused by bacterial infections, viral infections, parasitic infections, or autoimmune problems such as inflammatory bowel diseases (Sweetser, 2012).

Exudative diarrhea occurs with the presence of blood and pus in the stool, this occurs with inflammatory bowel diseases, such as Crohn's disease or ulcerative colitis, and other severe infections such as *E. coli* or other forms of food poisoning (Moon *et al*., 2015).

Dysentery diarrhea occur when there is blood visible in the stools, the blood is a trace of an invasion of bowel tissue, Dysentery is a symptom of, among others, *Shigella, Entamoeba histolytica, and Salmonella* (Moon *et al*., 2015).

Based on aetiologic classification, diarrhea can be classified as infectious or non-infectious,infectious diarrhoea is far more common and can be viral, bacterial, parasitic or fungal, other basis of classification include association with underlying clinical conditions such as systemic autoimmune diseases and congenital disorder (Nguyen *et al*., 2006).

1.2.3 Factors associated with diarrhea

Several factors affect the occurrence of diarrhea; these include child's age, maternal education, household income, hygiene of feeding practices, breastfeeding status, malnutrition, personal hygiene, environmental sanitation, water availability and quality, and latrine utilization (Anteneh *et al.*, 2017 and Tuky & Semender, 2019).

1.2.4 General mechanisms of diarrheal infection

Diarrhea develops when intestinal absorption is impaired or secretion is enhanced,intestinal absorption and secretion are complex processes that are carried out by the enterocyte linning the small intestine (Anbazhagan *et al*., 2018). Sodium is actively absorbed primarily by villus tip cells via both

electroneutral (NaCl) and electrogenic (Na^+ / glucose or Na^+/amino acid co-transport) . Mechanisms, in contrast, intestinal fluid secretion occurs largely as a result of active $C1^-$ and/or HCO_3^- secretion by intestinal crypt cells, often combined with diminished Na^+ absorption by villus tip cells,numerous intracellular mediators regulate intestinal absorption and secretion by modification of ion transport across the enterocytes.These mediators include cyclic nucleotides (cAMP and cGMP) and calcium as well as lipid and arachidonic acid metabolic products.In addition to the enterocytes, several other cell types in the intestinal epithelium and lamina propria, including mucosal endocrine cells, enteric nerves, smooth muscle cells, fibroblasts, mast cells, and immune cells leg. Polymorphonuclear neutrophils (PMNs), lymphocytes and macrophages also play an important role in the regulation of intestinal fluid secretion, these cells may produce effector molecules such as serotonin, histamine, prostaglandins, interferon- γ/(IFN- γ) and tumor necrosis factor-a (TNF-a), which are known to modify ion transport and/or epithelial permeability, Infectious organisms can alter intestinal ion transport by. For example, producing toxins that stimulate intestinal secretion or by adhering to, or invading, the intestinal epithelial cells. The pathogen may directly alter the transport properties of the intestinal epithelial cells and/or stimulate a neurohormonal, enteric nervous system or immune response that alters the balance of intestinal absorption and secretion (Clark & Sears, 1996;Ammoury & Ghishan, 2012 and Camilleri *et al*., 2017).

1.2.5 Etiological agent of infection diarrhea:

Acute diarrhea could be defined as excess stool water with loss of electrolytes. It could be infectious or non-infectious,the infectious diarrhoea is

due to viruses, bacteria and parasites,and non-infectious diarrhoea is due to food poisoning, medications and inflammatory or ischemic bowel diseases (Chattopadhyay, 2012).

Infectious diarrhea is mainly caused by enteric pathogens including viruses, bacteria, and parasites,the most common of infectious agent are *rotavirus* and diarrheagenic *Escherichia coli (*DEC*)* were found to be common causes of childhood diarrhea (Bonkoungou *et al*., 2013).

Rotaviruses were soon confirmed as a major cause of life-threatening diarrhoea in infants and children <5 years of age worldwide (Crawford *et al*., 2017). *E. coli* strains involved in diarrheal diseases are one of the most important of the various etiological agents of diarrhea (Gomes *et al*., 2016) , *Salmonella and Shigella spp*. continue to be the major cause of acute diarrhea in resource-limited countries (Zhu *et al*., 2016).

Although there could be many other causes of diarrhea, the enteric protozoa *E. histolytica*, *G. lamblia and Cryptosporidium spp*. have been recognized as important causes of diarrhea among human beings (Paulos *et al*., 2019).

1.2.6 Parasites that cause diarrhea

1.2.6.1 *Gairdia lamblia*

Giardia duodenalis, also known as Giardia intestinalis and Giardia lamblia, is a flagellated parasitic microorganism. It colonizes and reproduces in the small intestine, causing a diarrheal condition known as giardiasis (Cacciò *et al*., 2018). Giardia lamblia is the causative agent of the diarrheal

disease,giardiasis,which is the leading cause of death in developing countries for children below five years of age (Mulatu *et al*., 2015).

The intestinal protozoan parasite *Giardia* intestinalis is distributed worldwide and estimated to cause 280 million diarrhea infections annually (Einarsson *et al.*, 2016). Giardiasis infection is prevalent in all age groups, but young children are at the greatest risk for contracting giardiasis especially those attending childcare centers (nurseries) (Boontanom *et al*., 2011). .

The clinical symptoms of giardiasis are abdominal cramping, bloating, malabsorptive diarrhea (steatorrhea) and weight loss (Bartelt & Sartor, 2015). The first signs of infection appear after 6–15 days and giardiasis is usually treated with metronidazole or other nitroimidazoles (Watkins&Eckmann,2014). This parasite transmits via fecal-oral route through direct or indirect ingestion of infectious cysts. The incubation period varies from 9 to 15 days after ingestion of cysts (Hooshyar *et al*., 2019).

1.2.6.2 Pathogenesis of *Giardia lamblia*:

The clinical manifestation of giardiasis vary from asymptomatic infection to chronic diarrhea with malabsorption,patients with symptomatic giardiasis have diarrhoea with loose foul smelling stool that is greasy, frothy, or bulky, other common gastrointestinal symptoms include abdominal cramps, bloating, nausea , decreased appetite, Malaise and weight loss is seen in majority of patients and fever is occasionally present, especially in the early part of the infection (Ciccarelli *et al*., 2013).

There is mounting evidence that the characteristics of both the parasite and the host may determine the outcome of infection. In symptomatic cases of

Giardia, trophozoites attach to the microvillus brush border by virtue of their ventral disc and produce a barrier effect or a damage to the brush border. This may result in decreased brush border enzymes, which ultimately leads to malabsorption of fat, Vit B-12, lactose and proteins (Ganguly&Raj,2016).

Several *Giardia* parasitic factors are believed to be involved in the induction of host pathophysiology, 58 kDa‘‘enterotoxin’’ produced by *Giardia* trophozoites causes excessive ion secretion and intestinal fluid accumulation via the induction of several signal transduction pathways in host enterocytes , while host disaccharidase deficiencies have been induced following exposure to parasite soluble extracts ranging from 32 to 200 kDa in size (Cotton *et al*., 2011) .

A number of predisposing factors in the host may also take part in causing the disease some of these host factors are age, nutritional status of the host, genetic predisposition as suggested by frequency of infection in blood group A and various states of immunodeficiency (Einarsson *et al*., 2016) . *Giardia lamblia* infection has been found to be associated with extraintestinal manifestations (such as food allergy, urticaria, reactive arthritis, and inflammatory ocular manifestations), can develop and possibly persist beyond detectable parasite shedding (Bartelt & Sartor, 2015).

1.2.7 *Entamebea histolytica* :

Entamoeb histolytica is a pseudopod-forming,non-flagellated protozoan parasite that causes proteolysis and tissue lysis (Sateriale *et al*., 2012). *E. histolytica* infection is a pathogenic protozoan parasite that causes amebiasis in human, and the infection can be a symptomatic or lead to severe disease with amebic colitis and complication is amebic liver abscess.Worldwide, amebiasis

remains a significant cause of morbidity and mortality worldwide,the infection of parasite is estimated to kill more than 55,000 people each year (Shirley *et al*., 2018). .

Globally, diarrheal disease is the third leading cause of death in children under five years of age with amebic colitis being a leading cause of severe diarrhea in low sanitation countries (Shirley *et al*., 2018). *Entamoeba histolytica* usually causes asymptomatic infection but in a minority of cases causes symptoms ranging from a few loose stools to profuse bloody diarrhea (Chalmers, 2014). Transmission generally occurs by contaminated water or food with fecal excretion of quadric nucleated natural cycts,or fecal-oral transmission within household and during male homosexual activity (Cheepsattayakorn&Cheepsattayakorn,2014).

1.2.7.1 Pathogenesis of *E. histolytica*:

Entamoeba histolytica causes intestinal and extraintestinal amoebiasis,they cause disease only when trophozoites invade the intestinal tissues, the trophozoite penetrates the epithelial cells in the colon, aided by its movement and histolysin, a tissue lytic enzyme, which damages the mucosal epithelium, amoebic lectin mediates adherence (Mahmud *et al*., 2017).

Recent studies have indicated that *E. histolytica* trophozoites can kill various kinds of host cells via apoptosis, including neutrophils (Sim *et al*., 2007), T cells (Huston *et al*., 2000), and macrophages (Blazquez *et al*., 2007). Trophozoites colonize the human gut mainly as commensals that feed on bacteria, these are able to invade the mucosa then initiating the acute phase of the disease,intestinal amoebiasis has multiple clinical manifestations ranging from frequent mild diarrhoea to dysentery with blood and mucus in the stool.A

major research interest on *E. histolytica* is driven by the observation that only one out of five infected persons develops the disease (Watanabe & Petri Jr, 2015 and Carrero *et al*., 2020).Trophozoites disrupt the intestinal mucosa and spread to other organs, causing various forms of extra intestinal amebiasis, of these, amebic liver abscess (ALA) is the most common (Faust & Guillen, 2012).

In the liver, the amoebae generate an inflammatory reaction and cause necrosis of hepatocytes, producing an abscess, there is a relative paucity of inflammatory cells from biopsy specimens, which is believed to be due to lysis of cells by *E histolytica* (Wuerz *et al*., 2012).

1.2.8 *Cryptosporidium Parvum*

Cryptosporidium spp.is a coccidian protozoan parasite that causes waterborne outbreaks worldwide using the fecal-oral route for the transmission of infection, *Cryptosporidium* is one of the leading pathogens which are responsible of the majority of the diarrheal infections (Bilung *et al*., 2017). Itis considered as a major diarrheal cause of diarrhea in children and immune-compromised people with life threating for those children under 5 years of age (Squire & Ryan, 2017).

Cryptosporidium is second only to rotavirus as an agent of moderate to extreme diarrhea in children <2 years of age, there is a (2–3) times higher risk of death among children aged 12–23 months with cryptosporidiosis than in subjects of a similar age group without diarrhea (Kotloff *et al*., 2013).It is a particular public health concern because the ID50, or the number of parasites required to establish infection in a human, is low for human volunteers who

received three different isolates of *C. parvum* oocysts.The ID50 was found to be as few as 9 oocysts (Hogan, 2012).

Fecal-oral route is considered as the normal way of inducing the infection by *Cryptosporidium* spp, by ingesting viable oocysts of animal and/or human origin, emitted with feces that contaminated food or water (Gerace *et al*., 2019). The main symptoms of Cryptosporidiosis include:profused or prolonged watery diarrhea , nausea, vomiting and low-grade fever (Carter *et al*., 2020).

1.2.8.1 Pathogenesis of *C.parvum*:

the infection begins when the ingested oocysts release sporozoites, which subsequently attach to and invade the intestinal epithelial cells causing direct injury to epithelial cells or indirect damage through the effect of inflammatory cells and cytokine recruited to the site of infection, leading to impairment in the absorptive and secretory functions of the gut, oocysts are mainly located in the jejunum and terminal ileum and bind on the apical surface of the intestinal epithelium , the parasite can cause damage to the microvilli where it attaches (Bouzid *et al*., 2013). After infection, the parasite alters the function of the intestinal barrier, increasing its permeability, absorption, and secretion of fluid and electrolytes (Gerace *et al*., 2019).

Cryptosporidiosis in immunocompetent hosts is usually self-limiting with mild to moderate diarrhea, or asymptomatic (Allison *et al*., 2011). The incubation period ranges from one to two weeks , where most common clinical manifestation is profuse watery diarrhea containing mucus, nausea, abdominal cramp-like pain, vomiting, anorexia, weight loss and mild fever (Borad & Ward, 2010).

There is the difference in infectivity based on *Cryptosporidium* species ingested and oocyst dose, for the species that commonly infect humans, *C. hominis* and *C. parvum* the lowest infectious dose has been calculated to be 10 oocysts.Although,in reality, one oocysts could be sufficient to cause infection in humans through direct or indirect routes of transmission (Zakir *et al*., 2021).

The effect of *cryptosporidium* on metabolic activities cause decrease in lactase which secrete from intestinal cells and responsible for conver disaccharide to monosaccharide, causing in intestinal lumen that high concentration lead to absorption the water from blood and tissues toward intestinal lumen, then causing diarrhea (Certad *et al*., 2017).

1.2.8.2 Diagnosis of parasite:

1.2.8.2.1. Macroscopic Examination

The fecal samples were examined by eye investigating color, consistency, bloody, mucus, smell and other substances (David and William, 2006). Bacillary dysentery is most commonly caused by microorganisms belonging to the genus shigella, whereas amebic dysentery is caused by the protozoan parasite *Entamoeba histolytica*. Both of them characterized by bloody diarrhea ,so that the diagnosis of amebiasis is difficult because of the nonspecific nature of symptoms. It is easily confused with shigellosis (Haque *et al*., 2003;Tanyuksel & Petri Jr, 2003 and Chávez-Ruvalcaba *et al*., 2021).

1.2.8.2.2. Microscopic Examination

The earliest diagnosis method of amebiasis is the microscopic examination of stool samples whereby *E. histolytica* trophozoites can be seen containing red blood cells. However, this method is prone to cause misdiagnosis as other

morphological similar strains, such as *E.dispar* and *E.moshkovskii*, are indistinguishable from *E.histolytica* under the microscope (Othman *et al*., 2020). Also, the sensitivity of microscopy can be confounded with false-positive results due to misidentification of macrophages as trophozoites and polymorphnuclear cells (PMNs) as cysts can also be seen in cases of amebic dysentery (bloody stool), that can also be seen in bacterial infection such as *Shigella* and *Salmonlla* (Singh *et al*., 2009 and Kotloff, 2017). Sensitive and specific detection of *E. histolytica* infection is required to minimize the unnecessary treatment of individuals who are based simply on microscopic examination of stool (Fotedar *et al*., 2007 and Buss *et al*., 2008).

While current methods of diagnosis *o*f *Cryptosporidium* species include identification of *Cryptosporidium* oocysts by microscopy, antigen detection by enzyme-linked immunosorbent assay (ELISA), and DNA detection by polymerase chain reaction (PCR). Stool microscopy has low sensitivity in detecting *Cryptosporidium* species (Nazeer *et al*., 2013), and acid-fast staining is a prerequisite to differentiate the *Cryptosporidium* oocysts from other parasites and also from the yeast cells that are frequently present in stool (van der Giessen, 2015).

Also,correct diagnosis of giardiasis is important for treatment and prevention of diseases.The laboratory diagnosis of *Giardia spp.* is mainly based on finding and demonstration of microscopic cyst in stool samples, but immunological-based assay and molecular methods also are available and are used for diagnostic or research proposes in developed countries. All diagnostic methods provide different sensitivity and specificity. This condition depends on some factors such as the method of test, the skill of operations and the stage that the test has been performed (Elmi *et al*., 2017).

Finally,detection of trophozoites, cysts, or oocysts in fresh or preserved stool specimens using microscope examination is the most common method of diagnosis particularly in resource limited countries. Though microscopy is fairly inexpensive, it can be time consuming and labor intensive, and diagnosis usually depends on the microscopist's level of expertise and training (Swierczewski *et al*., 2012).

1.2.8.2.3. Culture

Although cultivation of human intestinal protozoa is a useful method for detection and diagnostic purpose, routine culture techniques were not established for Giardia spp. in the clinical diagnostic laboratory. Cultivation of *Giardia spp*. is applied in the research laboratory for many types of studies that require a large number of trophozoite,the *Giardia spp*. is grown in the monoxenic and xenic type of culture system. In monoxenic system, the parasite has been grown in the presence of a single additional flora organism species and in axenic, parasite has been grown in the absence of any other accompanied alive cell,the most common and suitable used medium for *Giardia* axenic culture is Diamond's medium "TYI-S-33" which modified by Keister DB (Hooshyar *et al*., 2019).

Culture of *E. histolytica* in a clinical diagnostic laboratory is not feasible as a routine procedure and is less sensitive than microscopy as a detection method.Amajor problem that may arise during *E.histolytica* culture is the overgrowth of bacteria, other protozoan or fungi, parasite cultures are difficult,expensive and labor-intensive (Saidin *et al*., 2019).

1.2.8.2.4. PCR

The diagnostic methods becoming more widely used therefore are based on either fecal antigen detection or parasite DNA, but both require considerable

technical expertise. polymerase chain reaction (PCR) methods for detecting intestinal parasites are increasingly available and exhibit excellent sensitivity and specificity compared to conventional methods such as microscopy and antigen detection assays .PCR is also expensive and requires skilled personnel, which limits its use (Nazeer *et al*., 2013) .

1.2.8.2.5. Fecal Immunoassay

Several immunological method such as direct fluorescent antibody (DFA) ,ELISA, rapid dipstick-like tests and enzymatic immunoassays (EIA) used to the visualization of the whole parasite through fluorescence, where enzymatic immunoassays (EIA) permit an objective result by the obtained optical densities (ODs), these tests can be performed quickly and do not require an experienced and skilled microscopist,but require more than an hour to generate a result and are optimally used in settings with a lot of samples allowing one to test the samples in batch (Uyar, 2009 and van der Giessen, 2015).

Laboratory diagnosis of these parasite is usually achieved by microscopic stool examination which is still considered as the gold standard in spite of being time-consuming and highly dependent on operator's skills, to overcome these limitations, there is currently a growing interest for alternative methods such as multiplex PCR or antigen-based detection tests (RDTs) mostly relying on immunochromatographic methods. Compared with PCR assays, RDTs, sometimes used as rapid point of care tests (POCs) are easy to use being technically less complex, requiring a limited training. RDTs also offer a shorter hands-on time, reducing diagnostic delays, so that the results are usually available within 15–20 minutes. Giardia and Cryptosporidium spp. are two of the most common protozoan infections in the United States, and multiple combined tests have been developed to facilitate rapid screening for both

organisms simultaneously. Such tests include EIAs, IC assays, DFA assays, and multiplex PCR assays (McHardy *et al*., 2014), where some of studies were used immunocromatographic in diagnosis of *Giardia* and *cryptosporidium spp*. Such as solid-phase immune chromatographic test card system (Immuno Card STAT *Cryptosporidium/ Giardia* rapid assay), Although a growing number of RDTs for *Cryptosporidium* spp. and *G. intestinalis* have been developed in the last years and are now commercially available, their performances are often difficult to assess in part due to the low number of positive samples included in validation studies, that could therefore prevent their implementation in the clinical laboratory . In addition, some RDTs have a dramatically reduced sensitivity with unusual *Cryptosporidium* species that is of concern in the clinical setting (Goudal *et al*., 2019). Some of studies showed that EIAs are sensitive and specific for antigen detection in human fecal eluates. They might be useful additions for the diagnosis of giardiasis, cryptosporidiosis, and amebiasis, and for differentiating *E. histolytica* from *E. dispar* (Gaafar, 2011).

The Triage parasite panel (TPP) is the first immunocromatographic assay for simultaneous detection of antigens specific for *Giardia lamblia, E.histolytica/E.disbar,and Cryptosporidium parvum*.TTP provides diagnostic laboratories with a simple,convenient alternative method for performing-simultaneous, discret detection of *Giardia, Cryptosporidium,and E.histolytica/E.disbar*-spesific antigens patient fecal specimens (Garcia *et al*., 2000;Sharp *et al*., 2001;Leiva *et al*., 2006;Gaafar, 2011;Swierczewski *et al*., 2012 and Atia *et al*., 2016).

1.2.9 Viruses that cause diarrhea

1.2.9.1 *Rota virus*

Rota virus which was firstly discovered in 1973 in duodenal biopsy of nine children who suffered from acute diarrhea, these non-enveloped viruses of the Reoviridae family (Sadiq *et al.*, 2018). The RV genome contains 11 segments of double-stranded RNA (dsRNA), segments which encode six structural proteins (VP1–VP4, VP6, and VP7) and six non-structural proteins (NSP1–NSP5/NSP6) (Estes & Greenberg, 2013),thay are ten different *rotavirus* species (A–J) have been classified on the basis of sequence and antigenic differences of VP6 (Bányai *et al*., 2017).

RVA remains a leading cause of diarrhea morbidity and mortality (Iturriza-Gómara *et al*., 2019), resulting in an estimated 128,500 deaths annually among children under-5-year-olds, a majority occurring in low-income settings (Troeger *et al*., 2018).

Because of the frequency of the virus in the winter season, it was called winter diarrhea before the virus was discovered (Fenjan *et al*., 2020). *Rotavirus* is transmitted as a result of contamination of the hand with the stool of infected people, and then it reaches the mouth or respiratory system (Shaheen *et al*., 2017).

The symptoms often start with fever, nausea, and vomiting, followed by abdominal cramps and frequent watery diarrhea, which may last for 3-8 days,the infected children may also have a cough and runny nose (Mohammed *et al*., 2016).

1.2.9.2 Pathogenesis of *Rotavirus*

Rotavirus is the most common causes of moderate to severe diarrhea among children <5 years of age (Odiit *et al*., 2014).

The incubation time is 24 to 48 hours and illness usually lasts from 3 to 5 days, longer in immunocompromised individuals (Estes & Greenberg, 2013).

Initially Non structural protein 4 (NSP4) was thought to play a role primarily in viral replication , but recently it has generated a lot of attention in viral research on being designated as the "first viral enterotoxin" and the major virulence factor responsible for the watery diarrhoea of *Rota virus* infection (Srivastava & Jain, 2015).

NSP4 secreted from cells infected with *Rotavirus* binds to intestinal epithelial cells and signals through phospholipase C to increase cytoplasmic calcium levels, which activates calcium-dependent chloride channels, activation of these channels causes excessive secretion of chloride ions into the intestinal lumen, creating an osmotic gradient that facilitates the transport of water into the lumen, leading to secretory diarrhoea (Crawford *et al*., 2017).

The enterotoxin domain of the NSP4 is capable of making intestinal crypt cells permeable to Cl-, which in turn, mobilizes calcium ions (Ca2+), Ca2+ is very important to the *Rotavirus* infection,whereas Ca2+ plays a role in outer capsid stabilization and shedding during entry, it also acts as a messenger, indicating cellular damage and inducing cell death, as cells making up the upper intestinal microvilli die, the absorption properties of the intestine are hampered, resulting in a high amount of fluid remaining in the intestine,the influx of intercellular calcium ions into the cytoplasm causes cell death at high concentrations (Srivastava & Jain, 2015). NSP4 can also induce plasma

membrane permeability to calcium ions in healthy cells, causing fluid loss to the intestine (Dian *et al*., 2021).

All mechanisms contributing to the increase in fluid in the intestine contribute to diarrhea, NSP4 can also bind microtubules on the cellular membrane, and binding microtubules may result in the lack of activity in intestinal brush border disaccharidases,which can result in the sustenance of diarrhea. Finally, NSP4 interact with several cellular calcium ion chaperone and regulatory proteins,which effects calcium distribution and possibly the enteric nervous system (Ball *et al*., 2005 and Bialowas *et al*., 2016).

Currently, three mechanisms for RV-induced diarrhea are proposed: (1) osmotic diarrhea due to malabsorption, (2) secretory diarrhea due to the effects of NSP4 expression as enterotoxin agent, and (3) activation of the enteric nervous system(ENS) (Mary K Estes *et al*., 2020).

1.2.9.3 Diagnosis of *Rotavirus*

The diagnosis of infection with *Rotavirus* normally follows diagnosis of gastroenteritis as the cause of severe diarrhea. Most childreen admmited to hospital with gastroenteritis are tested for *Rotavirus* A, specific diagnosis of infection with *Rotavirus* A is made by finding the virus in the child's stool by enzyme immunoassay.Other methods such as electron micoscpoy and PCR are used (Beards, 2017). It can be diagnosed by antigen detection such as latex agglutination (LA) techniques, which have a sensitivity and specificity above 90% (Pereira *et al.*, 2011).

Rotavirus is fastidious in nature and difficult to cultivate and most of the group A human *Rotavirus* can be cultivated if they are pretreated with a proteolytic enzyme like trypsin (Dbaibo *et al*., 2013).

The immunochromatographic assay (ICA) is an attractive diagnostic tool because the test can be run individually, and the results are generally available in less than 30 min, has recently been developed for one-step,rapid and simultaneous detection of *Rotavirus* in human stool samples (Kim *et al*., 2014).

Molecular methodologies such as Reverse transcriptase polymerase chain reaction (RT-PCR) has been founed to be a highly sensetive and specific method for diagnosis of *Rotavirus* in fecal samples (Das *et al*., 2018).

1.2.10 Bacteria that causes diarrhea

1.2.10.1 *Escherichia coli*

The name of genus *Eschrichia,* was determined after the German pediatrician Theodor Escherich. It consists of facultative anaerobic gram-negative bacilli,non-sporing and rod shape that belong to the family enterobacteriaceae (Gupta & Pandey, 2019). Although most *E. coli* strains live harmlessly in the colon and seldom cause disease in healthy individuals, a number of pathogenic strains can cause intestinal and extraintestinal diseases both in healthy and immunocompromised individuals, diarrheal illnesses are a severe public health problem and a major cause of morbidity and mortality in infants and young children,it is transmitted by fecal oral route or direct person to person contact (Gomes *et al*., 2016).

E. coli strains involved in diarrheal diseases are one of the most important of the various etiological agents of diarrhea, where strains have evolved by the acquisition, through horizontal gene transfer, of a particular set of characteristics that have successfully persisted in the host (Croxen *et al*., 2013). It is transmitted by fecal oral route and direct person to person contact,which classified into six main categories depend on the basis of distinct

epidemiological and clinical features,as well as specific virulence determinants :which include *Enteropathogenic E. coli (EPEC), Enterotoxigenic E. coli (ETEC), Enteroinvasive E. coli (EIEC), Enterohemorrhagic E. coli (EHEC) or Shiga-toxin producing E. coli (STEC), Enteroaggregative E. coli (EAEC)* and Diffusely adherent *E. coli(DAEC)* (Lozer *et al*., 2013).

1.2.10.2 Pathogenesis of *E.coli*

Major enteric pathogen *E. Coli* causes several diseases by using different mechanisms and virulence factor such as toxin production, colonization factors, hemolysins, siderophores, capsules, and adherence pili in extraintestinal infections,the bacterium *E. coli* causing diarrheal infection is contracted orally via digestion of food or water contained a pathogenic strain,it was shed by an infected person (Darko, 2012) . Several categories of *E. coli* are associated and regarded as the most common causes of infection , First group that produces enterotoxins is called enterotoxigenic *E. coli* (ETEC) which is the most important cause of diarrheal diseases in infants in developing countries, some strains of ETEC produce major virulence factors such as heat Label enterotoxin(LT) and/or heat-stable enterotoxin (ST- which promotes guanylyl cyclase in enteric epithelial cells and stimulates fluids excretion) . The other varulence factor such as colonization factors (CFs-by which permit the bacterium to attach the epithelium). Extremely severe diarrhea caused after exposure to strain has the ability to produce both types of toxins (Tobias & Vutukuru, 2012). .

A second group of Enteroinvasive *E. coli* strains (EIEC) have invasion ability and causing an infection similar to that caused by *Shigella spp*, the EIEC invaded intestinal mucosal epithelial cells and causes diseases such as dysentery

and diarrhoeae in humans, these diseases occur usually in kids in developing countries (Gomes *et al*., 2016).

A third group of enteropathogenic *E coli* (EPEC), are important small group which caused watery diarrhea Infection and epidemic hemorrhagic colitis. These strains have the ability to link with the epithelial surface of the intestine via the adhesive BFP (Bundle Forming Pili). Most strains of EPEC produced small amounts of a potent Shiga-like toxin that has both enterotoxin and cytotoxin activity instead of both ST and LT production, which are considered negative for them (Ochoa & Contreras, 2011). Other serogroups(the fourth group) of *E.coli* strains are Enterohemorrhagic *E.coli* (EHEC) that producing Verotoxin (VTEC) and this naming came from their cytotoxic effect on vero cells (a line of African green monkey kidney cells),these group poses a serious public health concern ,but only a small number of serotypes are associated with human diseases such as O157:H7 which is represent a main source of *E. coli* food poisoning.This type caused enteritis or gastroenteritis and associated with hemorrhagic colitis, a severe form of diarrhea, and with hemolytic uremic syndrome (Sperandio & Nguyen, 2012 and Majowicz *et al*., 2014).

Other serogroups are Enteroaggregative *E.coli* (EAEC) which are produced ST-like toxin (EAST 1). A hemolysin and several diseases such as acute and chronic diarrhea and also food-borne illnesses in developed countries(Jensen *et al*., 2014). The diffusely adherent *E.coli* (DAEC) which are considered as causative agents of infection such as watery diarrhea in children. Clinical manifestations of diarrhea episodes caused by diarrhea-causing DAEC may include watery or bloody diarrhea,abdominal pain, dehydration and fever (Le Bouguénec & Servin, 2006 and Abbasi *et al*., 2016).

1.2.10.3 Diagnosis of *E.coli*

The identification of *E.coli* cannot be based only on stool culture and bio chemical criteria. Since E.coli indistiguishable from the non pathogenic *E.coli* commonly found in human feces and time consuming,moreover,specific sertyping is not always correlated with pathogenecity.Since several virulence factors and DNA sequences of *E.coli* have been identified, they can be determined by the presence of genes coding for specific varulence factor,which are absent in non-pathogenic strain, polymerase chain reaction (PCR) is commonly used method that gives rapid,reliable results,and shows high sensitivity and high specificity, several PCR methods with both single and multiple target genes,have been reported detecting the different *E.coli* pathotypes (Croxen *et al*.,2013 and Hamada *et al*., 2016). .

1.2.11 *Salmonella*

Salmonella is a genus of the family *Enterobacteriaceae*, composed of Gram-negative, nonspore-forming, rod shaped bacteria that measures 2-5 x 0.7-1.5 μm in size,motile by peritrichous flagella (Fàbrega & Vila, 2013). *Salmonella* are facultative anaerobes that can grow in a temperature range of 5 - 45°C (optimum 35 - 37°C) and have the ability to grow at low pH (optimum 6.5 – 7.5), all *salmonellae* are facultative intracellular pathogen and can invade macrophages,dendritic and epithelial cells (Bhunia, 2018).

Salmonellae are catalase positive, reduce nitrate to nitrite and do not produce cytochrome oxidase, *Salmonellae spp*. are negative for indole and urease, they are methyl red positive and negative for Voges-Proskauer reaction, produce hydrogen sulphide from inorganic sulphur, decarboxylate lysine, arginine and ornithine (Wain & Hosoglu, 2008).

The *salmonella* genus is made up of two species, *S.bongori* and *S.enterica*. Strains of *S.bongori* and *S.enterica* have been further classified into serovars based on antigenic epitopes in the O-antigen and flagellin (Diaz-Ochoa, 2015).

Human infections with *S. enterica* subspecies I pathovars are generally distinguished based on their pathogenic potential; either those causing typhoid fever or para-typhoid fever are termed *typhoidal Salmonella* serovars, and those associated with a localized gastroenteritis in immunocompetent individuals are termed *nontyphoidal Salmonella* (NTS) serovars (Crawford *et al*., 2013).

Non-typhoidal salmonella spp. (e.g., *Salmonella enterica serovar typhimurium*) are the most burdensome food-born diarrheal pathogens,of the approximate 2.8 billion cases of diarrheal illness occuring worldwide annually,non-typhoidal salmonella is estimated to be responsible for 3%, or 93.8 million of these cases (Majowicz *et al.*, 2010 and Jayaratne, 2017).

Salmonella spp. infections range from gastrointestinal infections that are accompained by inflammation of intestinal epithelia,diarrhea, vomiting and abdominal cramps lasting for an average of 1 week to typhoid fever,a life threatening systemic infection (Hensel, 2004 and Drancourt, 2010). *Salmonella* has been listed as one of the bacteria that cause diarrheal cases in large number of people (Rowe *et al*., 2010 and Ao *et al*., 2015).

Non-typhoidal salmonella spp. transmission to humans can occur by consumption of animal food products, non-animal food products,contaminated water or by contact with animals (Sánchez-Vargas *et al*., 2011). Compared to adults even when it comes to *non-typhoidal Salmonella* infections, children and elderly people are at more risk just as any immunocompromised patients which leads to developments of more severe symptoms than normal healthy individuals who acquire *non-typhoidal salmonella* infection (Hedberg, 2011).

1.2.11.1 Pthogenesis of *Salmonella*:

Salmonella cause localized infection of the gastrointestinal tract and also have the ability to multiply in the reticuloendothelial system and cause systemic infection (Gillespie & Hawkey, 2006 and Monack, 2013) .

Salmonella is an enteric pathogen that is usually acquired through the consumption of a contaminated food or water source,once ingested, *Salmonella* makes its way to the intestines, where it can enter the epithelium via two routes, *salmonella* enters the epithelium either via M cells, which are specialized cells in the intestinal epithelium that allow immune cells to sample antigens in the lumen, or through intestinal epithelial cells (Broz *et al*., 2012). After *Salmonella* has crossed the intestinal barrier, resident immune cells located in the epithelium such as macrophages,will phagocyte the pathogen (Broz *et al*., 2012).

These infected phagocytes predominantly macrophages carry the bacteria to the mesenteric lymph nodes, multiply there and reach blood stream via thoracic duct resulting in transient primary bacteremia, during this transient bacteremia, *Salmonella* are seeded in the liver, gall bladder, spleen, lymph node and bone marrow where further multiplication takes place. After that, the bacteria are released into the bloodstream initiating secondary bacteremia and this marks the onset of clinical disease (Huang & DuPont, 2005 and Feasey, 2013).

Following invasion, *salmonella* is thought to induce an inflammatory diarrhea caused by the influx of neutrophils and subsequent disruption of intestinal barrier function by neutrophil activity (Zhang *et al*., 2003 and Jayaratne, 2017).

1.2.11.2 Diagnosis of *Salmonella*:

The routine method to detect *Salmonella* in stool is the standard cultural method which entails a non-selective pre enrichment step followed by a selective enrichment (to enhance the number of *Salmonella* cells versus the competitor microorganisms), isolation on selective agar medium, bacterial identification by biochemical and serological tests, to confirm the suspect colonies grown on the selective agar, although this method is very sensitive and inexpensive, it is labor-intensive, extremely time consuming (up to five days to obtain results), and not suitable for testing a large number of samples (Melo *et al*., 2016).

There are several alternative methods were proposed for the easy detection of *Salmonella* such as immunodetection technology relying on the specific binding of antigen and antibody, and molecular biological detection, such as that using Polymerase Chain Reaction (PCR) (S. Li *et al*., 2020). Enzyme-Linked Immunosorbent Assay (ELISA) (J. Li *et al*., 2013),it have become relatively mature detection technologies in the laboratory. However, limitations in these systems, such as insufficient information, complicated methods, and false positive results caused by competitive substances or the structural analogues of target bacteria in samples (S. Li *et al*., 2020).

Some of studies revealed that nucleic acid based methods by polymerase chain reaction proved to be the best known, fast ,sensitive assay and simple operation compared to the culture method (Kumar *et al*., 2008 and S. Li *et al*., 2020).

CHAPTER TWO
MATERIALS &METHODS

2.1 Materials

2.1.1 Equipments and Instruments

The equipments and instruments used through this study were listed in table (2-1) and (2-2).

Table (2-1): The equipments and their origin.

No.	Equipments	Origin
1	Autoclave	Hirayamy/Japan
2	Centrifuge	Hettich/Germany
3	Distillatory apparatus	LabTech/ Korea
4	Electrophoresis apparatus	Bio-Rad/Italy
5	Hood	LabTech/ Korea
6	Hot plate	
7	Incubator	Memmert/ Germany
8	Light microscope	Human/Germany
9	Nanodrop	Thermo Scientific/ UK
10	Oven	Hirayama /Japan
11	Refrigerator	LG /Korea
12	Sensitive balance	Sartorius /Germany
13	Thermo cycler	Syngene/ England
14	UV- Trans illuminator	Stuart/UK
15	Vortex mixer	Memmert /Germany
16	Water bath	Techen/ England

Table (2-2): The instruments and their origin.

No.	Instruments	Origin
1	Bunsen burner	Shndon/England
2	Beakers	Iwaki glass/Japan
3	Conical flask	Marienfeld/Germany
4	Digital camera	Canon/ Japan

5	Disposable syringe	Changazhou medical appliances/China
6	Disposable plastic cup (50 ml)	
7	Disposable tips	CAPP/Denmark
8	Disposable tips with filter	Bioneer/Korea
9	Disposable gloves without powder	Bioneer/Korea
10	Eppendrof tube (1.5)ml	Heittch/Germany
11	Eppendrof rack	Eppendrof/Germany
12	Loop	Shndon/England
13	Micro centrifuge (1.5 ml tube)	Hettich/ Germany
14	Micropipettes	Slamid/Germany
15	PH meter	Radiometer /Denmark
16	Petri dishes Plates	Sterilin/England
17	Slides	Sail Brand/China
18	Sterile Mask	Bioneer/Korea
19	Swabs	Arth Al-Rafidain/China
20	Test tubes	Arth Al-Rafidain/China

2.1.2 Chemicals

Chemicals used in this study were listed in table (2-3)

Table (2-3): Biological and Chemical materials.

No.	Biological and Chemical materials	Origin
1	α-naphthol (C1OH80)	BDH/ England
2	Agarose	BDH/ England
3	Benedict Reagent	Syrbio/Switzerland
4	DNA loading dye	BDH/ England
5	Ethidium bromide	BDH / England
6	Hydrogen peroxide (H2O2) 70%	SDI (Iraq)
7	Gram's Stain	Drugs and medical appliances/Iraq

8	Iodine stain	BDH/England
9	Kovac's Indole	Himedia/India
10	DNA ladder(100)bp	KAPA/South Africa
11	Methanol	GCC/UK
12	Methyl red	BDH/England
13	Normal saline	Haidylena/Egypt
14	N,N,N,N-tetra-Methyl-P- Phenylen Diamine Dihydro Chloride	BDH
15	Oil immersion	BDH/UK
16	Tris borate EDTA	Difco/ USA
17	Urea Solution	Mastdiagnostic/UK
18	Voges – proskauer reagent	Biomerieux/France
19	Ziehl-Neelsen Stain	SYRBIO/Switzerland

2.1.3. Culture Media:

The following culture media were used in the present study

Table (2-4) : Culture media used in present study.

No.	Medium	Origin
1	Eosin-Methylene Blue Agar	Himedia/India
2	MacConkey Agar	Himedia/India
3	MR-VP broth	Oxoid/UK
4	Nutrient broth	Himedia/India
5	Peptone water broth	TM MEDIA/India
6	Salmonella Shigella Agar	Himedia/India
7	Selenite F Broth	Himedia /India
8	Simmon Citrate Agar	Oxoid/England
9	Triple Sugar iron agar	Difco/USA
10	Xylose Lysine Deoxycholate Agar	Himedia/India

2.1.4 Amplification PCR Primers

The primers sequence which used in the present study listed in table (2-5).

Table (2-5): Primers used in this study.

Microorganism	Target gene	Primer Sequence 5´- 3´	Product Size (bp)	Reference
Rotavirus	*VP4-F* *VP4-R*	TATGCTCCAGTNAATTGG ATTGCATTTCTTTCCATA ATG	663	(Durmaz *et al*., 2014)
E.histolytica	*EHCP8-SIF* *EHCP8-SIR*	ATTTGTTAAGTATTGTAAATGGG ATTGTAACCTTTCATTGTAACAT	605	(Bairami *et al*., 2018)
G.lamblia	*GLCP6-SIF* *GLCP6-SIR*	AATCTGTTGACTTAAGGGAGTA ATTGAGTCATTATAGGGATTGT	463	(Bairami *et al*., 2018)
C.parvum	*CRYI8$_S$-SIF* *CRYI8$_S$-SIR*	TAAACGGTAGGGTATTGGCCT CAGACTTGCCCTCCAATTGATA	240	(Bairami *et al*., 2018)
Salmonella	*16SrRNA-F* *16SrRNA-R*	TGT TGT GGT TAA TAA CCG CA CAC AAA TCC ATC TCT GGA	574	(Ziemer and Steadham, 2003)
E.coli	*PHO-F* *PHO-R*	GTGACAAAAGCCCGGACACCATA AATGC TACACTGTCATTACGTTGCGGATT TGGCG	903	(Aklilu and Raman, 2020)

2.1.5.The Molecular Kits

2.1.5.1 RNA Extraction kit

The contents of the kit that used for the extraction of viral RNA from stool samples are explained in tables (2-6)**.**

Table (2-6): RNA purification kit

RNA Purification kit	Contents	Origin
GENEzolTM Tri RNA extraction kit	GENEzol Reagent Wash Buffer 1 Wash Buffer 2 RNase-free Water Dnase I DnaseReactionBuffer RB Column 2 ml Collection Tube	Geneaid(USA)
GoScript™ Reverse Transcription System	Reverse Transcriptase 5X Reaction Buffer $MgCl_2$ PCR Nucleotide Mix Oligo(dT)15 Primer Random Primers Nuclease-Free Water Recombinant RNasin® Ribonuclease Inhibitor	Promega(USA)
Accupower® PCR premix	Top DNA polymerase dNTP (dATP, dCTP, dGTP, dTTP) Reaction buffer, with 1.5mM $MgCl_2$ Stabilizer and tracking dye	Bioneer(Korea)

2.1.5.2 DNA Extraction kit

The contents of the kit that used for the extraction of DNA from stool samples is explained in tables (2-7)**.**

Table (2-7): DNA purification kits

DNA Purification kit	Contents	Origin
Presto™ Stool DNA Extraction Kit	ST1 Buffer ST2Buffer ST3Buffer Washing Buffer Elution Buffer Inhibitor Removel Column GD Colmn Bead beating tube 2ml collection tube 2 ml centrifuge tube	Geneaid(USA)

2.2 Stains and Solutions Preparation :

2.2.1.Benedict Solution

Benedict's reagent is a deep-blue aqueous solution. Each liter contains:

17.3g. copper sulphate

173g. sodium citrate

100g. anhydrous sodium carbonate or, equivalently, 270g. sodium carbonate decahydrate

The sodium carbonate and sodium citrate are mixed first, and then the copper sulphate was added slowly with constant stirring (Benedict, 1909).

2.2. 2.Gram's Stain Solution according to (Coico, 2006).

It consists of crystal violet stain, iodide solution, ethanol, and safranin stain.

Counterstain

2.5 g safranin was mixed with 100 ml of 95% ethanol.

Crystal violet solution

20 g crystal violet (85% dye) was dissolved in 100 ml of 95% ethanol. Store up to 1 year, at room temperature (25 °C).

Decolorizing solution

Equal volumes of 95% ethanol and acetone were mixed.

Gram's iodine solution

1 g iodine crystals (Sigma) and 2 g potassium iodide (Sigma) were dissolved in 5 ml water, then 240 ml water and 60 ml of 5% (w/v) sodium bicarbonate solution were added and mixed well and stored up to 6 months in an amber glass or foil-covered bottle, at room temperature (25 °C).

.2.2.3. 1X Tris Borate EDTA (TBE) Buffer:

Prepared by diluting the concentrated TBE buffer (10X). It was used to dissolve agarose powder and in electrophoresis procedure. 100 ml of TBE (10X) was added to 900 ml of distilled water to reach to 1X TBE concentration (Sambrook and Russell, 2001).

2.2.4. Urea Solution (20%):

This solution was prepared by dissolving 20 g. of urea in small volumes of D.W. and then completed to 100 ml by D.W. Then Millipore filter paper was used to sterilize this solution. The purpose of this solution was to detect of urease enzyme in urease test (MacFaddin, 2000).

2.2. 5.Normal Saline Solution:

This solution was prepared by adding 0.85g of NaCl to 90 ml of D.W and completed by distilled water to 100 ml (Collee *et al*., 1996).

2.2.6. Ziehl-Neelsen Stain according to Shyamasundari and Rao, (2019).

Solution 1 carbol fuchsin

Basic fuchsin 4g

Ethanol 95% 20ml

Phenol 8ml

Distilled water 100ml

Basic fuchsin was dissolved in alcohol and then water added slowly while shaking, first melt phenol crystals at 56 ˚C and then 8ml was added to the stain.

Solution2 declorizer

Ethanol 95% 9.7ml

HCL 3ml

HCL was added into Alcohol slowly

Solution3 counter stain

Methylene blue 300mg

Distilled water 100ml

2.2.7. Lugul's, Iodine Solution:

This stain can be prepared by adding:

5 gm Iodine crystals.

10 gm Potassium iodide.

100 ml D.W.

Iodine crystals first heated in D.W. then, potassium iodide was added until dissolved completely (WHO, 1991).

2.2.8. Methyl red Reagent

It was prepared by dissolving 0.1 gm of methyl red in 300 ml of 96% ethanol, and then, completed to 500 ml with D.W. It is used to identify the complete fermentation of glucose (MacFaddin, 2000).

2.2.9. Voges-Proskauer reagents

Two solution were mixed to prepare this reagent:

Solution1(a-naphthol):5 g of this material was dissolved in 100 ml of ethanol.

Solution 2(40% KOH):40 g of KOH was dissolved in 100 ml of D.W. Then 6 drops of 5% of a-naphthol and 2 drops of 40% KOH was added to both test tubes. This reagent used to identify the formation of acetoine in the culture media (MacFaddin, 2000).

2.2.10 Kovac´s Reagent:

This reagent was prepared by adding 5g of P-dimethyl amine Benz aldehyde to amyl alcohol (75ml), then 25 ml of concentrated hydrochloric acid (HCl) was added, This reagent used to identify the formation of indole (MacFaddin, 2000).

2.2.11 Catalase Reagent

This reagent was prepared in 3% concentration of H_2O_2 and used to identify bacterial ability to produce catalase enzyme (MacFaddin, 2000).

2.2.12 Oxidase Reagent

The reagent was prepared directly by dissolving 0.1g of tetramethyl-ρ-paraphenylene diamine dihydrochloride in 10 ml of distilled water, to be stored in a dark container. Every time used, the reagent has been freshly prepared (MacFaddin, 2000).

2.3. Method

2.3.1. Study Design

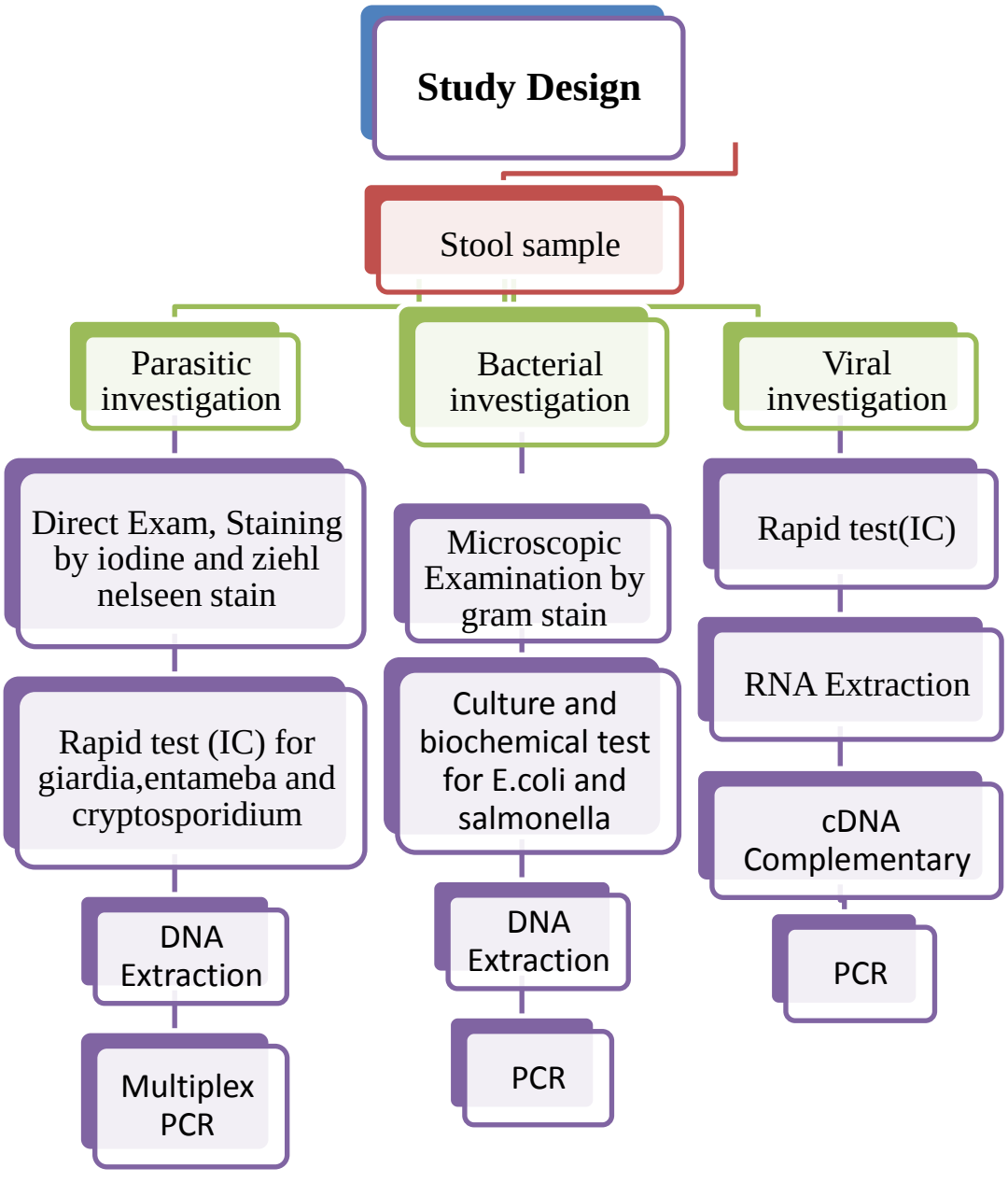


Figure (2-1): Study Design

2.3.2. The Studied groups

The current study was conducted in Babylon Teaching Hospital for Maternity and Children, and the number of children included in the study was (320) suffering from diarrhea, with ages ranged from (1 week - 6 years), during the period from November 2020 to June 2021, to identify the most important causes of diarrhea in children.This stage included many steps, after collecting exit samples from children with acute diarrhea, lying in emergency rooms and children's lounges, and recording the required information in the questionnaire list assigned during the study.

2.3.3. Exclusion Criteria

Study had to report original data on the aetiology of viruses, bacteria and parasites associated with gastrointestinal diseases with a particular emphasis on diarrhoea before they could be included in this study. Study was excluded if they focused on participants above 6 years. The study was again excluded if quantitative data was not available or accessible. The process of data search and inclusion was summarized in Appendix (1).

2.3.4. Ethical Approval :

A consent from patients parents was taken .

2.3.5. Samples Collection

The stool samples were collected in suitable clean plastic container, and each container was labeled by a special number. Samples were stored in clean Eppendrof tube at deep freeze -20 ˚C for molecular study. The samples should be examined within (30 min to1 hour) from the time its gained from the patient, because delaying the examination for a longer period of time leads to disintegrate of trophozoite (Garcia, 2001).

2.3.6. General Stool Examination

This test involves two steps, macroscopic and microscopic examinations, the macroscopic examination of stool sample was done visually. For consistency (formed, unformed 'soft', or liquid), color (white, yellow, brown, or black), smell and presence of any abnormal components (mucus or blood). While the Microscopic examination of stool sample was done to demonstrate RBCs, pus cells, Monillia, bacteria, intestinal protozoa, fatty drops, undigested food, and normally small to moderate epithelial cells. The presence of large number of epithelial cells indicates that the intestinal mucosa was irritated (Guerrant *et al*., 2002 and David and William, 2006).

2.3.6.1. The pH measurement of stool

The pH of the stool was measured using special paper tapes.

2.3.6.2. Detection of sugars in the stool

This detection was done by using a blue-colored Benedict solution, by adding an amount of (3ml) to a quantity of stool of (0.5-1.0 gm), in a glass tube and shaking it well, then it was placed on a heat flame to a boiling point (Simoni *et al*., 2002).Appendix (4)

2.3.6.3. Microscopic Examination:-

Direct Method

From each stool sample, smear with normal saline and Lugols iodine was examined. Two direct smears were examined from each faecal sample, by preparing two clean dry microscope slides, each with normal saline and lugols iodine solutions.

By using clean fine wood stick, the stool specimen was touched in different sites, especially where streaks of blood or pus were noticed, then mixed thoroughly with each drop of normal saline and lugols iodine solutions on the prepared 2 slides, then each half of the slides was covered by cover slip.

The smear was examined thoroughly under the low (x10) power and high (x40) power of the microscope (Cheesbrough and McArthur, 1976).

2.3.6.4. Ziehl-Neelsen Stain

Modified ziehl neelsen-acid fast stain has been used in the current study for identification of *cryptosporidium* oocytes. Briefly, a small drop of the concentrated stool sample was used to prepare thin smear on a clear microscope glass slide. The slides were allowed to air dry and then fixed for 3-5 min in absolute methanol. Fixed slides were placed on staining racks and flooded with ZN carbol fuchsin for 20-25 min and the slides were rinsed under slow ran tap water. Five percent acid Alcohol was added approximately for 20-30 s. for de-colorization and the slides were rinsed under slow ran tap water. The slides were then flooded with methylene blue for 2-3 min. for counter staining and then slides were rinsed under slow ran tap water and allowed to air dry. After drying, slides were examined microscopically with a drop of oil under high power (100x oil immersion) lens. Positive *cryptosporidium* oocytes slides stain bright red with a blue background (Garcia *et al*., 2003).

2.3.7. CERTEST Crypto+Giardia+Entamoeba COMBO CARD TEST Immuno-Chromatography (IC)

2.3.7.1. Specimen Preparation

The parasite immunochromatography kit contents were listed in Appendix (2). The specimen preparation by immunochromatography kit done in steps:

1. The stool collection tube cap was took out, and the stick was used to pick up sufficient sample quantity. Then, the stick was introduced once into 4 different parts of the stool sample, faecal sample was collected (approx. 125 mg) and added to the stool collection tube. For liquid samples, added approx. 125µL in the stool collection tube by using a micropipette.
2. The tube was closed with the diluent and stool sample. The tube was shaked in order to assure good sample dispersion.

2.3.7.2 Test Procedure

All tests, stool samples and controls have been allowed to reach room temperature (15-30 °C) prior to testing. pouches not opened until the performance time of the assay according to manufacture company.

1. The stool collection tube was proceeded to shake in order to assure good sample dispersion.
2. CerTest Crypto+Giardia+Entamoeba combo card test was removed from its sealed bag just before using it.
3. The stool collection tube was taken , the end of the cap was cut and it was dispensed 3 drops in the circular window marked with the letter A, 3 drops, using the same tube, in the circular window marked with the letter

B (6), and 3 drops, using the same tube, in the circular window marked with the letter C. The solid particles were avoided adding with the liquid.

4. The results were read at 10 minutes.

2.3.7.3.Interpretation of Results

Positive result: a distinct red colored band appears on test line regions, in addition to a green line on the control line region.

Negative result: no line appear in the test line region. A distinct green line showed on the control line region.

Invalid: The control line next to the test line does not become visible within 10 minutes after the addition of the sample.

2.3.8. Detection of *Rota virus*

2.3.8.1. LumiQuick, Adeno-Rota Virus Antigen Comb Test Card Immuno Chromatography (IC)

The specimens were screened for *Rotavirus* antigen through Immuno Chromatography Test (ICT). The *Rotavirus* immunochromatography kit contents listed in Appendix (3).

2.3.8.2. Reagents Preparation

All specimen Preparation carried out as the following steps:

1. The sample bottle was unscrewed, the attached applicator stick was attached on the cap to transfer small piece of stool (4-6 mm in diameter; approximately 50 mg – 200 mg) into the sample bottle containing specimen preparation buffer. For liquid or semi-solid stools, 100 microliters of stool was added to the vial with an appropriate pipette.

2. The stick in the bottle was replaced and securely tighten . Stool sample was mixed with the buffer thoroughly by shaking the bottle for a few seconds.

2.3.8.3. Assay Procedure

1. The test card was removed from the sealed foil pouch.
2. The sample bottle was holden upright with the tip point toward the direction away from the test performer, the tip was removed out.
3. The bottle was holded in a vertical position over the sample well of the test card, delivered 3 drops (120-150 µL) of diluted stool sample to the sample well.
4. The result was read between 5 - 10 minutes. A strong positive sample may show result earlier.

2.3.8.4 Interpretation of Results

1. Positive result: A distinct pink colored band appears on test line regions, in addition to a pink line on the control line region.
2. Negative result: No line appears in the test line region. A distinct pink line shows on the control line region.
3. Invalid: The control line next to the test line does not become visible within 10 minutes after the addition of the sample.

2.3.9 Diagnosis of Bacteria

2.3.9.1 Cultural Characteristics

Stool sample was cultured on selective media, then the positive culture was recognized according to the morphological properties like (Colony size, shape, color and natural of pigments, edge, texture and lactose fermentation on MacConkey agar) (Procop *et al*., 2020).

2.3.9.2.Preparation of culture media

2.3.9.2.1 MacConkey Agar

The media was prepared by dissolving 56 grams of macconkey agar in one liter of distilled water, then it was heated to a boiling until the media completely dissolved, then it was sterilized by autoclave at 121° C for 15 min, and then it was cooled to (47° C).Then ,poured in petri dishes. This media Utilize for the primary isolation of G^{-ve} bacteria and to distinguish between lactose fermenters bacteria and non-lactose fermenter (Winn Washington *et al*., 2006).

2.3.9.2.2 Nutrient Agar

The media was prepared by dissolving 28 grams of nutrient agar in 1000 ml of distilled water, then it was heated to a boiling until the media completely dissolved, then it was sterilized by autoclave at 121° C for 15 min , and then it was cooled to (45° C). Then ,poured in petri dishes. This media used for Subculture and growth of bacterial isolates (MacFaddin, 2000).

2.3.9.2.3 Nutrient broth

This medium was prepared by dissolving 13 grams of nutrient broth in one liter of distilled water, then it was heated to a boiling until the media completely dissolved, then it was sterilized by autoclave at 121° C for 15 min. Then ,poured in petri dishes. This media used to grow and preserve the bacterial isolates (MacFaddin, 2000).

2.3.9.2.4 Salmonella-Shigella Agar (S-S agar)

The media was prepared by dissolving 63 grams of salmonella-shigella agar in 1000 ml of distilled water, then it was heated to a boiling until the media

completely dissolved , and then it was cooled to (50 °C). Then ,poured in petri dishes. This is selective media for *Salmonella* and some *Shigella* spp (Forbes *et al*. 2016).

2.3.9.2.5 Eosin-Methylene Blue Agar (EMB)

The media was prepared by dissolving 35.96 grams in 1000 ml of distilled water, then it was heated to a boiling until the media completely dissolved, then it was sterilized by autoclave at 121 °C for 15 min, and then it was cooled to (45 °C),and poured in petri dishes.This media used for Isolation and differentiation of lactose fermenting and non–lactose fermenting enteric bacilli and used for isolation *E-coli* (Forbes *et al*. 2016).

2.3.9.2.6 Xylose Lysine Deoxycholate Agar (XLD)

The media was prepared by dissolving 56.68 grams of xylose lysine deoxycholate agar in 1000 ml of distilled water, then it was heated to a boiling until the media completely dissolved, we do not use Autoclave or Overheating, and then it was cooled to (45 °C) ,and poured in petri dishes (MacFaddin, 2000).

2.3.9.2.7 Selenite F Broth

The media was prepared by dissolving 4 grams of part B in 1000 ml of distilled water, then it was added 19 grams of part A and mix them well, then it was sterilized in a boiling water bath for 10 min. It is enrichment of isolation of *Salmonella spp.* (Forbes *et al*., 2016)**.**

2.3.9.2.8 Simmon Citrate Agar

The media was prepared by dissolving 23 grams of simmon citrate agar in one liter of distilled water, then it was heated to a boiling until the media

completely dissolved, then it was sterilized by autoclave at 121 °C for 15 min, and poured in petri dishes. The medium used for determining the ability of bacteria to utilize citrate as the sole carbon source (Atlas, 2005).

2.3.9.2.9 Triple Sugar Iron Agar (TSI)

The media was prepared by dissolving 65 grams in one liter of distilled water, then it was heated to a boiling until the media completely dissolved, then it was sterilized by autoclave at 121°C for 15 min, and poured in petri dishes. It is a differential medium used to know the ability of bacteria to carbohydrate fermentation and hydrogen sulfide production in the identification of gram-negative bacilli (Atlas, 2005).

2.3.9.3 Microscopic Examination

Gram stain is a method of staining used to distinguish and classify bacterial species into two large groups: gram-positive bacteria and gram-negative bacteria (Coico, 2006).

The specimen Preparation was carried out as following steps:

1. The material (single colony of bacteria) was made a thin film on a clean glass slide, used a sterile loop or swab for viscous specimens. Air dried, then heat fix the slide by passing it several times through a flame.
2. Crystal violet was applied to heat fixed slide for 20 seconds.
3. Gram's iodine was added to fix crystal violet 20 seconds.
4. Decolorizing agent such as alcohol/acetone was applied to remove the primary stain,timing is dependent on the thicking of the specimen.
5. Counter stain was applied (safranin) for 20 seconds.

6. The slide was rinsed and allow to air dried, observed with light microscope. Gram positive bacteria were observed as purple colored cells while Gram negative bacteria were pink in color (Wanger *et al*., 2017).

2.3.10 Biochemical Tests

2.3.10.1 Oxidase Test

This test was used to investigate the ability of bacterial isolates to produce cytochrome oxidase enzyme,this test was done by transport a single colony from bacterial culture growing within 24-28hrs,to filter paper and spread by wooden stick, the drops of oxidase reagent (tetra methyl-ρ-phenylenediamine di hydrochloride) that prepared immediately was added on it, the turning of the color of the smear from rose to purple within 10 seconds was indicated a positive result (Tille, 2015).

2.3.10.2 Catalase Test

This test was used to detect the ability of bacterial isolates to produce catalase enzyme that release oxygen from hydrogen peroxidase, a little amount of bacterial growth was transported by wooden stick on glass slide, and then a drop of catalase reagent (3%H_2O_2) was added to it. The formation of gas bubbles indicated the positive result (Tille, 2015).

2.3.10.3 Indole Test

Peptone water broth medium was inoculated by bacterial growth by loop, and it was incubated for (24-48) h, at37°C,then 2-3 drops of Kovac's reagent (p-dimethyl amino benzaldehyde in amyl alcohol) was added to it,a positive result

was recognized by formation of red color ring at top of broth ,otherwise a yellow color ring indicated negative result (MacFaddin, 2000).

2.3.10.4 Methyl Red Test

This test was used to investigate the ability of bacterial isolates to ferment glucose and produce differential acids that reduce pH of the broth. The test was performed by inoculating MR-VP broth with a young bacterial isolates and incubated for 24-48 hrs. at 37°C, then five drops of methyl Red reagent were added to it and mixed. The color turning from yellow to red considered appositive result (MacFaddin, 2000).

2.3.10.5 Vogues –Proskauer Test

This test was carried out by inoculating MR-VP broth by young bacterial growth and incubated for 24hrs. at 37°C, then 0.6ml of alpha nephthol (reagent A) and 0.2ml of 40% KOH solution indicated (reagent B) was added to it,the change of the medium color to pink after 15-20 minutes indicated a positive results due to formation of acetate or acetyl - methyl – carbonyl form glucose hydrolysis (MacFaddin, 2000).

2.3.10.6 Citrate Utilization Test

This test was used to detect the ability of bacterial isolates to utilize citrate as the sole carbon source,the positive result was indicated by change of the color of medium from green to blue with streaks of growth, while unchanged original green color indicated negative results (Atlas, 2005).

2.3.10.7 Urease Test

The specific media for detecting urease production was streaked with single bacterial colony of overnight cultured and incubated for 24hrs. at 37^0C, a positive results was indicated by changing the color of media to pink (MacFaddin, 2000).

2.3.10.8 Triple Sugar Iron Test

This test was done by inoculating the test tubes that were contained triple sugar iron agar with selective bacterial colonies by a stabbing and streaking method, and then they were incubated at 37^oC for 24hrs. with loosened cap, the results were configured in table (2-8) (MacFaddin, 2000).

Table (2-8): Reactions of triple sugar iron

Reaction	Color
Acid/acid	Yellow color
Acid/alkaline	Yellow+red color
Alkaline/alkaline	Red color
Alkaline/alkaline with H_2S	Red color+black color

2.3.11. Molecular Method

2.3.11.1.Molecular detection of *Rota virus* from children suffering from diarrhea

2.3.11.2.Preparation of Fecal Suspension

10-20% of fecal suspension was prepared in 50 mM Tris buffer, pH 7.4, and mixed well (200 µl) of the mixture, then the suspension was centrifuged at

2,000 xg for 3 min, the supernatant was transferred to another tube (Santos and Vera, 1994).

2.3.11.3.RNA Extraction kit

GENEzol™ was used for isolation viral nucleic acid from supernatant stool samples.

Table (2-9): RNA extraction kit contents

No.	Contents	Amount
1	GENEzol™ Reagent	80 ml
2	Pre-Wash Buffer1	35 ml
3	DNase I	550 µl
4	DNase I Reaction Buffer	5 ml
5	Wash Buffer3	50 ml
6	RNase-free Water	6 ml
7	RB Columns	100
8	2 ml Collection Tubes	200

2.3.11.4. Protocol

A. Sample Homogenization and Lysis

1. 200µl of liquid sample was transferred up to a 1.5 ml of microcentrifuge tube (RNase-free).
2. Three volumes of GENEzol™ Reagent was added per 1 volume of sample (3:1) then, mixed well by vortex.
3. The sample mixture was incubated for 5 minutes at room temperature.

B. RNA Binding

1. The sample was centrifuged at 16,000 x g for 1 minute to remove cell debris then, the clear supernatant was transferd to a new 1.5 ml microcenterifuge tube (RNase-free).
2. One volume of absolute ethanol was added directly to 1 volume of sample mixture (1:1) in GENEzol™ Reagent.
3. The mixture was mixed well by vortex then a RB Column was placed in a 2 ml collection Tube.
4. 700µl of the sample mixture was transferred to the RB Column. Centrifuged at 16,000 x g for 1 minute then, the flow-through was discarded.
5. The RNA binding step was repeated by transferring the remaining sample mixture to the RB column.
6. The mixture was Centrifuged at 16,000 x g for 1 minute then the flow-through was discarded , the RB Column was placed in a new 2 ml Collection tube.

In Column DNase I Digestion

1. 400µl of Wash Buffer was added to the RB Column then was centrifuged at 16,000 x g for 30 seconds.
2. The flow-through was discarded and the RB Column was placed back in the 2 ml Collection tube.
3. DNase I solution was prepared in a 1.5 ml microcenterifuge tube (RNase-free) as follows:

a-5µl (2 U/µl) of DNase I .

b-45µl of DNase I Reaction Buffer.

c-50µl of total volume.

4. The DNase I solution was mixed gently by pipette (without vortex) then (50 µl) of DNase I solution was added into the CENTER of the RB column matrix.
5. The column was incubated for 15 minutes at room temperature (25ºC) then, proceeded with RNA wash.

C. RNA Wash

1. 400 µl of Pre-Wash Buffer was added to the RB Column then, centrifuged 16,000 x g for 30 seconds.
2. The flow-through was discarded then, the RB Column was placed back in the 2 ml Collection Tube.
3. 600 µl of Wash Buffer was added to the RB Column.
4. The mixture was Centrifuged at 16,000 x g for 30 seconds then the flow-through was discarded . The RB Column was placed back in the 2 ml Collection Tube.
5. 600 µl of Wash Buffer was added to the RB Column.
6. The mixture was centrifuged at 16,000 x g for 30 seconds then, the flow-through was discarded .
7. The RB Column was placed back in the 2 ml Collection Tube.
8. The tubes was centrifuged at 14-16,000 x g for 3 minutes to dry the column matrix.

D. RNA Elution

1. The dry RB Column was placed in a sterile 1.5 ml microcentrifuge tube (RNase-free).
2. 50 µl of RNase-free water was added into the CENTER of the column matrix.
3. 3 minutes left to ensure the RNase-free Water is completely absorbed by the matrix.

4. The tubes were Centrifuged at 16,000 x g for 1 minute to elute the purified RNA.

2.3.12. Reverse Transcription Kit

2.3.12.1.Synthesis of cDNA

A. First-Strand cDNA Synthesis

The following procedure was designed to convert up to 5µg of total RNA or up to 500ng of poly(A) RNA into first-strand cDNA.

1. Each component was mixed and centrifuged briefly before use,the compenent of cDNA synthesis kit were:

Table (2-10): RTmaster mix components for Rota virus detection

Compenets	Volume
Experimental RNA (up to 5µg/reaction)	3µl
Primer [Oligo (dT)15 (0.5µg/reaction) and/or Random Primer (0.5µg/reaction) or gene-specific primer (10–20pmol/reaction)]	1µl
Nuclease-Free Water	1µl
Final volume	5µl

2. Each tube of RNA was closed tightly, the tubes were placed into a preheated 70°C heat block for 5 minutes,the tubes was chilled them in ice-water for at least 5 minutes, each tube was centrifuged for 10 seconds in a microcentrifuge to collect the condensate and maintain the original volume. The tubes were kept closed on ice until the reverse transcription reaction mix is added.
3. The reverse transcription reaction mix was prepared by combining the following components of the GoScript™ Reverse Transcription System in

a sterile microcenterifuge tube on ice. Sufficient mix was prepared to allow 15µl for each cDNA synthesis reaction to be performed. The volumes needed was determined for each component, and combined them in the ordered list,the tubes were mixed gently by vortex, and kept on ice prior to dispense into the reaction tubes.

Table (2-11) :components of RT-PCR in the preparation of cDNA.

Components	Volume
Nuclease-Free Water (to a final volume of 15µl)	5.5µl
GoScript™ 5X Reaction Buffer	4µl
MgCl2 (final concentration 1.5–5.0mM)1	3µl
PCR Nucleotide Mix (final concentration 0.5mM each dNTP)2	1µl
Recombinant RNasin® Ribonuclease Inhibitor (optional)	0.5u
GoScript™ Reverse Transcriptase	1µl
Final volume	15.0µl

4. 15µl aliquots was added of the reverse transcription reaction mix to each reaction tube on ice and 5µl of RNA and primer mix were added to each reaction tube for a final reaction volume of 20µl per tube, the reaction tube was overlay with a drop of nuclease-free mineral oil to prevent evaporation and condensation.

Table (2-12): Reverse Transcription Reaction Thermal Profile.

Steps	Temperature	Time
1	37°C	10 min
2	42°C	60 min
3	70°C	15 min
4	4	Hold

The synthesized cDNA was added directly to PCR amplifications. RT-PCR using either 1µl or 5µl of the reverse transcription reaction in a 25µl PCR. The volumes of PCR components assembled were taken into account the carryover of buffer, magnesium and dNTP from the reverse transcription reaction to achieve the final concentration of each component.

2.3.13 DNA Extraction kit

The PrestoTM Stool gDNA Extraction Kit is designed for rapid isolation of genomic DNA from microorganisms, such as bacteria and parasite…etc, in stool samples.

Table (2-13): DNA extraction kit contents.

No.	Contents	Amount
1	ST1 Buffer	85 ml
2	ST2 Buffer	30 ml
3	ST3 Buffer	160 ml
4	Wash Buffer2	25 ml

5	Elution Buffer	30 ml
6	Inhibitor Removal Column	100 pcs
7	GD Column	100 pcs
8	Bead beating Tube (Type C)	100 pcs
9	2 ml Centrifuge Tube	100 pcs
10	2 ml Collection Tube	100 pcs

2.3.13.1. Protocol

1. Sample Lysis

200 mg of stool sample was transferred to a bead beating tube containing ceramic beads, then 800 µl of ST1 Buffer was added .The samples were vortexed briefly then incubated at 70 °C for 5 minutes, the bead beating tubes were attached horizontally to a standard vortex by taping or using an adapter,the samples were vortexed at maximum speed for 10 minutes at room temperature. The bead beating tubes were centrifuged at 8,000 x g for 2 minutes at room temperature to eliminate the foam caused by detergents present in ST1 Buffer, 500 µl of supernatant was transferred to a new 1.5 ml microcenterifuge tube.

2. PCR Inhibitor Removal

150µl of ST2 Buffer was added to each tube, then vortexed for 5 seconds,and incubated at 0-4 °C for 5 minutes,the tubes were centrifuged at 16,000 x g for 3 minutes at room temperature to precipitate insoluble particles and PCR inhibitors. Inhibitor Removal Column (purple ring) was placed in a 2 ml centrifuge tube, 500 µl of clear supernatant was transferred to the Inhibitor

Removal Column,the tubes centrifuged at 16,000 x g for 1 minute at room temperature then the column was discarded ,the flow-through was saved in the 2 ml centrifuge tube for DNA binding.

3. DNA Binding

800 µl of ST3 Buffer was added to the flow-through then mixed by shaking vigorously for 5 seconds, GD Column (green ring) was placed in a 2 ml Collection Tube.,700 µl of sample mixture was transferred to the GD Column,the tubes were centrifuged at 16,000 x g for 1 minute at room temperature then the flow-through was discarded , the GD Column was placed back in the 2 ml Collection Tube, the remaining sample mixture was transferred to the GD Column,the tubes were centrifuged at 16,000 x g for 1 minute at room temperature,the flow-through was discarded then the GD Column was placed back in the 2 ml Collection Tube.

4. Wash

400 µl of ST3 Buffer was added to the GD column,the tubes were Centrifuged at 16,000 x g for 30 seconds at room temperature, the flow-through was discarded then, the GD column was placed back in the 2 ml collection Tube. 600 µl of wash Buffer was added to the GD Column,the tubes were centrifuged at 16,000 x g for 30 seconds at room temperature, the flow-through was discarded then, the GD Column was placed back in the 2 ml collection tube. 600 µl of Wash Buffer was added to the GD column again, the tubes were centrifuged at 16,000 x g for 30 seconds at room temperature, the flow-through was discarded then, the GD Column was placed back in the 2 ml collection tube,the tubes were centrifuged at 16,000 x g for 3minutes at room temperature to dry the column matrix.

5. Elution

The dried GD Column was transferred to a new 1.5 ml microcentrifuge tube. 100 µl of preheated Elution Buffer was added into the center of the column matrix,the tubes were let stand for at least 2 minutes to allow Elution Buffer, then, the tubes were centrifuged at 16,000 x g for 2 minutes at room temperature to elute the purified DNA.

2.3.13.2. PCR Thermocycler Conditions

The PCR thermocycler conditions for genes of *Rotavirus*,parasite and bacteria show in table(2-14),(2-15),(2-16) and (2-17).

Table (2-14): PCR Thermocycler Conditions for *Rota virus.*

PCR Steps	Temp.	Time	Repeat
Initial Denaturation	95 ºC	5min	1
Denaturation	95 ºC	30sec	30cycles
Annealing	50 ºC	30sec	
Extension	72 ºC	30sec	
Final extension	72 ºC	5min	1

Table (2-15): Multiplex PCR Thermocycler Conditions for parasites (*E.histolytica,G.lamblia* and *C.parvum*)

PCR Steps	Temp.	Time	Repeat
Initial Denaturation	95 ºC	5min	1
Denaturation	95 ºC	30sec	30cycles
Annealing	55 ºC	55sec	
Extension	72 ºC	30sec	
Final extension	72 ºC	5min	1

Table (2-16): PCR Thermocycler Conditions for *E. coli*.

PCR Steps	Temp.	Time	Repeat
Initial Denaturation	95 ºC	5min	1
Denaturation	95 ºC	30sec	30cycles
Annealing	60ºC	55sec	
Extension	72 ºC	30sec	
Final extension	72 ºC	5min	1

Table (2-17): PCR Thermocycler Conditions for *Salmonella*.

PCR Steps	Temp.	Time	Repeat
Initial Denaturation	95 ºC	5min	1
Denaturation	95 ºC	30sec	30cycles
Annealing	55ºC	55sec	
Extension	72 ºC	55sec	
Final extension	72 ºC	5min	1

2.3.13.3. PCR Product analysis

The PCR products were analyzed by agarose gel electrophoresis as

the following steps:

1. 1 gm. of agarose powder was dissolved in 100 ml of 1X TBE. Then , boiled in water bath at 100 ºC for 15 minutes for complete dissolving,then, left to cooled at 45 ºC.
2. Then 5 µl of Ethidium bromide stain were added into agarose gel solution.

3. Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 5 µl of DNA sample were added in to each comb well and 100 bp DNA size marker was loaded along with experimental samples.
4. The gel tray was fixed in electrophoresis chamber and filled by 1XTBE buffer .Then electric current was performed at 50 volt (5V/cm between electrodes) for 1hour.
5. PCR products were visualized by using UV Transilluminator.

2.3.14. Measurement the purity of DNA

Nanodrop was used to measure the extracted DNA purity by measuring the absorbance at (260/280 nm) and quantifying the DNA concentration (ng/ml) (Adams and Otárola-Castillo, 2013).

The DNA purity was examined as the following steps:

1- The Nanodrop software was opened, and a suitable application was selected (Nucleic acid, dsDNA).
2- A dry wipe was used to clean all device parts, particularly those connected to measurements, and then the system was blanked by carefully pipetting free nuclease water 2 µl on the surface of the lower measurement platforms.
3- After closing the sampling cover and clicking the okay button to start the Nanodrop process, 1 µl of DNA was put to the measuring pedestal after it was cleaned.

2.3.15.The Preparation of Primers :

All primers used in this study (Table 2-5) were prepared according to the recommendation of the manufactured by dissolving a lyophilized primers in appropriate volume of nuclease free water to yield 100 pmol/ µl as a stock solution . A working solution was prepared with the final concentration 10 pmol/ µl by dilution methods.

2.3.16. Statistical analysis :

The statistical analysis was done for diarrheal patients groups , using Chi-square(χ^2-test) , Sensitivity and Specificity according to (Armitage *et al*., 2008). The following statistical tests were used:

1- **Chi-square test** was used to evaluate association between any two categorical variables, have expected count of less than 5.

2- **Sensitivity** was calculated as true positive X100/ total positive.

3-Specificity was calculated as true negative X100/total negative .

CHAPTER THREE
RESULTS &DISCUSSION

3.1 Results and Discussion

3.2 Distribution of microorganisms that causing diarrhea according to gender

The table (3-1) showed the distrubution of diarrhea causes according to gender with pathogens, it was found (200 males and 120 females),the table was recorded distribution of bacteria,viruses and parasites was 62(57.94%),81 (67.5%) and 53 (61.62%) in males and 45 (42.05%),39 (32.5%) and 33(38.37%) in females ,respectively.

Table (3-1) The distribution of microorganism that causing diarrhea according to gender

Gender	Total	Bacteria	Viruses	Parasite	Others
Male	200	62(57.94%)	81(67.5%)	53(61.62%)	4(57.14%)
Female	120	45(42.05%)	39(32.5%)	33(38.37%)	3(42.85%)
Total	320	107(33.43%)	120(37.5%)	86(26.87%)	7(2.18%)
Statistical analysis		X^2=1.42 P>0.05	X^2=2.048 P>0.05	X^2=0.038 P>0.05	-

The results recorded no significant differences among gender,it was founded increasing prevelance of parasite infection in males was (61.62%) compared to females (38.37%),this result was in agreement with many studies that confirmed the high prevelance of intestinal parasite in males rather than females (Mumtaz *et al*., 2010;Waqar *et al*., 2014;Hussein & Meerkhan, 2019 and Al-Saqur *et al*., 2020). But disagreement with Al-Rumaidh & AL-Aboody, (2017),who was found the high prevelance

infection of parasite in females (51.75%) rather than males (48.24%) in Thi-Qar.

The present work founded high prevelance of *Rotaviruse* in male (67.5%) rather than female (32.5%),this results was in agreement with Kargar *et al*., (2012), who said that *Rotavirus* was more frequently in boys (16.31%) than in girls (12.05%) and he noticed that it doesn't statistical different ($p<0.05$) in the sex distribution among Iranian population.

Many studies improved that gastroenteritis are usually prevalent in countries with issues of poor hygiene and sanitation and it was associated with death of more than 1.4 million of children due to a consequence of water borne gastroenteritis (Brown *et al*., 2013).Many studies also recorded the association between *Rotavirus* infection and diarrhea (Kargar *et al*., 2012;Habash & Habeeb, 2018 and Lafta *et al*., 2019).The *Rotavirus* infection was highly distributed among males (Zaman *et al.*, 2012;Narci *et al*., 2013 and Nasab *et al.*, 2016).Some studies carried out in Al-Dywania and Thi-Qar recorded a high percentage of *Rotavirus* infection among children suffering from gastroenteritis which were recorded was 46.09% and 45%, respectively (Abood *et al*., 2014 and Fenjan *et al*., 2020).

In the case of bacteria ,there was an increasing in prevelance of bacterial infection in males was (57.94%) compare to the distribution of bacterial infection in females (42.05%),this result agreement with Rathaur *et al*., (2014), who was founded the high prevelence of bacterial infection in males was (54.6%) rather than females was (45.4%) . Another study in Thi-Qar confirmed the high prevelance of bacterial infection in males was (56.3%) rather than females was (43.7%) (Harb *et al*., 2017).

Through the advanced results, it was founded that the prevelance of diarrhea was different in males from that in females,where the percentage of infection is high in males than females in children, and these results were in agreement with the results of other studies (Moyo *et al*., 2011 and Mengistie *et al*., 2013), the study conducted by the researcher de Melo and his workers, in Brazil, in which they confirmed that, in addition to the high incidence of infection, males take a longer period to recover (Melo *et al*., 2008).Environmental theories suggested different exposure by gender, for example, older boys may be allowed more freedom to roam from home, or go to work with his fathers, unequally exposing them to infectious pathogens ,the biological hypothesis assumes that there may exist pathophysiologic sex differences between girls and boys with regard to acute diarrhea that make boys more susceptible to infection (Jarman *et al*., 2018).

3.3 Distribution of microorganism that causing diarrhea according to age groups

Table(3-2) showed the distribution of children age with infectious agents,it was found significant differences in the parasites 16(18.60%), 26(30.23%), 44(51.16%) and virus 59(49.16%),45(37.5%),16(13.33%) in <2y,2-4y and 4-6y respectively.

Table (3-2) The distribution of microorganisms that causing diarrhea according to age groups

Age (years)	Total	Bacteria (n)%	Viruses (n)%	Parasite (n)%	Other (n)%
2y<	116	39(36.44%)	59(49.16%)	16(18.60%)	2(28.57%)
2-4 y	109	35(32.71%)	45(37.5%)	26(30.23%)	3(42.85%)
4-6 y	95	33(30.84%)	16(13.33%)	44(51.16%)	2(28.57%)
Total	320	107(33.43%)	120(37.5%)	86(26.87%)	7(2.18%)
Statistical analysis		X^2=0.16 P>0.05	X^2=26.8 P<0.05	X^2=28.87 P<0.05	-

The results found the highest prevelance of parasites in the age group (4-6) years was (51.16%),this result was an agreement with Tiwari *et al*., (2013), who found that the highest rate of parasitic infection (38.18%) and they observed the positiviy among children in the age group (4-6) years old, also the present study was compatible with Al-Taei, (2019) who found a highest percent of infection (44.35 %) in (1-10) years, but disaggree with Moyo *et al*., (2011) who found the highest detection of parasites in children aged 7-12 months as (26.1%).

Curent sudy demonstrated the highest number of *Rotavirus* occurred in children who have aged group less than two years was (49.16%), it was slightly higher as compared with other groups, this result has an agreement with Durmaz *et al.,* (2014) who were found that the highest rate of *Rotavirus* infection (38.7%) and they observed the children in the (13 to 24) months age group have positive *Rotavirus*,but disaggre with Samarbafzadeh

et al., (2005) who found the highest detection of *Rotavirus* in children aged (7-12) months.

The prevalence of diarrhoea has been reported to be higher among children below five years (Walker *et al*., 2013;Charyeva *et al*., 2015 and Zhang *et al*., 2016).Some of studies reported that the prevalence of diarrhea was highest in the age group below 24 months and declines with an increase in age (Walker *et al*., 2012 and Akinnibosun & Nwafor, 2015).These observations could easily explain as children in this age group start complementary foods and a large portion of children at this age start crawling, and can touch contaminated materials in unclear environment and immediately return their hand to their mouth,so it may cause them to easily infect with causative agent for diarrhoea,also poverty,lack of safe dringing water,poor hygiene (Emile *et al*., 2013;Al-Taei, 2019 and Sahiledengle *et al*., 2021).

3.4 Distribution of microorganisms that causing diarrhea according to residence

The present study found significant differences (X^2=6.08,p<0.05), (X^2=0.05,p<0.05) and (X^2=4.43,p<0.05) for bacteria,parasites and viruses respectivelly,it was found the rural area more prevalence in the parasite bacteria, and viruses infection than urban area as shown in table (3-3).

Table (3-3) The distribution of microorganism that causing diarrhea according to residence

Residence	Total	Bacteria (n)%	Viruses (n)%	Parasite (n)%	Other (n)%
Rural	**197**	76(71.02%)	65(54.16%)	52(60.46%)	4(57.14%)
Urban	**123**	31(28.97%)	55(45.83%)	34(39.53%)	3(42.85%)
Total	**320**	107(33.43%)	120(37.5%)	86(26.87%)	7(2.18%)
Statistical analysis		X2=6.08 P<0.05	X2=4.43 P<0.05	X2=0.05 P<0.05	-

Precent data recorded that the highest number of parasitic infection in rural area with percentage (60.46%) rather than urban area (39.53%),this result has an agreement with Salim & Al-Aboody, (2019),who were founded the highest infected patients with *C.parvum* (64.5%) in rural area and lowest infected patients found (35.5%) in urban area ,but disagreement with Ranjbar-Bahadori *et al*., (2011),who was discovered that prevalence of *C.parvum* infection in urban and rural children was similar. Also it has an agreement with another study in Thi-Qar,which showed the majority (69.4%) of infected patients with amoebiasis resided in rural areas, whereas 30.6% was recorded in urban areas (Flaih *et al*., 2021).Another studies confirmed the prevelance of *E.histolytica* and *G.lamblia* was higher in rural area rather than urban (Tyoalumun *et al*., 2016 and Samie *et al*., 2020) .This due to ignorance, poverty, poor environmental and personal hygiene, shortages of clean potable water and indiscriminate defecation as most vegetable farmers use excreta as manure which was a veritable source of infection since children and their mothers often go to the farm to tender to the vegetables poor sanitation, insufficient water treatment, day-care

centers and in institutions with children who were not toilet trained (Al Saeed & Issa, 2006 and Tyoalumun *et al*., 2016).

In current study found the highest number of *Rotavirus* infection in the rural area with percentage (54.16%) rather than urban area (45.83%). The results has an agreement with Haruki & Sherchand, (2004) Who become discovered the maximum not unusual infection of *Rotavirus* during one-yr incidence of *Rotavirus* in people and home animals in rural communities of Nepal. Also agreement with Azemi *et al*., (2013) who was found the higher prevelence of *Rotavirus* in rural area with percentage (57.0%) rather than urban area (38.7%).As well as agreement with Alani *et al*., (2012) that found the high prevelence of *Rotavirus* in rural area as (72.7%)in western Iraq, but disaggree with Jaff *et al*., (2015) who were founded the highest detection of *Rotavirus* was made in urban area as 19(86.36%).

For bacteria such as *Escherichia coli* and *salmonella* spp. the highest prevelence of infection was in the rural area (71.02%) rather than urban (28.97%),in current study found the highest prevelence of *salmonella* occurred in rural area ,this was compatible with Gaensbauer *et al*., (2019) that found the prevelance of *salmonella* in rural area (4.1%) rather than urban area (2.9%).While the researcher Gong and his groups confirmed that the prevelance of bacteria was high in rural areas, as the prevelance of *E.coli* in the rural area (3.86%) compared to the urban (3.16%), and the prevelence of *salmonella* (3.10%) compared to the urban (2.77%) (Gong *et al*., 2018). Another study confirmed that the prevelance of diarrhea was highly in rural area (Mengistie *et al*., 2013 and Pirsaheb *et al*., 2017).

The impact of the educational level of mothers in the health status of their children is important because it is directly related to their awareness levels, mothers without formal education are more likely to have children who will suffer diarrhoeal diseases when compared to educated mothers(Jolaiya Tolu *et al*., 2016;Desmennu *et al*., 2017 and Ugboko *et al*., 2020).Another study confirmed that the higher rates of diarrhea might be because the households in these regions were less favorable in terms of improved water, sanitation and hygiene (WASH) coverage and access to healthcare services (Sahiledengle *et al*., 2021).

3.5 Distribution of microorganisms that causing diarrhea according to period of study(months)

The results found a significant decrease (x^2=45.9,p<0.05) of parasite causes diarrhea in the winter season compared to hot season.The most prevelance parasite infection in April was (26.74%)(table3-4).This result agreement with Jaran, (2016) who found prevalence of different parasites varied according to season, on average the summer months showed the highest incidence of parasitic infection (62%) compared with the winter months (16%), *Giardia lamblia* and *Entameba histolytica* were most prevalent in the summer months in Jordan. Also many studies confirmed that the infection of parasite is higher during the hottest months compared to the coldest months of the year (Amin, 2002;Ponce-Macotela *et al*., 2005;Khoshnood *et al*., 2015 and Al-Rumaidh & AL-Aboody, 2017).

Table (3-4): The distribution of microorganism that causing diarrhea according to months study

Seasonal period	Total	Bacteria (n)%	Viruses (n)%	Parasite (n)%	Other (n)%
December	53	16(14.95%)	30(25%)	5(5.81%)	2(28.57%)
January	79	24(22.42%)	46(38.33%)	8(9.30%)	1(14.28%)
February	56	25(23.36%)	17(14.16%)	13(15.11%)	1(14.28%)
March	46	15(14.01%)	12(10%)	18(20.93%)	1(14.28%)
April	52	18(16.82%)	10(8.33%)	23(26.74%)	1(14.28%)
May	34	9(8.41%)	5(4.16%)	19(22.09%)	1(14.28%)
Total	320	107(33.43%)	120(37.5%)	86(26.87%)	7(2.18%)
Statistical analysis		X^2=4.53 P>0.05	X^2=41.45 P<0.05	X^2=45.9 P<0.05	-

The variance of parasitic infection according to the months may be due to the socio-economic level of the community, geographic, sanitary, feeding pollution factors which may effect on the incidence of pathogenic intestinal parasites(Spinelli *et al*., 2006;Balcioglu *et al*., 2007 and hamady obeid Al-Taei, 2019). Another study confirmed that there are many factors could account for seasonal variations in the occurrence of Cryptosporidiosis, including factors affecting the numbers of oocysts present in the environment such as rainfall or agricultural practices, factors affecting oocyst survival, such as humidity or temperature, and factors promoting exposure to oocysts such as contact with animals or attendance at child care centers (Areeshi *et al*., 2007). Also the difference between our results and theirs may be a reflection of the difference in sample size and duration of the study.

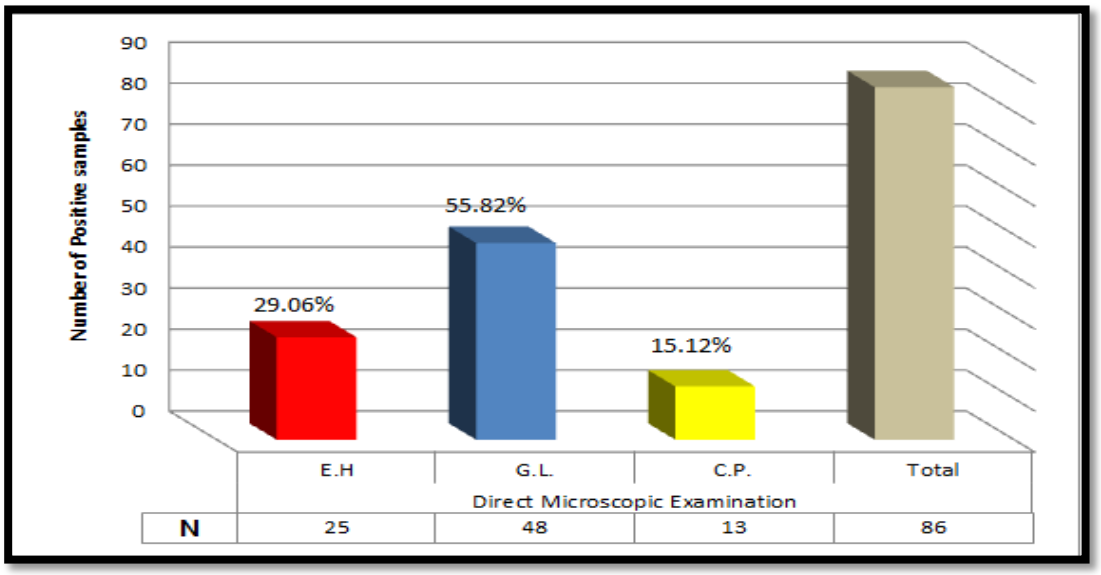
While the results found a significant increase (x^2=41.45,p<0.05) in viral infection (*Rotavirus*) that cause diarrhea in children in the winter

season compared with hot season,the most prevelance about rotavirus infection in January with percentage (38.33%), this result has an agreement in with Levy and his group who were conclude that *Rotavirus* responds to changes in climate in the tropics, with the highest number of infections found at the colder and drier times of the year (Levy *et al*., 2009).Also agreement with Narci *et al*., (2013) that found the high numbers of cases were found in winter months (43%), with the highest peak in January.As well as with Azeez & Alsakee, (2017),who were founded the highest detection rate (38.6%) and he recorded positive *Rotavirus* during winter season (January and February) in Erbil.Many studies also recorded a seasonal variation of *Rotavirus* infection in which a high percentage of *Rotavirus* infection were reported during winter (Gleizes *et al*., 2006;Moyo *et al*., 2014 and Fenjan *et al*., 2020).

The results of present study showed a high prevalence of *Rotavirus* during Winter (Table 3-4) , this may be due to the possibility of the surviving of the virus in the environment that is characterized by low humidity,low temperature,rainfall, altitude, population density and behavioral factors, and perhaps the economic income of the country,the economic status of countries does not explain the seasonal or the year-round occurrence of *Rotavirus* because in countries with similar income levels, seasonality exists or it does not , depend on the country‟s distance to the equator (Patel *et al*., 2011;Jagai *et al*., 2012 and Ureña-Castro *et al*., 2019). On the other hand we don't found a significant difference (x^2=4.53,p>0.05) in the children suffering from diarrhea caused by bacterial infection as shown in table 3-4. Al-Abbas, (2018) found the high prevelance of bacterial infection in May as (49.24%) in Iraq.

3.6 Distribution of parasitic causes of diarrhea by using direct examination :

The current data showed the prevelance of *Giardia lamblia* as (55.82%),this result disagreement with the study in Dohuk that found the prevelance of *G.lamblia* as (38.5%) (Al Saeed & Issa, 2006). The present study found that the prevelance of *G.lamblia* (55.82%) is higher rather than *E.histolytica* (29.06%),this result agreement with Nazeer et al., (2013) who was confirmed the high prevelance of *G.lamblia* (27.8%) compared with *E.histolytica* (8.0%) in Egypt and agreement with Jaeffer, (2011) who was found the infectivity rate of *G.lamblia* was higher than the infectivity rate of *E.histolytica* as their rate was 10.72% and 2.92%, respectively in Baghdad.



Figure(3-1) The distribution of parasitic causes of diarrhea according to direct microscopic examination

But disagreement with Al Saqur *et al*., (2017) , who were founded the high prevelance of *E.histolytica* in Iraq.

The infection rate of Giardiasis which recorded in present study is higher than other studies conducted in Al-Karkh side of Baghdad ,where Giardiasis was 1.77% (Ibrahim, 2012) ,in AL-Mahmoudyia area in Baghdad ,which was 34% (Hammadi, 2012) ; in Wasit province 11% (Mahmud, 2009). The results showed the prevelance of *Entameba histolytica* as (29.06%),this result agreement with Butera *et al*., (2019) ,who was founded the prevelance of *E.histolytica* as (25.95%),but disagreement with the study that found the prevelnce of *E.histolytica* as (7.4%) in Erbil (Mahmood & Bakr, 2020). The recent results found the prevelance of *C.parvum* as (15.12%), this result agreement with Yahya Jirjees Salman *et al*., (2015) ,who was found the prevelance of *C.parvum* as (16.28%) .But disagreement with some of studies (Al-zubaidi, 2012 and Al-Zubaidi, 2017), who were founded the prevelance of *C.parvum* (35.44%), (48.88%) ,respectively.

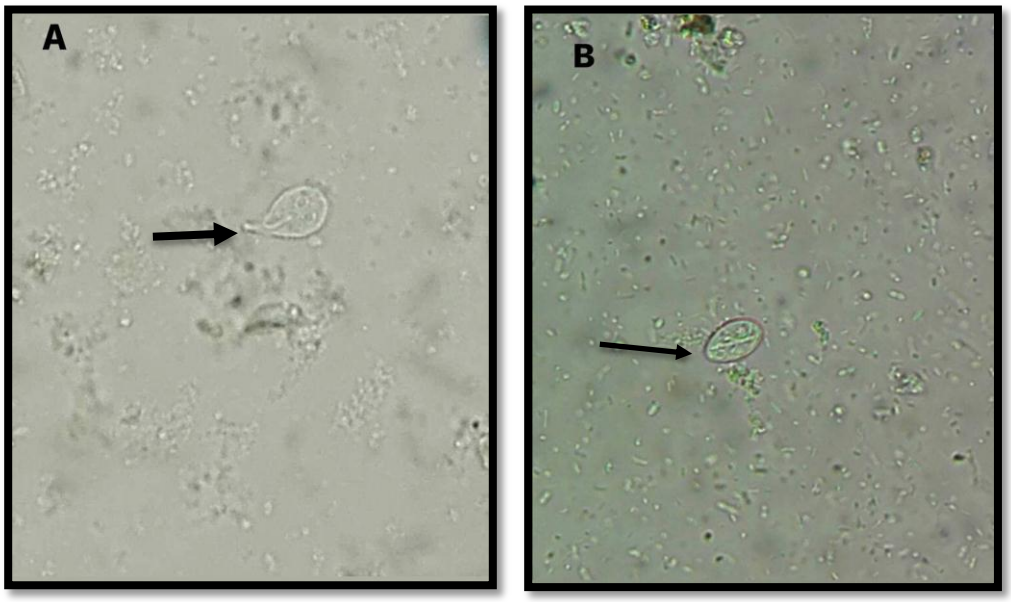


Fig. (3-2a): (A)Trophozoite of G. lamblia without staining(B)Cyst of G. lamblia without staining (40 X).

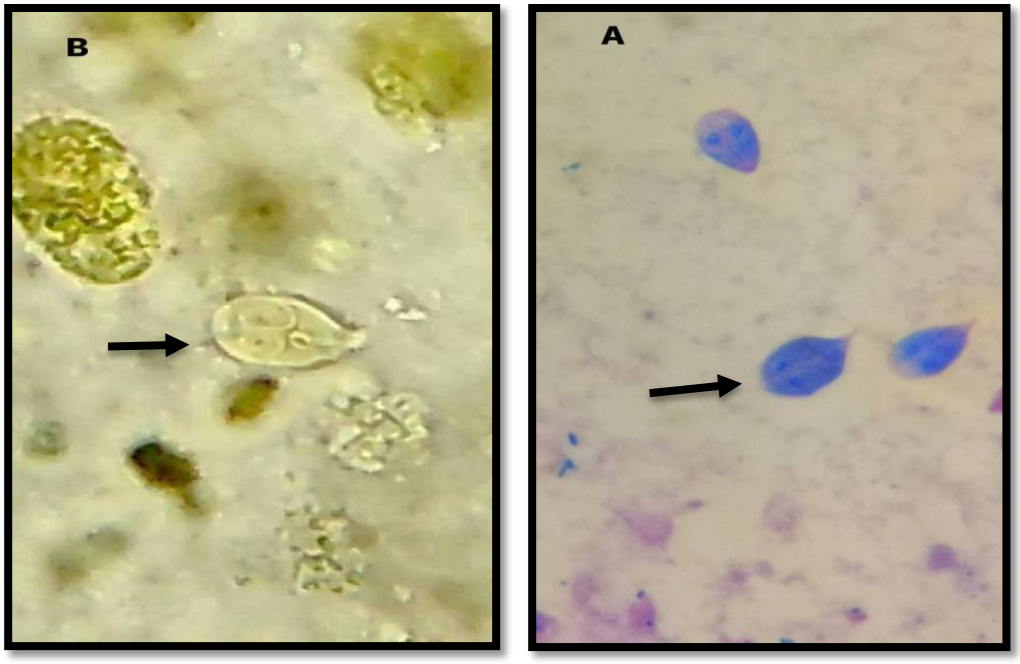


Fig. (3-2b)(A) Trophozoite of G. lamblia stained with modified Ziehl-Neelson stain (B)Trophozoite of G. lamblia stained with Lugol's iodine stain (40 X).

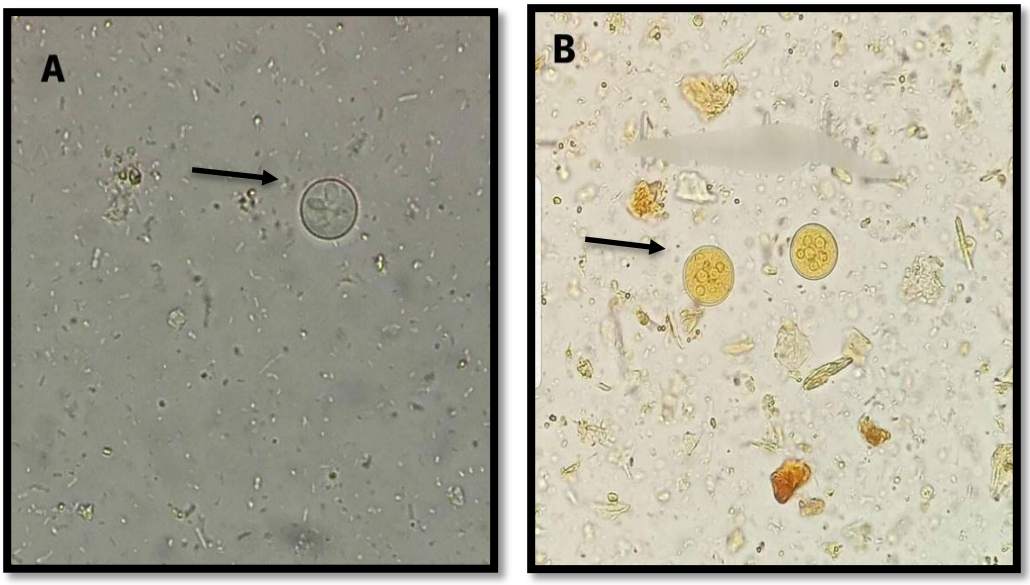


Fig. (3-2c): (A)Cyst of E. histolytica without staining and (B) Cyst of E. histolytica stained with Lugol's iodine stain (40 X).

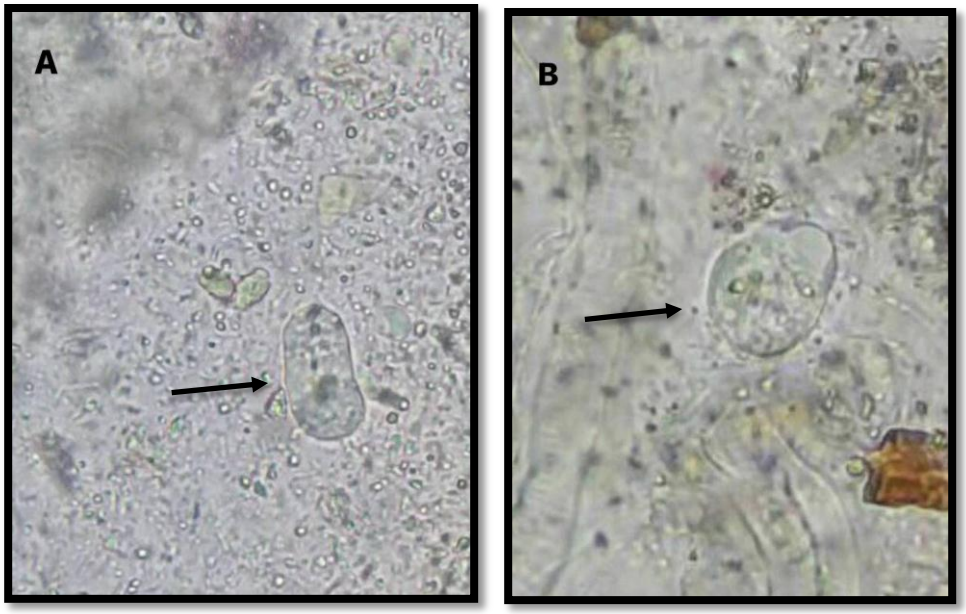


Fig. (3-2d): (A)Trophozoite of E. histolytica without staining and (B)Troph of E. histolytica stained with Lugol's iodine stain (40 X)

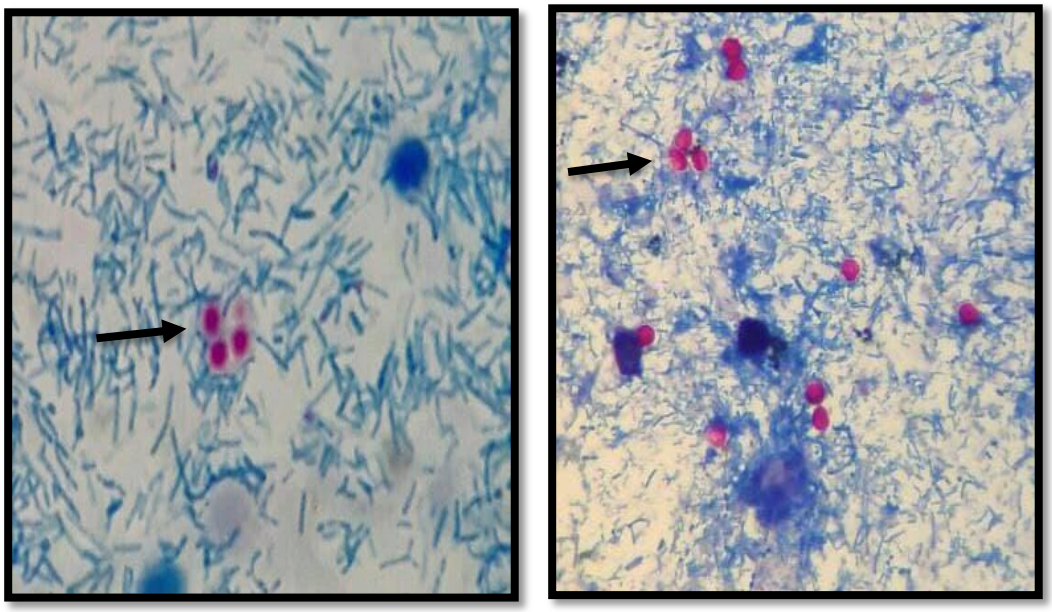


Fig. (3-2e): Oocyst of Cryptosporidium spp. stained with modified Ziehl-Neelson stain (100 X).

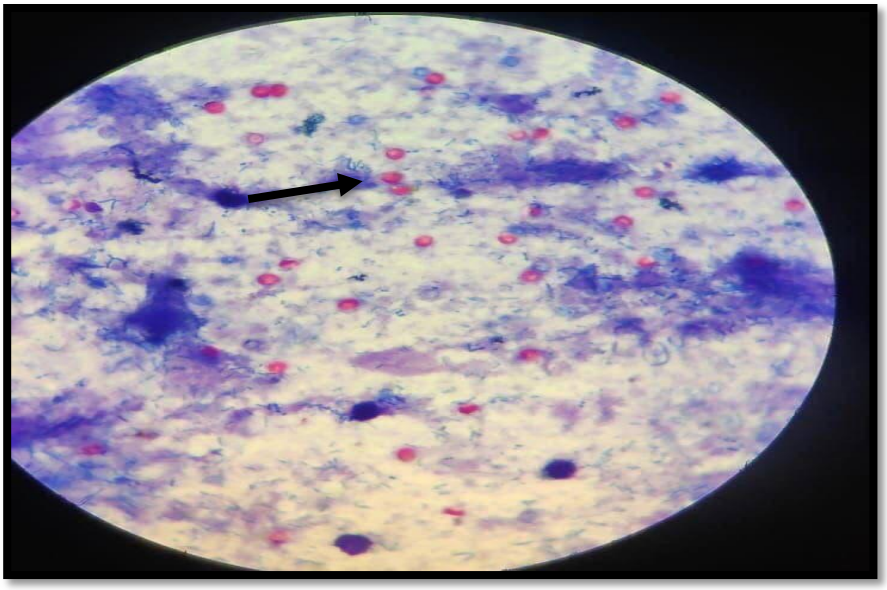
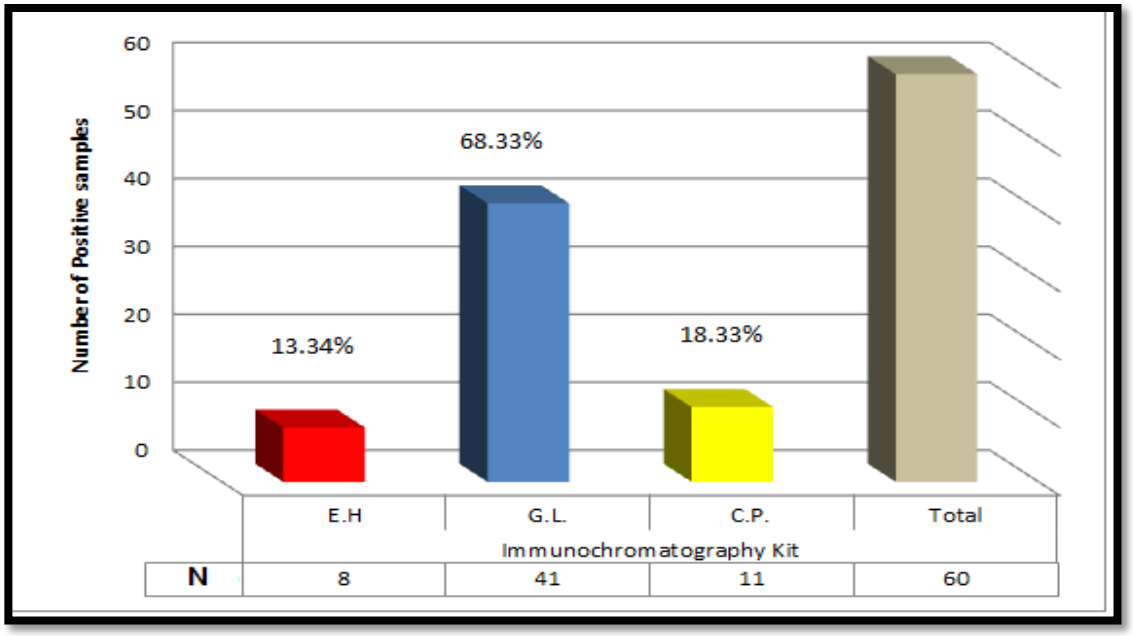


Fig. (3-2f): Oocyst of Cryptosporidium spp. stained with modified Ziehl-Neelson stain (100 X).

3.7 Distribution of parasitic causes of diarrhea by using triage immunocromatography assay

The figure (3-3) showed the distribution of intestinal causes of diarrhea by using Trige test,where the results was discovered the high prevelance of *G.lamblia* was (68.33%) followed by *C.parvum* (18.33%) and *E.histolytica* (13.34%),this result has an agreement with Gaafar, (2011) who was found the prevelance of *G.lamblia* ,*C.parvum* and *E.histolytica* were (23%),(5%) and (2%), respectively.Also agremeent with Atia *et al*., (2016) ,who were confirmed the high prevelance of *G.lamblia* followed by *C.parvum* and *E.histolytica* were (30.07%),(19.8%) and (11%), respectively. But,Goni *et al*.(2012) found that *E.histolytica* (25.63%) was the most common followed by *G.lamblia* (19.38%) and then *C.parvum* (13.75%), which might be due to different in environmental conditions.



Figure(3-3) The distribution of parasitic causes of diarrhea by using triage immunocromatography test

3.8 Distribution of single and mixed parasitic causes of diarrhea by using triage immunocromatography test

The result found the most common prevelence of intestinal parasite in the single infection,it was found were (88.48%),(62.5%) and (27.27%) in *G.lamblia* ,*E.histolytica* and *C.parvum* respectively , Our result has an aggrement with Swierczewski *et al*., (2012) who were confirmed the most prevelance of parasite infection in *G.lamblia* followed by *E.histolytica* and *C.parvum* in Kenya . Also,some of study confirmed that the most prevelance parasite in G.lamblia followed by *E.histolytica* and *C.parvum* respectivilly (Garcia *et al*., 2000 and Sharp *et al*., 2001), as shown in table (3-5).

Table (3-5) The distribution of single and mixed parasitic causes of diarrhea by using immunocromatographic assay

Type of parasite	*G.lamblia*	*E.histolytica*	*C.parvum*	Negative
G.lamblia	33(88.48%)	-	-	-
E.histolytica	-	5(62.5%)	-	-
C.parvum	-	-	3(27.27%)	-
G.lamblia+E.histolytica	1(2.43%)	1(12.5%)	-	-
G.lamblia+C.parvum	6(14.63%)	-	6(54.54%)	-
E.histolytica+C.parvum	-	1(12.5%)	1(9.09%)	-
G.lamblia+C.parvum + E.histolytica	1(2.43%)	1(12.5%)	1(9.09%)	-
Total	41	8	11	26

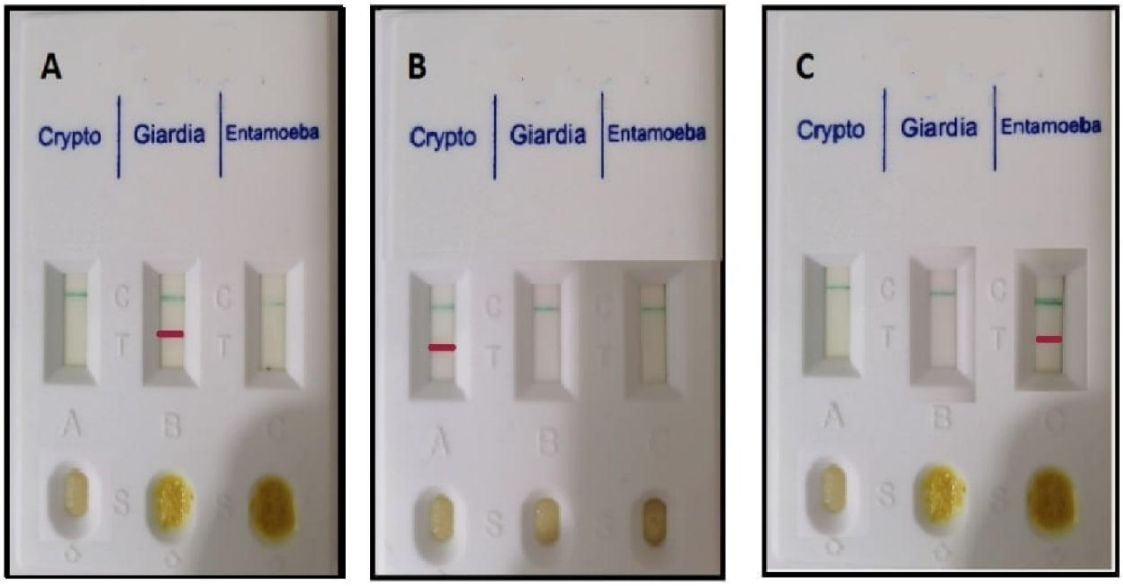
The mixed infection involving two or three intestinal parasite are also observed,the most prevelence mixed infection was found in the *G.lamblia* and *C.parvum*,it was found two parsites (*G.lamblia* and *C.parvum*) in six children suffered from diarrhea followed by three parasites (*G.lamblia,C.parvum* and *E.histolytica*),it was found in one children suffering from diarrhea followed by two parasite (*C.parvum* and *E.histolytica*),it was found in one child suffered from diarrhea followed by two parasites (*G.lamblia* and *E.histolytica*), were found in one child suffered from diarrhea as shown in figure (3-4) ,the study was founded one parasitic infection was more incidence than mixed parasite infection ,this result agreement with Atu *et al.*, (2011) ,who was confirmed that immunocromatography assay was used to identify single and mixed infection, Atu *et al*.(2016) found parasite infection of 13(n=105,12.38%) single infection and 10(n=105,9.52%) mixed infection by using Rida Quick

test for three parasite infection. Atia *et al*. (2016) confirmed that single infection was more incidence than mixed parasite infection in Egypt.

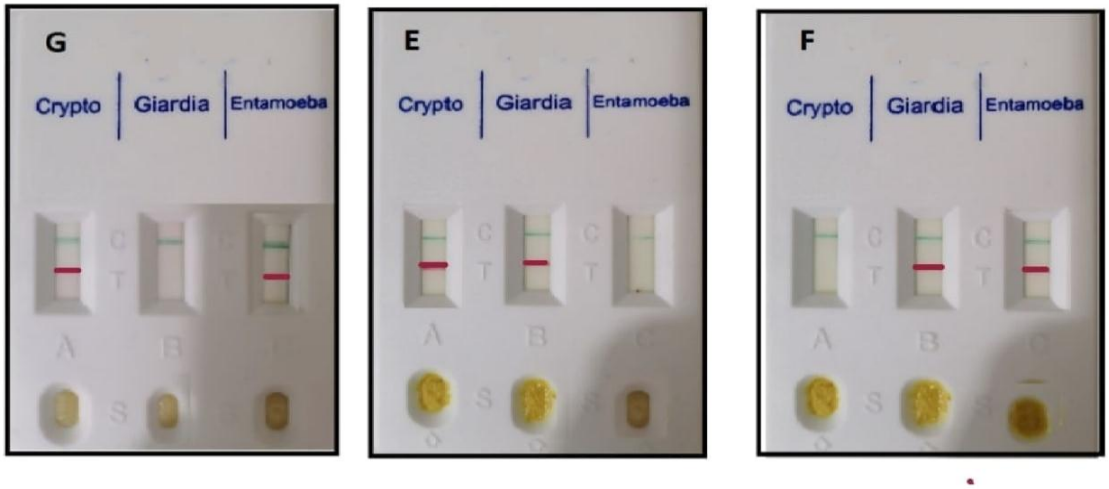
Laboratories in the developing world continue to rely on microscopic examination as the gold standard for detection of intestinal parasites as it is relatively inexpensive and appropriate for resource-limited countries in the developing world. However, accurate diagnosis of intestinal parasites mostly is dependent on the level of expertise of the microscopist, and therefore the sensitivity and the specificity of microscopic examination can vary from laboratory to another (Platts-Mills *et al*., 2012). As *E. histolytica, C. parvum,* and *G. lamblia* are considered the three most common parasitic causes of acute diarrhea worldwide, it is imperative that accurate diagnosis is correct as misdiagnosis can lead to missed treatment resulting in morbidity/mortality and the continuous shedding of the parasites leading to increase transmission. For these reasons, EIA kits such as the Triage Micro Parasite Panel offer an acceptable alternative method to microscopic examination for diagnosis of *E. histolytica, C. parvum,* and *G. lamblia*. Also, Gaafar (2011) reported that Triage Micro parasite Panel test did not diagnose any parasite other than *G. lamblia, C. parvum* and *E. histolytica* with no cross reactivity with other intestinal parasites.

The Triage immunocromatography test is an EIA kit which is able to simultaneously detect specific antigens of *E. histolytica/E. dispar, C. parvum,* and *G. lamblia* in stool. The assay can be read within 15 min using fresh or frozen unfixed stool samples. The assay has been compared to microscopic examination for the detection of *E. histolytica/E. dispar, C. parvum,* and *G. lamblia* in several previous studies reported sensitivities and specificities ranging from 91.5% to 100% (Garcia *et al*., 2000;Sharp *et*

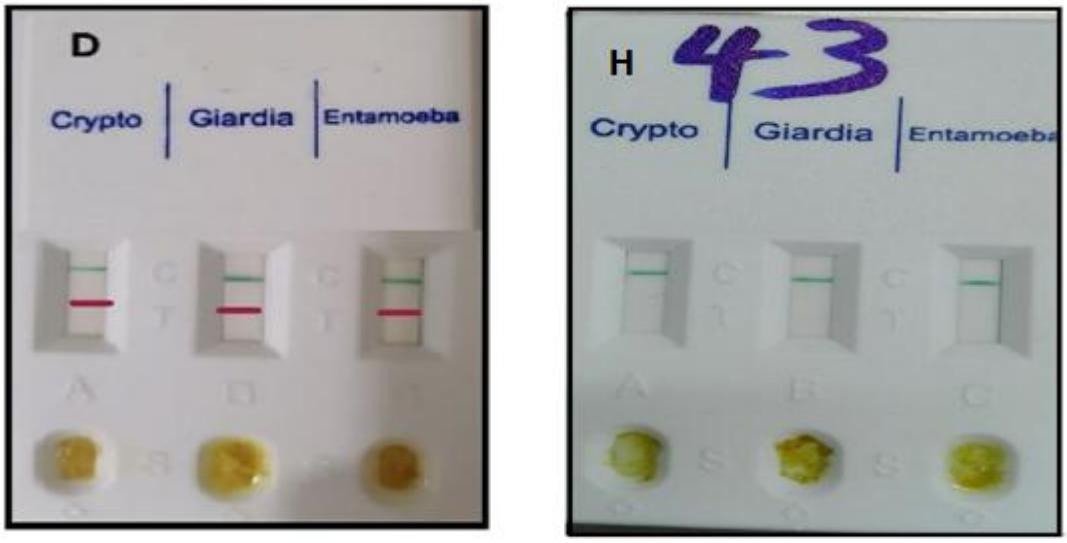
al., 2001;Leiva *et al*., 2006;Gaafar, 2011;Swierczewski *et al*., 2012 and Atia *et al*., 2016).



Figure(3-4a): Triage parasite panel demonstrating positive results. (A) Positive and negative controls and positive test zone for G. lamblia, B) positive and negative controls and positive test zone for C. parvum ; (C) positive and negative controls and positive test zone for E. histolytica.



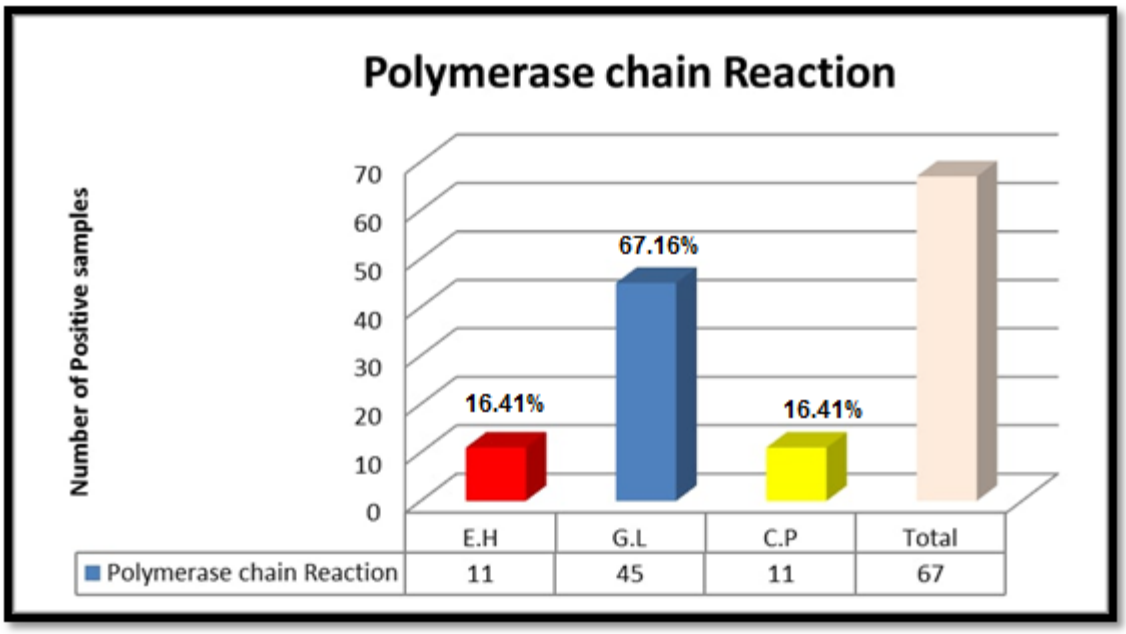
Figure(3-4b): Triage parasite panel demonstrating positive results. (G) positive and negative controls and mixed infection with Crypto+Entameba, (E) positive and negative controls and mixed infection with Crypto+ Giardia; (F) positive and negative controls and mixed infection with Giardia+Entameba.



Figure(3-4c): Triage parasite panel demonstrating positive results.; (D) positive and negative controls and mixed infection with Crypto+ Giardia+Entameba ; (H) negative result with controls.

3.9 Distribution of parasitic causes of diarrhea by using PCR

The Figure (3-5) Showed the distribution of parasitic causes of diarrhea by using PCR,where the result of this study found the high prevelance of *G.lamblia* (67.16%) compared with other parasite ,this result has an agreement with many studies form developing and developed countries indicating that *G.intestinalis* is the most common protozoan parasite-causing diarrhea (Foronda *et al*.,2008;Sabry *et al*., 2009;Stark *et al*., 2009;Laude *et al*., 2016 and Mero *et al*., 2017). In present study found the positivity rate for *Cryptosporidium spp.* was (16.42)% ,this result has an agreement with Altamimi & Al-Zubaidi, (2020),who were founded the higher rate of *C.parvum* was (11%) in Babylon. In present study found the prevelence of *E.histolytica* was (16.42%), this result disgreement with another study in Thi-qar,who was founded the prevelence of *E.histolytica* was (35%) by using real –time PCR (Flaih *et al*., 2021).



Figure(3-5) The distribution of parasitic causes of diarrhea by using PCR

PCR detection of enteric protozoa is largly dependent upon the method used for DNA extraction from the stool specimens.Most of the previously developed PCR assays have reported high levels of sensitivity and specificity using pure genomic DNA samples (Won *et al*., 2016).They were some studies used multiplex PCR assay developed for simultaneous detection of *E.histolytica,G.lamblia* and *C.parvum* in stool sample (Verweij *et al*., 2004;McAuliffe *et al*., 2013;Ramírez *et al*., 2015 and Al-Talib *et al*., 2019).

Table (3-6) Comparison of the Triage Micro Parasite Panel, direct examination and PCR results for diagnosis intestinal parasite.

Parasite / Test	*E.histolytica* (n)%	*G.lamblia* (n)%	*C.parvum* (n)%	Total
Direct fecal examination	25(29.06%)	48(55.81%)	13(15.11%)	86
Immunocromatography test	8(13.33%)	41(68.33%)	11(18.33%)	60
PCR	11(16.41%)	45(67.16%)	11(16.41%)	67

The PCR technique that revealed high rate of *Giardia* infections 67.16%, followed by 55.81 % for direct microscopy as shown in table 3-6 , this result agreement with Yahya J Salman, (2014) ,who was founded high rate of *Giardia* infections 18.43% in PCR, followed by 15.20 % for direct microscopy in Kirkuk .While *C.parvum* was (15.11%) in direct microscopic examination followed by (16.41) in PCR ,this result has an agreement with Gawad *et al*., (2018) ,who was founded the high rate *C.parvum* as(21%) in PCR followed by (9.5%) in direct microscope examination in Egypt. In current study found the high prevelance of *E.histolytica* as (29.06%) in direct microscope examination and lower prevelance (13.33%),(16.41%) in immunocromatography and PCR respectively,this result has an agreement with many studies by microscopy that exhibited many false positive results ,this may be due to misdiagnosis of other *Entamoeba* species such as *Entamoeba coli, Entamoeba hartmanni*, or the morphologically identical *Entamoeba moshkovskii*, Also

the trophozoites of other several nonpathogenic intestinal amebas or fecal macrophages being misdiagnosed as *E. histolytica/E. dispar* (Kebede *et al*., 2004; Leiva *et al*., 2006; Hamzah *et al*., 2010 and Nazeer *et al*., 2013). .

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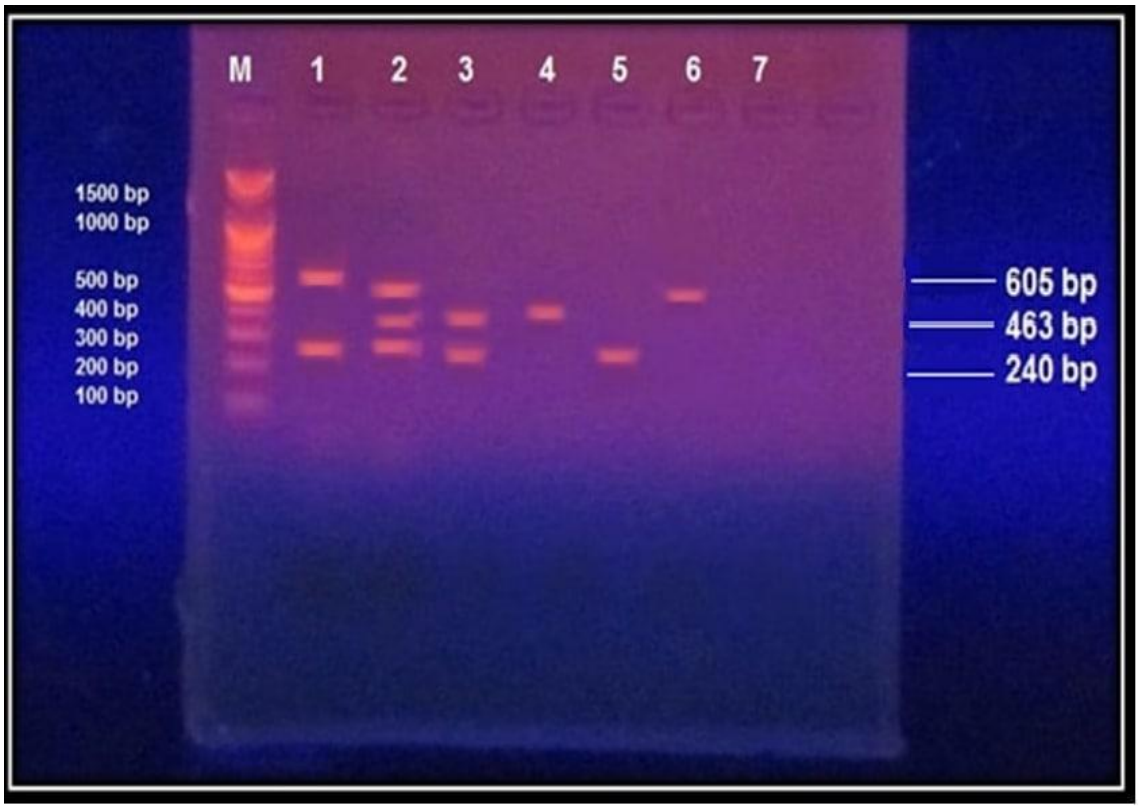


Figure (3.6): Agarose gel electrophoresis image showed multiplexPCR product analysis for *G.lamblia,E.histolytica* and *C.parvum* gene M (Marker ladder 100bp). Lane 1: *C.parvum* and *E.histolytica* represented 240 and 605 bp resepctivelly, Lane 2: *C.parvum, E.histolytica* and *G.lamblia* represented 240, 605 and 463 bp respectivelly, lane3; *C.parvum* and *G.lamblia* represented 240 and 463 bp respectivelly; lane 4; *G.lamblia* represented 463 bp, lane 5: *C.parvum* represented 240 bp, lane 6: *E.histolytica* repesrented 605 bp lane 7: negative control.

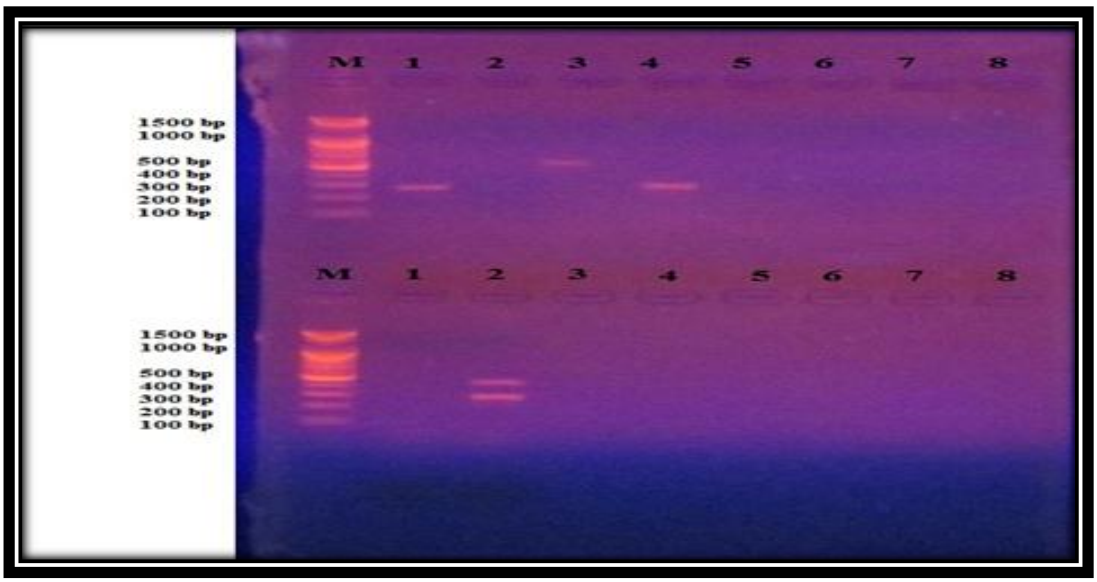


Figure (3.7): Agarose gel electrophoresis image showed monoplex product analysis for M (Marker ladder 100bp). Lane 1: *C.parvum* represemnted 240 bp , Lane 2: negative control, lane3; *G.lamblia* represented 463 bp; lane 4; *C.parvum* represented 240 bp, lane 5-8 ; negative control, while the bottom picture represented multiplex PCR *G.lamblia* and *C.parvum* parasites represented 463 and 240 bp in the lane 2.

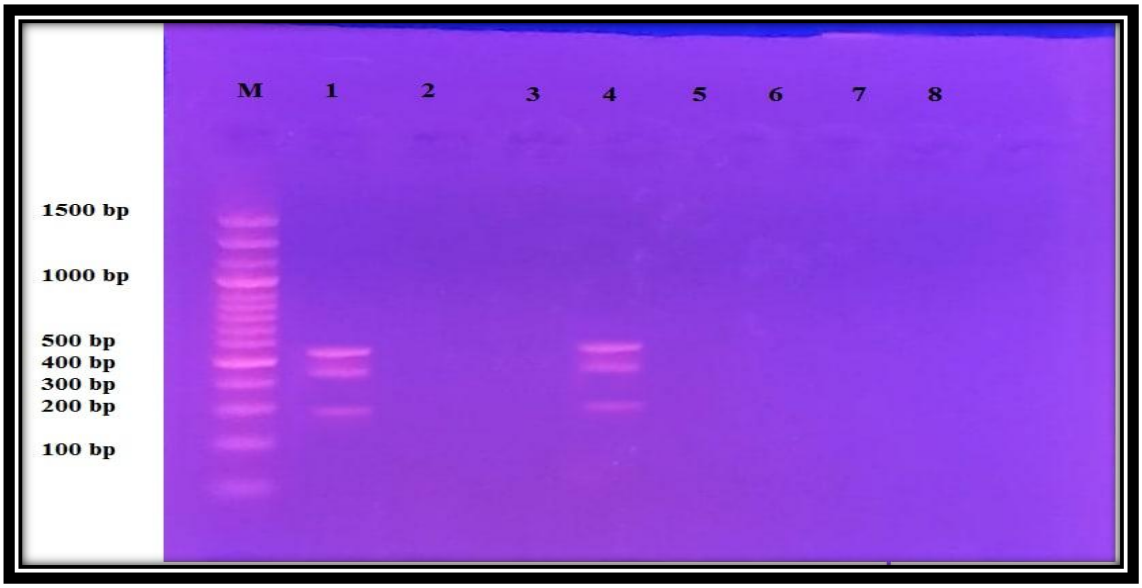


Figure (3.8): Agarose gel electrophoresis image showed multiplexPCR product analysis for *G.lamblia,E.histolytica* and *C.parvum* gene M (Marker ladder 100bp). Lane 1: *C.parvum, E.histolytica* and *G.lamblia* represented 240, 605 and 463 bp respectivelly, lane2; negative control ; lane 3; negative control , lane 4; *C.parvum, E.histolytica* and *G.lamblia*, represented 240, 605 and 463 bp respectivelly, lane 5; negative control.

Table(3-7) showed the immunocromatography test have sensitivity (66.67%, 93.18% and 100%),for detection of intestinal parasites (*E.histolytica*,*G.lamblia* and *C.parvum*) respectively,this result agreement with Goni *et al*., (2012),who were found the sensitivity for detection *E.histolytica* 62.5%,*G.lamblia* 97% and *C.parvum* 72% , Also disagreement with Swierczewski *et al*., (2012) ,who was found the sensivity of *G.L*, *E.H* and *C.P* 100%,100% and 73% respectivily, As well as disagree with Yahya J Salman, (2014) ,who was discoverd the sensitivity of *G.lamblia* was 40.10%.

Table (3-7): The Sensitivity and specificity results for the Triage Micro Parasite Panel

	Imunocromatography (Triage panal test)	
Type of parasite	**Sensitivity**	**Specificity**
E.histolytica	**66.67%**	**94.55%**
G.lamblia	**93.18%**	**82.61%**
C.parvum	**100.00%**	**100.00%**

Polymerase chain Reaction :gold standard

The specificity of precent result ,by immunocromatography test was 94.55%, 82.61% and 100% for *E.histolytica* ,*G.lamblia* and *C.parvum* respectivilly,the result agreement with Nazeer *et al*., (2013) who found 91%,85.5% and 100% for *E.H*,*G.L* and *C.P* respectively .

Table (3-8): The Sensitivity and specificity results for the Triage Micro Parasite Panel and PCR.

	Imunocromatography (Triage panal test)		PCR	
Type of parasite	**Sensitivity**	**Specificity**	**Sensitivity**	**Specificity**
E.histolytica	**72.73%**	**77.33%**	**78.57%**	**80.56%**
G.lamblia	**93.18%**	**83.33%**	**93.75%**	**92.11%**
C.parvum	**78.57%**	**97.22%**	**78.57%**	**97.22%**

Direct microscope :gold standard

While the results found the sensivity of *E.H* ,*G.L* and *C.P* (78.57,93.75% and 78.57%) in multiplex PCR (table 3-8),this result has an agreement with (Bairami *et al*., 2018 and Yahya J Salman, 2014) ,who was founded sensitivity 90.91% and 92.37% for *G.lamblia*, respectivilly. On the other hand it was different with Nazeer *et al*., (2013),who was recorded the sensitivity for detection of *E.histolytica*,*G.lamblia* and *C.parvum* 100%,57.8% and 33.3% by using molecular technique (RT-PCR) respectivilly.

3.10 Prevalence of *Rotavirus* that cuases diarrhea

In figure 3-10, the comparsion between two test for detection of *Rotavirus*. In this study, stool samples of all children were less than six years old with acute diarrhea were screened for *Rotavirus* antigen.The results showed 120 samples out of 320 were positive for *Rotavirus* infection by using immunogromatography from pediatric teaching hospital in Babylon province.

In order to Immunochromatography assay results analysis, conventional PCR was carried out to detect the gene of outer layer protein *VP4* of *Rotavirus* 663bp. The study enrolled 120 samples from *Rotavirus* positive samples by an Immunochromatography assay , while the result of RT-PCR demonstrated 84 samples from 120 *Rotavirus* positive samples by an Immunochromatography assay.

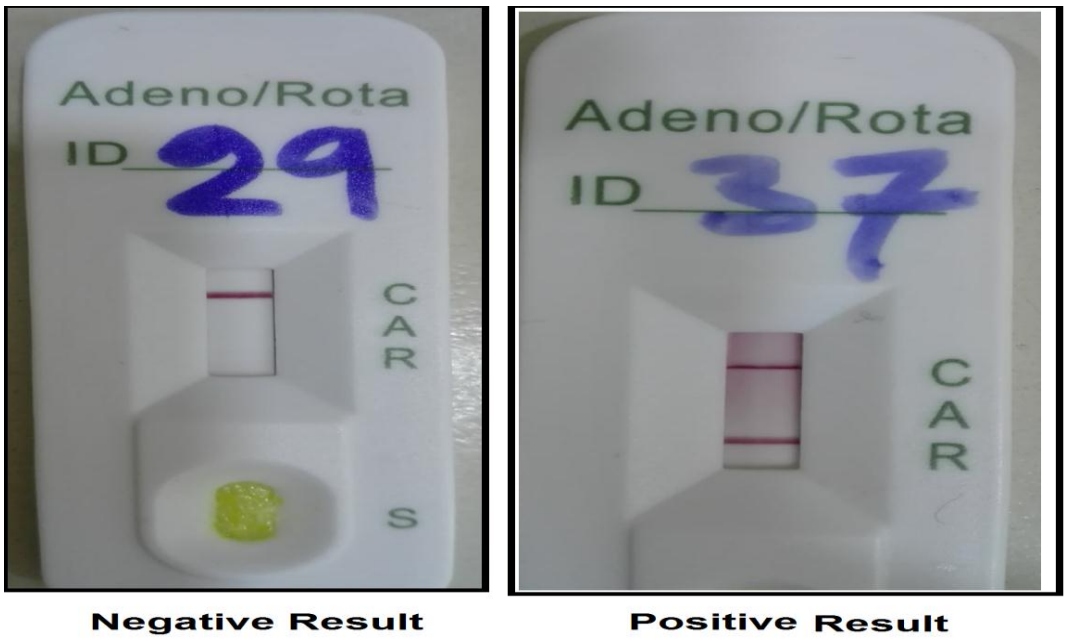
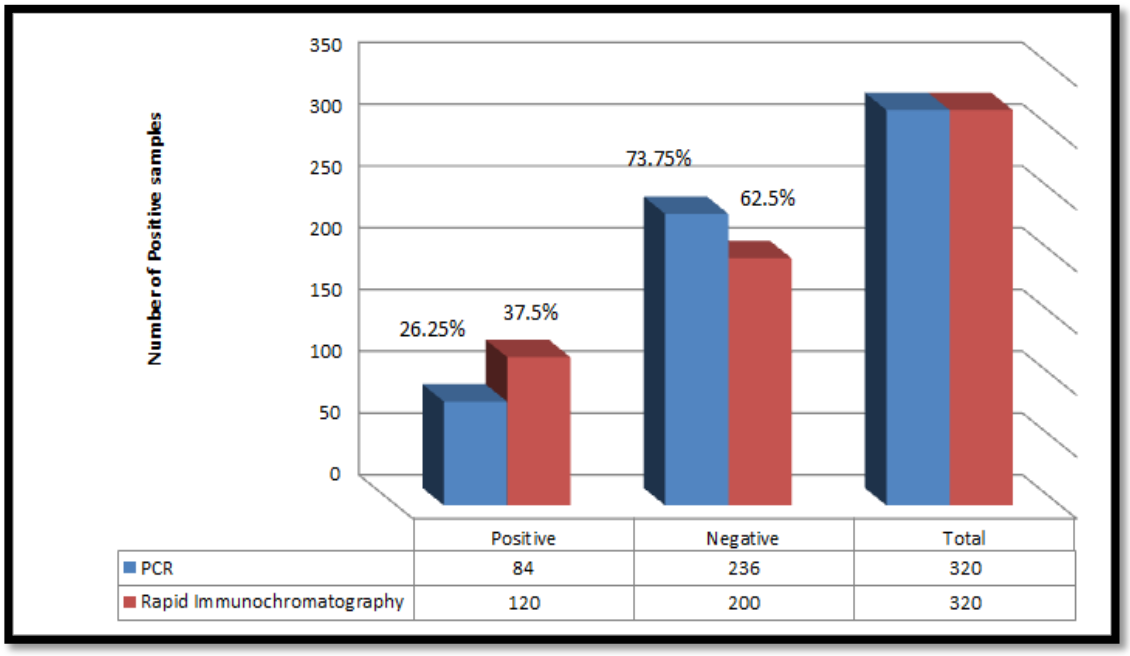


Figure (3-9):Immunochromatography assay for *Rota virus*, positive and negative results

The present study of *Rotavirus* infection in children was detected by rapid Immunochromatographic test (ICT) showed 37.5% figure (3-9), recent results disagree with Abdulazeez & Abed, (2017) who collected the samples from five Iraqi provinces (Babil, Kerbala, Missan, Qadissiya, and Wasit) they found a similar prevalence (47.2%), by using (IC) test,but agreement with other studies conducted in Basrah city/Iraq by (Yaqoob *et*

al., 2016) he found the prevalence of *Rotavirus* infection (31%) among hospitalized children ,this percentage was closes to the percentage that recorder in Basra 32.5% and Babylon 33.3% (Habash & Habeeb, 2018 and Lafta *et al*., 2019). The high differences in the occurrence of diarrhea caused by *Rotavirus* infection due to variant in age, detection protocol, prevalence of disease according to month and season of year as well as variant region in different countries (Zeng *et al*., 2012). Fenjan *et al*., (2020) showed the occurrence of *Rotavirus* more common in children under 5 years. On the other hand ,among PCR recorded the prevelance of *Rotavirus* was (26.25%) in PCR,this result disagree with (Durmaz *et al*., 2014) who was found (78.2%) of *Rotavirus* in tested samples from children by using RT-PCR in Turkey.Also disagree with Marmash *et al*., (2007) who revealed (44%) in Kuwait children.



Figure(3-10) Comparsion between rapid immunocromatography test and PCR for detection of *Rotavirus*

The prevalence of documented study found lower to those described from neighboring city close related in 2017 (Monavari *et al*., 2017) showed closely rate 39.9% in Iranian Children suffering from diarrhea, whereas Karadag *et al*., (2005) showed (37%) in Turkey.

Two tests were used for detecting the *Rotavirus* infection in children suffering from diarreha, Rapid Chromatographic Immunoassay and RT PCR . Table (3-9) was explained the specificity and sensitivity; the result found Rapid Chromatographic Immunoassay was sensitive to detecting *Rota virus* infection; its show sensitive by 100% and specificity 84.75% .

Table 3-9: Specificity and sensitivity of Rapid Chromatographic Immunoassay for detection of *Rotavirus* infection.

Type of pathogen	Immunocromatography (Triage panel test)	
	Sensitivity	**Specificity**
Rotavirus	**100.00%**	**84.75%**

The sensitivity of IC (75%) and the specificity (95%) (Weitzel *et al*., 2007). Another study found the sensitivity and specificity of immunocromatography as (97.1%) (100%) for *Rotavirus* detection (Artiran *et al*., 2017). The RT-PCR assay's good performance was also expressed in its ability to detect *Rota virus* RNA in 84 of 320 children, with a prevalence of 26.25 % .

To detect Vp4 protein in *Rotavirus* infectious agents, two sets of oligonucleotide primers were used in a polymerase chain reaction (PCR)

assay. The efficiency of these primers was demonstrated on environmental isolates that had previously been verified by (Hassine-Zaafrane *et al*., 2015).

Rotavirus viral protein 6, a major core protein, comprises antigenic epitopes for group and subgroup specificity, and VP4, a coat protein that induces neutralizing antibodies, is the viral hemagglutinin and determines species susceptibility (Dormitzer *et al*., 2002).

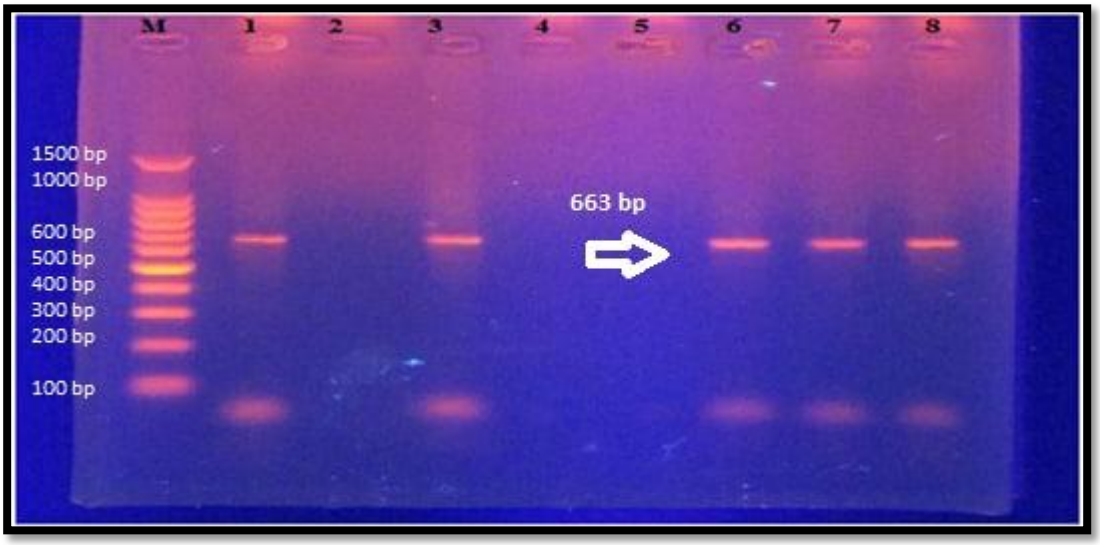
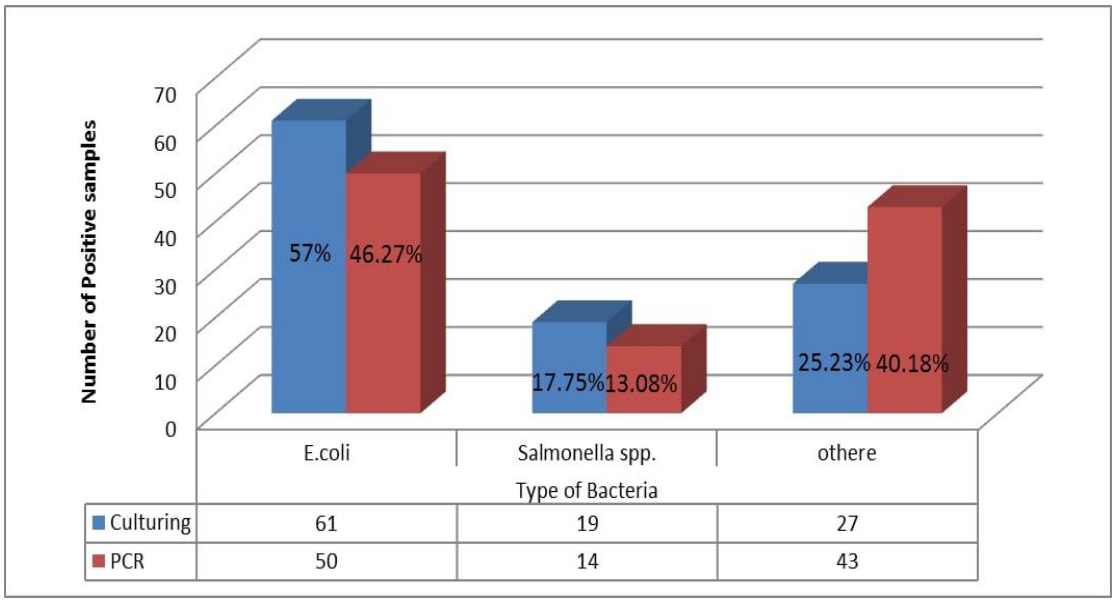


Figure (3-11): Agarose gel electrophoresis image showed RT-PCR product analysis for *VP4 gene* M (Marker ladder 100bp). Lane (1, 3, 6,7 and 8) represented positive *VP4 gene* in *Rotavirus* at 663 bp product., while lane 2, 4 and 5 represented negative samples.

3.11 Isolation and identification of bacterial causes of diarrhea

In figure (3-13), the comparsion between two tests for detection of bacterial infection, all samples were tested from children suffered from diarrhea by culture and PCR.In this study,the result showed 107 samples out of 320 were positive for bacterial infection from pediatric teaching hospital in Babylon province.



Figure(3-13) Comparsion between culture and PCR for detection of *E.coli* and *Salmonella*

The primary characteristics of cultured bacterial isolates depend on Maconkey agar, XLD agar,EMB agar, and S-S agar then study of microscopic and morphological characteristics and biochemical tests , figure (3-12) show the types of bacterial isolates. *E.coli* showed pink and dry colonies on Maconkey agar because it ferments the sugar lactose, while on EMB medium the *E.coli* appear a shiny metallic green color,while on XLD agar the bacterium *Salmonella spp* appear as pink (lactose non-fermenting colonies) with black center (due to H_2S production) or it's appear yellow colony without H2S production , *Salmonella spp* appear pink or colorless colony with and without black center on SS agar (Humphries & Linscott, 2015).The bacterial isolate was appearing as Gram negative rod ,and the biochemical test according to (MacFaddin, 2000).

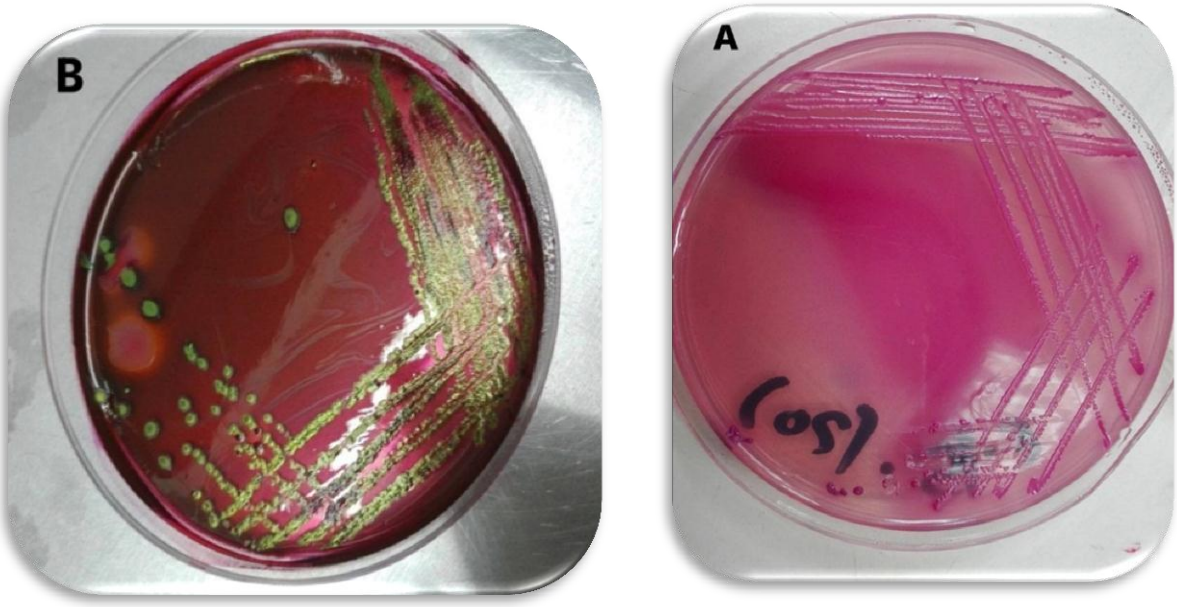


Fig. (3-12a): (A)E.coli on MacConkey agar (B)E.coli on Eosin methylene blue agar

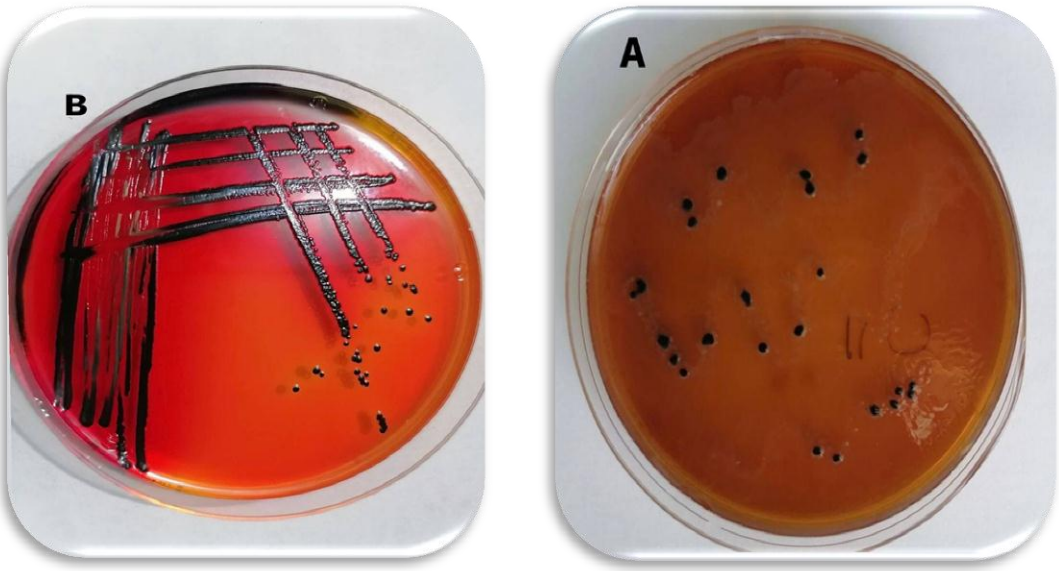


Fig. (3-12b): (A)Salmonella on (S-S) agar (B)Salmonella on (XLD) agar

3.12 Biochemical tests

To classify the isolates of bacteria, a number of biochemical tests were conducted. The results of biochemical tests confirm the diagnosis of bacterial isolates and their names, according to (MacFaddin, 2000) are shown in Table (3-10).

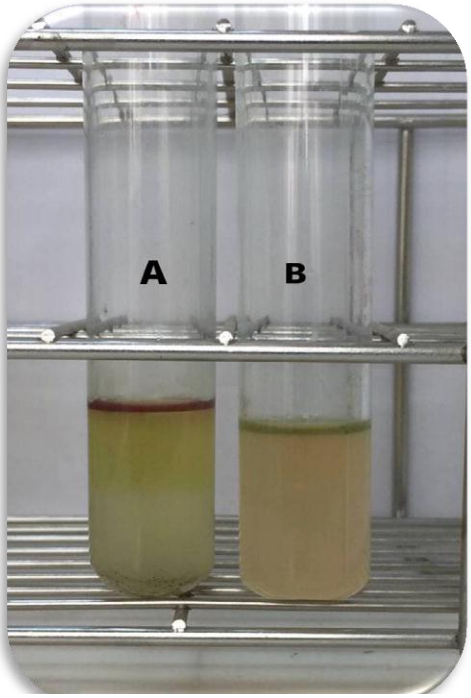
Table (3-10):Biochemical tests of gram negative bacteria

Test / Bacteria	Indole	Citrate	Methyl red	Catalase	Oxidase	Triple Sugar Iron			
						Gas	H_2S	Butt	Slop
E.coli	+	-	+	+	-	+	-	**A**	**A**
Salmonella	-	+	+	+	-	-	+	**A**	**K/R**

A: acid, K: alkaline, R: no change, +: positive , - : negative

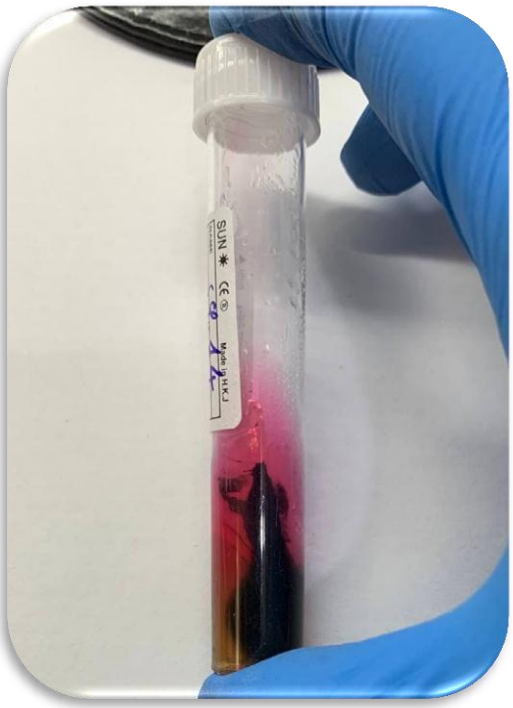
Current results found the high number of total positive isolates from cultured 107 stool samples were 61 isolates (57%) for *E.coli* rather than 19 isolate (17.75%) for *Salmonella spp* as shown in figure (3-13),this result has an agreement with Shatub *et al*., (2021),who was found the prevelance of *E.coli* (40.5%) in Tikrit. Also agreement with Tawfeeq *et al*., (2017) ,who was found that most of the isolates belong to *E.coli* enterobacteriaceae,this means that the enterobacteriaceae, which is present in a normal flora in the intestine, when occur disorder in the immune system, becomes an opportunistic pathogenic bacteria, they were found bacterial isolates distribution were (34.48 and 20.68)% for *Escherichia coli* and *Salmonella*, respectivilly.As well as agreement with (Rodulfo *et al*.,

2012;Yasir, 2017;Al-Abbas, 2018 and Sharif *et al*., 2020) who were confirmed the high prevelance of *E.coli* (25.8%),(75%),(33.6%) and (17.3%), respectivilly compared with other types of bacteria.Another study in Diyala found the high prevelance of *E.coli* was (82.80%) compared with *salmonella* (5.03%) (Abood *et al*., 2020).Similar findings were also reported in other studies (Rashedul, 2011 and McAuliffe *et al*., 2013). Libya country,it has found diarrheagenic *E.coli* was the most common detected bacterial pathogens (Rahouma *et al*., 2011). These high rates of recovery of *E coli* found in the current study may be due to low education of parents, low-grade of health hygiene, poor toilet training, lack of exclusive breastfeeding, artificial feeding, source of water supply, over crowding and climatic conditions (Hellard *et al*., 2000). .



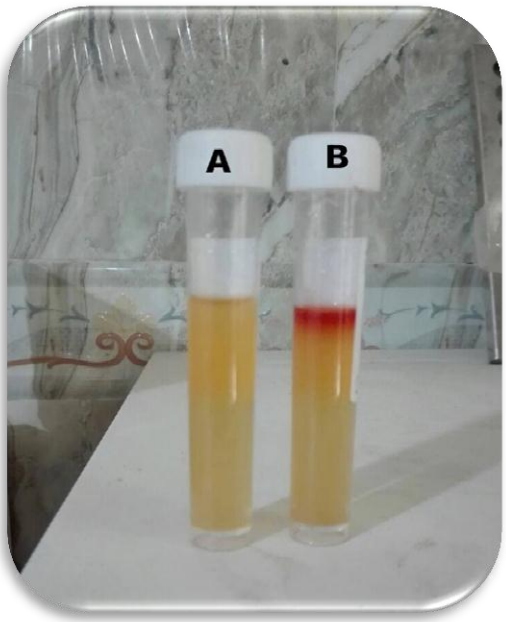
Figure(3-14a):Indole test

(A):Positive test
(B):Negative test

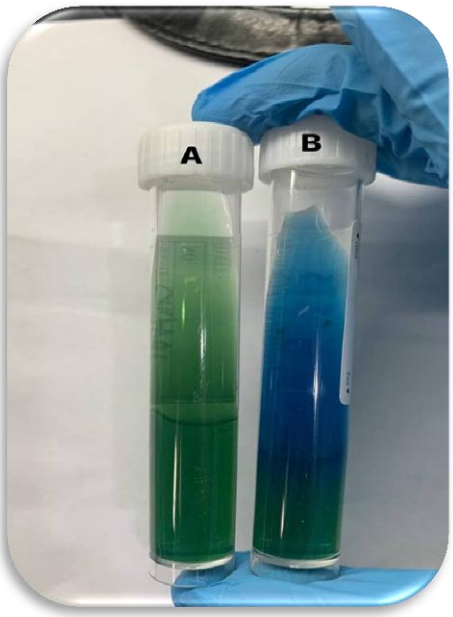


Figure(3-14b):Triple sugar iron test

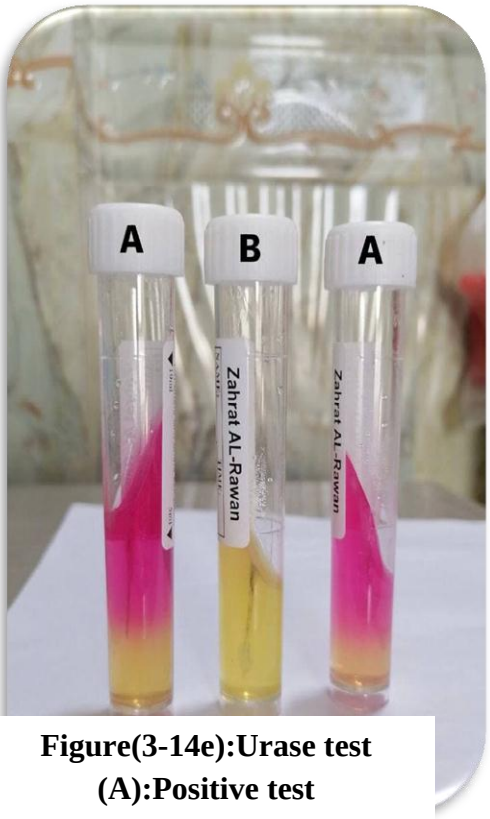
(K/A) hydrogen sulfide



Figure(3-14c):Methyl red test
(A):Negative test
(B):Positive test



Figure(3-14d):Cimmon citrate test
(A):Negative test
(B):Positive test



Figure(3-14e):Urase test
(A):Positive test
(B):Negative test

Figure (3-14f):
Catalase test

3.13 Molecular characteristics of bacterial causes of diarrhea

The PCR analysis was applied to DNA extracted from bacteria, *Salmonella* isolates showed that out of 19 *Salmonella* isolated from conventional culture , 14 (13.08 %) isolates were positive , showing the specific band for *16S rRNA* gene of *Salmonella.* Only the band with the expected size (574 bp) was observed (Fig 3-16).

While among *E.coli* the performing PCR reaction using the two primer pairs (PhoF and PhoR primers) on the PCR positive *Esherishia coli* strain isolates to the detection of *E.coli* of family species .The results of

Pho gene family amplification which produce a sharp band with the expected size (903 bp) have been observed in 50 (46.27 %) of PCR positive *Salmonella* isolates (figure 3-15).

In present study found the prevelence of *E.coli* (46.27%) in PCR,this result disagree with Shatub *et al*., (2021),who was found the preveance of *E.coli* (61.3%) by using PCR. The result found the prevelence of *salmonella* (13.08%),this result disagreement with McAuliffe *et al*., (2013) ,who was found the prevelence of *Salmonella* (1.3%). Another study in Egypt using pcr in diagnosis of *Salmonella spp* and *E.coli* (Younis *et al*., 2009).Polymerase Chain Reaction(PCR) is a major develop in molecular diagnostics of pathogenic microorganisms including *E coli*, which has a great sensitivity and high specificity in detection of target templates over classical bacteriological methods (Hellard *et al*., 2000).

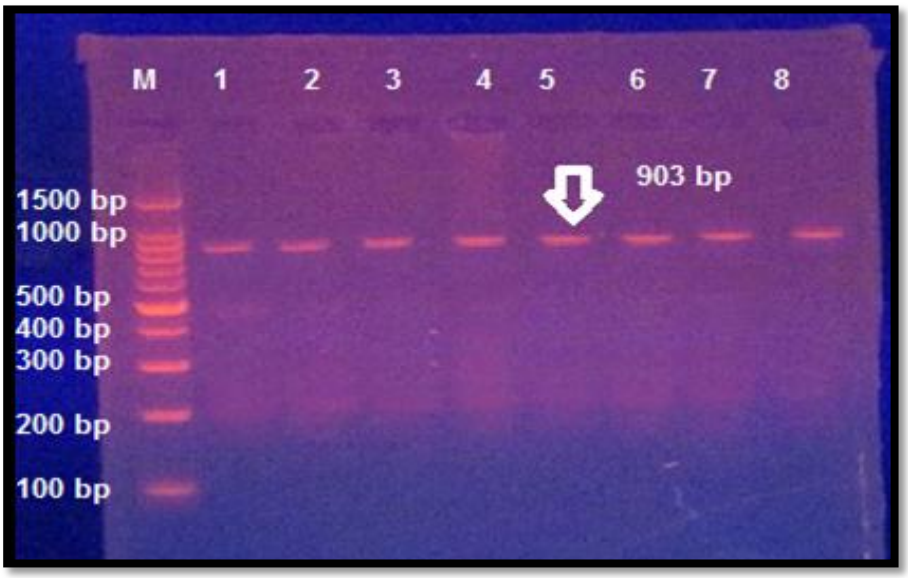


Figure (3-15): 1.5% gel electrophoresis for *E. coli* genes of family (*Pho*), M lane: DNA marker, Lane 1-lane8: represented 903 bp of *(Pho)* gene for *E.coli.*

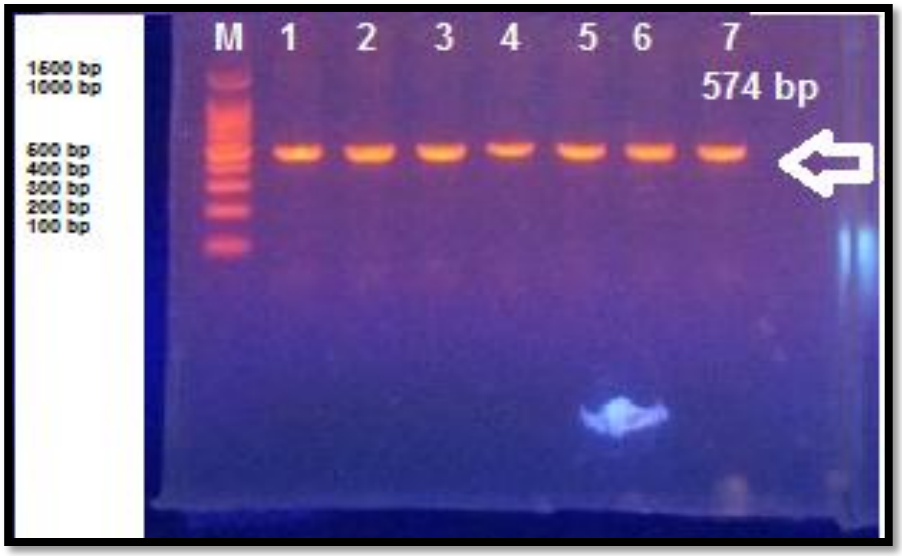


Figure (3.16): 1.5% gel electrophoresis for *Salmonella spp*. genes of 16S rRNA gene, M lane: DNA marker, Lane 1-lane7: represented 574 bp *of 16S rRNA* gene for *Salmonella spp.*

CHAPTER FOUR
CONCLUSIONS &RECOMMENDATIONS

4. Conclusions:

1.The highest infection rate of acute diarrhea in pre school children with viral (*Rotavirus*) more than bacterial *(E.coli,Salmonella)* and parasite infection *(G.lamblia,E.histolytica and C.parvum).*

2.Acute diarrhea caused by viruse and bacterial infection was widely occured among age group less than two years,while parasitic infection was widely occured among age group (4-6) years.

3.The high prevelance of parasitic infection in hot season compared with viral infection that occurred in cold season.

4.The rapid (Triage panel test kit) which used in this study ,which highly sensitivite and specific for *G.lamblia,C.parvum and E.histolytica* and this assay could be utilized by the person who do not have extensive training in manual parasitological methods. The kit used was good compared to the pcr and have high sensitivity in diagnosis of parasite in children stool samples.

5. Triage panel test is important in diagnosis of cryptosporidium parvum, which was not part of routine examinations in parasitological laboratory.

6. The specificity and sensitivity of the triage panel test, is approximate to the rotavirus kit that used for routine examination, which confirm the possibility of its using in the routine examination for the diagnosis of parasites.

4.1 Recommendations:

1. Many genotypic studies must be carried out to evaluate the differentiate between pathogenic and non pathogenic of *Entameba* species.

2. Immunocromatography rapid tests recommended as a rotine work in hospitals lab. for detection of parasites and *Rotavirus* infections.

3. The possibility of using immunocromatography rapid test to detect parasitic Ag in tissue, stool, urine ,saliva , CSF ,and blood .

4. Triage panel test can be used on frozen samples after thowing.

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APPENDICES

Appendix (1)The questionnaire of cases of intestinal disorders.

No.	Patient name	
1	Sample collection site	Hospital Clinical Laboratory
2	Gender	Male Female
3	Age of patient	Less than 2 years 2-6 years
4	Residency	Rural Urban
5	Feeding less than 2 years	Breast feeding Artificial feeding
6	diarrhea frequent ,(recurrent infection)	One Two More
7	the number of times of diarrhea /day accompanied by vomiting, fever	Three time defecation Five time with More with
8	the treatments used if any, type of drugs	Yes type of drugs no
9	The macro examination characteristic , color of the discharge	Watery with Bloody with Stetoreah with
10	Notes	

Appendix (2): Immuno Chromatography kit.

Purification kit	Contents	Origin
CERTEST Crypto+Giardia and Entamoeba COMBO CARD TEST	CerTestCrypto+Giardia+Entamoeba combo card tests Instructions for use Stool collection tubes with diluent	Zaragoza(Spain)

Appendix (3): Immuno Chromatography kit.

Purification kit	Contents	Origin
LumiQuick Adeno-RotaVirus Antigen Comb Test Card	Combo test card Each cassette contains a test strip with adenovirus and rotavirus specific antibody on the test region of the membrane and colored adenovirus and rotavirus antibody-gold conjugate pad. Combo test card. Sample bottle ,each sample bottle contain 1.5 ml of stool specimen collection buffer.	Netherlands

Appendix (4): Benedicts soulution

(A):Negative solution

(B):Positive solution

الخلاصة

تتضمن الدراسة الحالية بعض انواع الطفيليات والبكتريا والفايروسات المسؤولة عن الإصابة بالإسهال لدى الأطفال دون سن السادسة من العمر الذين يعانون من الإسهال الحاد خلال الفترة من اول كانون الأول ٢٠٢٠ حتى نهايه أيار ٢٠٢١الوافدين إلى مستشفى بابل التعليمي للولادة والاطفال في مدينة بابل.

تم جمع حوالي٣٢٠ عينة براز من كلا الجنسين من المرضى الراقدين في المستشفى لتحديد العامل المسبب للإسهال الحاد وتم إجراء الكشف الأولي عن الاصابة الطفيلية باستخدام الفحص المجهري والفحص الكروماتوغرافي المناعي (فحص سريع) وتم استخدام تفاعل البوليميراز المتسلسل (PCR) المتعدد من أجل تأكيد الاصابات بالطفيليات الثلاثة بواسطة الجين المستخدم (*EHCP8-S1*، *GLCP6-S1* و *CRY18s-S1*) ، (PCR) استخدم للتعرف على البكتيريا للكشف عن الجين (*pho*) لبكتريا (*E.coli*) والجين *16s rRNA* لبكتريا (*Salmonella spp.*) . ومن ناحية أخرى من بين الفايروسات التي تسبب الاسهال، تم إجراء الكشف الأولي عن الاصابة بفيروس (*Rotavirus*) باستخدام الفحص الكروماتوغرافي المناعي (فحص سريع) وباستخدام Reverse Transcriptase-PCR لتأكيد الكشف عن الاصابة بفايروس (*Rotavirus*) بواسطة الجين (*vp4*).

وأظهرت النتائج وجود الطفيليات في ٨٦ عينة من أصل ٣٢٠ عينة إسهال (مأخوذة من الأطفال)وهذه الطفيليات مقسمة إلى ثلاثة أنواع وهي (٢٩.٦٪) ٢٥ *E.histolytica* و (٥٥.٨٢٪) ٤٨ *G.lamblia* و (١٥.١٢٪) ١٣ *C.parvum* عن طريق استخدام الفحص المجهري المباشر بينما الفحص الكروماتوغرافي المناعي (١٣.٣٤٪) ٨ ، (٦٨.٣٣٪)٤١ و (١٨.٣٣٪)١١ لهذه الطفيليات على التوالي . وأظهر فحص PCR(١٦.٤١٪)١١ و (٦٧.١٦٪)٤٥ و(١٦.٤١٪)١١ لكل

من *E.histolytica* و *G.lamblia* و *C.parvum*على التوالي لتقييم الفحص الكروماتوغرافي المناعي .ومن بين عمليات الكشف عن فيروس (*Rotavirus*) وجد الفيروس في١٢٠ (٣٧.٥٪) عينة موجبة من ٣٢٠ طفلاً مصاباً بالإسهال عن طريق استخدام الفحص الكروماتوغرافي المناعي بينما في فحصPCR وجد (26.25%) 84عينة موجبة لفيروس (*Rotavirus*). كما وجدت النتائج حساسية لفيروس (*Rotavirus*) (١٠٠٪) ونوعية (٨٤.٧٥٪) أما العزلات البكتيرية(*E.coli* and *Salmonella spp.*) فقد أظهرت النتائج أن هناك ١٠٧ (٣٣.٤٣٪) عينة موجبة من ٣٢٠ طفل مصاب بالإسهال عن طريق زرع العينة على الاوساط الزرعية والفحوصات (Biochemical tests) أما في فحص PCR فقد ظهرت الإصابة البكتيرية في ٥٠ (١٥.٦٢٪) عينة موجبة فقط ووجدت النتائج فروقآ معنوية بين الاصابات الطفيلية والفيروسية والبكتيرية ووجدت النتائج ارتفاع نسبة انتشار الطفيليات والفيروسات والبكتيريا لدى الذكور بنسبة ٦١.٦٢٪ و ٦٧.٥٪ و ٥٧.٩٤٪ أكثر من الاناث ٣٨.٣٧٪، ٣٢.٥٪ و ٤٢.٠٥٪ على التوالي .

اظهرت الدراسه الحاليه وجود فروقات معنوية في الاصابة بالبكتيريا والطفيليات والفيروسات حيث وجد أن المناطق الريفية ٦٠.٤٦٪ و ٥٤.١٦٪ و ٧١.٠٢٪ أكثر انتشارًا للاصابات الطفيلية والفيروسية والبكتيرية مقارنة مع المناطق الحضرية بنسبة ٣٩.٥٣٪ و ٤٥.٨٣٪ و ٢٨.٩٧٪ للطفيليات والفيروسات والبكتيريا على التوالي.

كذلك أظهرت الدراسة الحالية علاقه بين توزيع الأطفال في العمر مع عامل الاصابة ووجدت فروق معنوية في الفيروس والطفيلي. أعلى نسبة إصابة بفيروس (*Rotavirus*) والاصابة البكتيرية حدثت في الأطفال الذين تقل أعمارهم عن سنتين بنسبة (٤٩.١٦٪) ، و (٣٦.٤٤٪) على التوالي

وكانت أعلى بقليل مقارنة بالمجاميع الأخرى بينما سجلت نسبة انتشار أكبر للطفيليات (٥١.١٦٪) بين الأطفال من عمر ٤-٦ سنوات.

توصلت النتائج الى وجود زيادة معنوية في الاصابة الفيروسية (*Rotavirus*) التي تسبب الإسهال لدى الأطفال في فصل الشتاء مقارنة بفصل الصيف بينما كانت الإصابة بالطفيليات عالية في فصل الصيف مقارنة مع فصل الشتاء.

أخيرا، اظهرت الدراسة الحالية أن الفحص الكروماتوغرافي المناعي يمتلك حساسية (٦٦.٦٧٪ ، ٩٣.١٨٪ و ١٠٠٪) للكشف عن الطفيليات المعوية (*E.histolytica*، *G.lamblia* و *C.parvum*) على التوالي . بينما خصوصية النتيجة التي توصلنا إليها عن طريق الفحص الكروماتوغرافي المناعي ٩٤.٥٥٪ ، ٨٢.٦١٪ و ١٠٠٪ لـ *E.histolytica* و *G.lamblia* و *C. parvum* على التوالي.

وجدت نتائجنا أن العدد الكبير من العزلات الموجبة الكلية من مجموع ١٠٧ عينة براز كانت ٦١ عزلة (٥٧٪) لبكتيريا (*E.coli*) و ١٩عزلة موجبة (١٧.٧٥٪) لبكتريا (*Salmonella spp.*).

نتائج الفحص الكروماتوغرافي المناعي أثبتت أنه يمكن تحديد كلا النوعين من الطفيليات *C.parvum* مع *G.lamblia* كأصابة مزدوجة بدلا من اصابة مفردة.

من خلال النتائج التي حصلنا عليها نستنتج ان أعلى معدل إصابة بالإسهال المائي لدى الأطفال هي الاصابة بالفيروسات تليها الاصابة البكتيرية ثم الطفيلية وكذلك العدة المستخدمه في هذه الدراسة يعتبر شديد الحساسية والخصوصية ل *G.lamblia* و *C. parvum* و*E.histolytica* ويمكن استخدام هذا الفحص المناعي من قبل الأفراد الذين ليس لديهم تدريب مكثف على طرق تشخيص الطفيليات مجهريا. خصوصيه وحساسيه عدة تشحيص كانت قريبه من عدة تشخيص الروتا فايروس الذي يستخدم بصوره روتينيه مما يؤكد امكانيه استخدامها في الفحص الروتيني لتشخيص الطفيليات.

وزارة التعليم العالي والبحث العلمي

كلية العلوم الطبية التطبيقية / جامعة كربلاء

قسم التحليلات المرضية

تقييم كفاءة اختبار الشرائط المناعية مقارنة بالتقنيات التقليدية والجزيئية في تشخيص بعض مسببات الإسهال الحاد في المرضى دون سن المدرسة في محافظة بابل / العراق

رسالة مقدمة

الى مجلس كلية العلوم الطبية التطبيقية - جامعة كربلاء

وهي جزء من متطلبات نيل شهادة الماجستير في التحليلات المرضية

من قبل

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