**Republic of Iraq**
**Ministry of Higher Education**
**And Scientific Research**
**University of Baghdad**
**College of Veterinary Medicine**
**Department of Parasitology**

# Study the prevalence and molecular identification of *Cryptosporidium* in Ostriches( *Struthio camels*) at central parts of Iraq.

**A Thesis**

**Submitted to the Council of the College of Veterinary Medicine / University of Baghdad as a Partial Fulfillment of the Requirements for the Degree of Ph.D. in Veterinary Medicine / Parasitology**

**By**

**Haleem Hamza Hussain Al-Zubaidei**

**Supervised by**

**Prof. Dr. May Hameed Kawan**

**2020 A.D.** **1441 A.H.**

## Introduction

The common Ostrich (*Struthio camelus*) large size and flightless bird ,the species name *Struthio camelus* comes from Latin. The word *camelus* is based on the similarities of Ostriches with camels, such as prominent eyelashes and remarkable tolerance to the desert habitat. (Cooper et al., 2009)

The classification of the Ostriches according to Abbas et al (2018) is :

**Kingdom:** Animalia

**Phylum:** Chordata

**Class:** Aves

**Order:** Struthioniformes( Ratites)

**Family:** Struthionidae

**Genus:** *Struthio*

**Species:** *S. camelus*

Ostriches are omnivores, diet consists mainly of plant , shoots, shrubs, leaves, seeds and fruit also eats invertebrates and lizards .Its preference open plains with short grasses, and semi-arid regions and deserts with annual grass,It lives in nomadic groups as families formed from 5 to 50 birds.( Hassan et al., 2004).

Common Ostriches occupied Africa north and south of the Sahara, distribution in to large areas around the world East Africa, and much of South-West Asia , the Arabian Ostrich ranged from southern Syria and the Arabian Peninsula to Iraq's Euphrates Valley , Negev Desert ( Shanawany and Dingle , 1999)

Ostriches are multi-benefits, valuable products which include feather dusters are effective by means of the tiny barbules on the feathers themselves that act as fingers to collect the dust, also used around electronics because of their resistance to static electricity. Each ostrich get (1.4 – 1.8 kg of feathers / bird), Ostrich leather has a high economic price in the world ,leather (1.1 - 1.3 $m^2$/bird), healthy red meat (34 – 41 kg/bird) , egg production (50 – 120/season) (El-Safty, 2015). Ostrich oil is more important , have used in economic purposes and traditional medicine for treatment of various disease, including asthma (Magige and Roskaft, 2017)

Farming Ostriches both wild and captive Ostriches is a new field of birds livestock production which infected with internal parasites : *Houttuynia struthionis* (cestoda) ,*Libyostrongylus douglassii* (Nematoda) ,also intestinal protozoans including *Hexamita, Giardia, Trichomonas, Cryptosporidium , Eimeria spp* .which they isolated from Ostrich chicks causing the most serious economic losses in ratites through the world. (Nemejc and Lukesova ,2012)

The genus *Cryptosporidium* from apicomplexan protozoal parasites that infected the microvillus surface of digestive and respiratory epithelial cells of many types of vertebrates .(Xiao and Ryan,2004). Avian cryptosporidiosis has been recorded in more than 30 birds species in the world (Behzadi *etal* ., 2009 ) It is important pathogens with highly distribution rate in livestock and wild life (fayer,2004). The parasite well be found in intestine ,respiratory ,urinary ,pancreatic and bile tracts of over 30 species of birds but the important *Cryptosporidium spp* are named: *C. galli*, *C. meleagridis* and *C. baileyi* which infected awide range of birds and the most third Cyptosporidiosis in man ( Dacunha *et al.*, 2018).the predilection sites of C. *meleagridis* and C. *baileyi* in the bursa, small and large intestine , while C. *baileyi* mostly found in respiratory tissues in contrast , *C. galli* presented in proventriculus of birds.(Xiao *et al* .,2009)

*Cryptosporidium* parasites are important from sanitary and economically in ratites birds , it has been infected ostrich chicks causes cloacal and phalus prolapse with enteritis ,pancreatic necrosis (Bezuidenhout *et al* .,1993 and Behzadi *et al.*, 2009) .The adult Ostriches infected with *Cryptosporidium* without clinical illness, while young Ostriches and other birds that infected with secondary bactrial and viral disease or imunocompromised show fatal disease.( Current, 1991). The transmission of *Cryptosporidium* oocysts via faecal- oral rout , direct or indirect contact , water borne, food borne , and airborne (fayer,2004). The genatic analysis of genus *Cryptosoridium* consist many different species and genotypes depending in the molecular technique's since oocysts are not recognized morphologically (khan etal., 2018). Cryptosporidium species distribution in Ostriches population depending in many factors : stressing condition ,poor husbandry practices, type of feed , water or hygiene management ( Godro *et al.,* 2002 ) The conventional methods for detection *Cryptosporidium* prtozoal parasite is based on morphology and diameters of oocysts by using modified Ziehl-Neelsen stain (mZN) ( Kar *et al*., 2011). This traditional staining method is not useful for differentiation between *Cryptosporidium* spp. because of the morphological similarity between them. While molecular , gentyping and subgenotyping tools of *Cryptospridium* has led to identification and unique characterization of *Cryptosporidium* species in animals and man( Feng and Xiao ,2017)

The epidemiology of most *Cryptosporidium* spp. that infected Ostriches are still un determined , The Ostriches farming is most prevalence in south Africa , united states of America , Zimbabwe and Australia Although Western Europe and Botswana ( Mushi *etal* ., 2003) . *Cryptosporidium* oocysts in Ostriches have been found in greece and highly prevalence in spanish and also present in france and birds imported from Portuguese , Netherland and Belgium (DeGraaf *etal.*,

1999 and Sotiraki *etal* .,2001) it could be a new species similar in size to the *C.bailey , C.meleagridis, C.galli* and *C. parvum* oocysts, ( Godro *etal*., 2002 ) . where in china Wang *et al* (2011) confirmed the *Cryptosporidium baileyi* by DNA sequencing.

There are many important factors affected in prevalence of *Cryptosporidium* in ostriches such as stress conditions ,immunosuppression ,poor husbandry practices were affecting as predisposing factors to the philus and cloacal prolapse with bacterial infection: *E.coli* and protus (Laatamna et al .,2017) .

The spreaded of *Cryptosporidium* spp. in wild and domestic birds in Iraq was determined in Al- Qadisiya province by genotypic characterization and phylogenetic analyze based on 18S rRNA sequences of *Cryptosporidium* obtained from these bird with rate (58.1%) of infected birds'. They was 54.5% of the turkey, 57.5% of the domestic chickens, 53.8% of the broiler chickens, 62.5% of the common ducks, 76.7% of the quails and 26.7% of the feral pigeon. Sequencing and further phylogenetic analyses identified *Cryptosporidium parvum* in all birds ,*C. meleagridis* isolated from turkey and quail , *Cryptosporidium baileyi* observed in domestic and broiler chickens, quail and feral pigeon ducks, *C.galli* only recorded from domestic chickens.( Jasim and Marhoon, 2015) . In Baghdad city the total infection rate of wild pigeons was 40% which divided in to 38.18% males and 41.53% in females. A high infection rate 76.66% was found in winter season, while a low infection rate 16.66% was recorded in Autumn. The three *Cryptosporidium* species oocysts were detected varied from large size *Cryptosporidium galli* , medium *Cryptosporidium baileyi* and small *Cryptosporidium meleagridis* ( Faraj, 2014).While in Mosul city the investigation of natural occurrince of Cryptosporidiosis in pigeons recorded *C. baileyi* infection in rate 30% (Al-Mahmood, 2011).

Although there are some studies on wild birds in Iraq, but not any study on *Cryptosporidium spp* that infected farming Ostriches .

**Aims of the study :**

1- Study the prevalence of some intestinal protozoa from Ostriches and the effects of age ,sex and months in central and south parts of Iraq.

2- Study the morphological characterization of *Cryptosporidium spp* .

3- Using molecular technique from fecal samples using nested PCR technique for detection Cryptosporidium by using (18SrRNA gene) .

4- Study the genotyping of *Cryptosporidium spp* in Ostriches by sequencing .

.

## 2.Literature Review

### 2.1 Historical background

The first recognized of *Cyptosporidium* by Clarke and Tyzzer (1907) in early years of the twentieth century which they described the development stages of parasites ( sporozoites , trophozoites , merozoites , microgametes , macrogametes and oocysts ) as well as that he commonly attached to the gastric glands and found in laboratory mice feces and they chose the name *Cryptosporidium muris* . *Cryptosporidium muris* ( cypticus , latin for hidden) have spheroid or ellipsoid oocyst shape that described by Tyzzer (1910) .and later he observed development stages in small intestine of labratory mice that shedding oocysts smaller than *C. muris* ,and that is first report for new species named *C.parvum* ( Tyzzer, 1912).

The first discovered of *Cryptosporidium* in birds by Tyzzer (1929) . and because it resembled their earlier description of *Cryptosporidium muris* in mouse, he did not named this protozoal parasite (Tyzzer, 1907, 1910). After that Slavin in1955 was reported a similar morphologically parasite in turkeys named *C.meleagridis* .In 1986 isolated organism from chickens and named *C. baileyi* as separated species from *C. meleagridis* which has been supported by further experimental studies in poultry (Lindsay et al ., 1987). The *Cryptosporidium* criteria used for differentiation between vialed species suggested that *C.meleagridis* isolated from domastic and wild birds is very closely with *C.parvum* that infestation wide range of mammals (Sreter and Varga , 2000) . *Cryptosporidium* have many species and genotypes which can be recognized depending on morphological , biological and molecular characteristics , On the other

hand host–parasite co-evolution is common in *Cryptosporidium* parasites and phylogenetic analysis suggest *C. meleagridis* is from rodents and mammals origin (Xiao et al., 2004) .The Ostrich *Cryptosporidium sp* characterization provided by molecular phylogenetic analysis of the 18S ribosomal DNA fragments, showed that the *Cryptosporidium baileyi* is genetically distinct from all other known *Cryptosporidium* species or genotypes. Although biological and molecular studies indicate that the Ostrich *Cryptosporidium* is a new species. ( Meireles et al 2006) .Recently the avian *Cryptosporidium* genotype II was reported, in china which has been described in Ostriches species in rat 0.78% . while the chickens that were experimentally infected did not observed oocyst in feces( Nakamura and Meireles , 2015) .

**2.2. Taxonomy of *Cryptosporidium***

The taxonomy and nomenclature is important in the public health of various *Cryptosporidium spp* ,Currently the oocyst morphology and measurements are essential foundation for apicomplexan taxonomy. large numbers of genera can be Measurements by different type of microscope to identify distinct species, Therefore, the structure of oocyst is the first step in requirements for establishing a new species (Xiao and Ryan,2004) however , the *Cryptosporidium* morphology is not adequate for naming a new species because the Oocytes of many species are closely similar in size, and oocyst structure similarities have been caused confusion about *Cryptosporidium* spp validity (Anderson, 1981) *Cryptosporidium spp* are eukaryotic protozoa parasite present intra cellular extra cytoplasm it is host specific but in experimental infection have shown that more than one host can be infected (Duszynski , 1969).The classification of *Cryptosporidium* depending on species of host,infection side and oocyst size ( fayer and Ungar , 1986)

**Classification of *Cryptosporidium*** (Smith and Corcoran, 2004).

Phylum: *Apicomplexa*

Class: *Sporozoasida*

Sub–class: *Coccidiasina*

Order: *Eucoccidiorida*

Sub-order: *Eimeriorina*

Family: *Cryptosporidiidae*

Genus *Cryptosporidium*

Molecular taxonomy have been commonly used epidemiology characterization and genotypes of *Cryptosporidium* isolates from animal and human which based on many markers such as (18S subunit of rRNA gene and of the actin gene , employing 292 positions of 18S rDNA, *Cryptosporidium* oocyst wall protein (COWP), gp60 and heat shock protein 70 (hsp 70) (Ryan, 2010 ; Sevá *etal* 2011 ; khan *etal*., 2018 )

The markers are amplified from DNA extracts made from samples that using specific PCR primers followed by sequencing or restriction fragment length polymorphism analyses (RFLP) or Small subunit (SSU) rRNA sequencing-based tools which commonly used to genotype *Cryptosporidium* in the environment (Xiao *et al.*, 1999 ) The genome made from many copies of the SSU rRNA gene throughout this

marker are more sensitive than single copy genes. However, minor genetic variations will be observed among different copies of the SSU rRNA gene in *Cryptosporidium* species and genotypes, therefore new species or genotypes should not be named based on those minor (one or two single nucleotide polymorphisms (SNPs) (Abeywardena *et al.,* 2014 ).

### 2.3. Species of *Cryptosporidium* in birds

There are three dominant avian *Cryptosporidium* spp. Which can infect a broad range of birds, with different predilection sites (Xiao and Ryan,2004)

( Table 2-1) *Cryptosporidium* species in ostriches and other birds

| Species | Location | References | Host | Oocysts size **(µm)** |
|---|---|---|---|---|
| *C. baileyi* | respiratory tissues conjunctiva, small intestine .ceaca, cloaca and kideny | Current *et al.*, 1986 | Ostriches and chickens | 6.2 × 4.6 \ 5.6 – 6.3 × 4.5 – 4.8 |
| *C. galli* | proventriculus | Pavlasek , 1999 Ryan etal., 2003 | Chickens , ostriches | 8.3 × 6.3 \ 8 – 8.5 × 6.2 – 6.4 |
| *C. meleagridis* | bursa,small and large intestine | Slavin , 1955 | chicken,turkeys,ducks, cockatiels,abrown quail, ostrich and human | 5.2 × 4.6 \ 4.5 – 6 × 4.2 – 5.3 |
| *C.parvum* | intestinal and respiratory epithelium | Tyzer, 1912 | chickens , mice ,man and ostriches | 5.2 × 4.3 \ 5.5 – 5 × 4.5 – 4.1 |

| | | | | |
|---|---|---|---|---|
| *C.tyzzeri* | small intestine | Tyzer, 1927 | Chicken | 5.2 × 4.6 \ 4.5 – 6 × 4.2 – 5.3 |

## 2.4. Developmental stages of *Cryptosporidium* spp

### 2.4.1. Oocysts

Oocysts are ovoid to ellipsoidal or sub spherical in shape, with four sporozoites and a residuum, fully sporulated . The residuum contains a large lipid body and a crystalline protein body alongside amylopectin granules (Harris *et al*., 2004). Smooth oocyst wall , measures approximately 50 nm in thickness, which composed of two electron dense layers separated by an electron-lucent space and it is resist mechanical and chemical disruption. Oocysts of *Cryptosporidium* lack sporocysts and other morphological structures such as a micropyle and polar cup and granules, which are often observed in other coccidian oocysts (Arrowood, 2002). The dimensions of the oocysts slightly vary among species of *Cryptosporidium*, but in general, the length ranges from 4.5 to 7.5 μm and the width ranges from 4.2 to 5.7 μm (Thigeel, 2016).

### 2.4.2. Sporozoites

Sporozoites characterized by crescent shape, with a slightly pointed anterior end and a rounded posterior end containing a prominent nucleus ( Aldeyarbi and Karanis ,2015). Sporozoites lie parallel to each other within the oocyst and upon excystation escape through a slit-like opening created upon dissolution of the unique suture in the oocyst wall. Excysted sporozoites move by flexing and gliding, and eventually penetrate the host cells (Borowski *et al.,* 2010). The dimensions of sporozoites are ranged 4.5 to 7.5 μm long and 1.2 to 1.8 μm wide (Thigeel, 2016).

### 2.4.3. Trophozoites

Trophozoites have circular to oval shape, uni-nuclear stages, epicellular, smooth surface, electron dense band, feeder-organelle, cytoplasmic granulation, hood like shape varied in size depending upon their developmental stage measured <1 µm to 2.5 µm in diameter, which represent the transitional stage between sporozoites and merozoites (Borowski *et al.,* 2010). The individual trophozoites undergo multiple mitotic divisions, resulting in the formation of type I meronts (Aldeyarbi and Karanis, 2015 ).

### 2.4.4. Meronts

Meronts have epicellular, smooth surface measured approximately 1.5 µm in diameter. Two different types of meronts have been described (type I and type II), the development of type I meronts occurs as a result of mitotic division of trophozoites, as well as aggregates of 'recycled' merozoites released from type I meronts variable in size ( Borowski *et al.,* 2010). Initial descriptions of type I meronts state that each can develop six or eight merozoites, and four merozoites from type II meronts (Valigurova et al ., 2008)

### 2.4.5. Merozoites

Merozoites have central nucleus and are motile, circular to oval in shape, displaying gliding and flexing movements. Type I merozoites are small in size and are

very active, measured (0.4 × 1 μm) with both a rounded and a pointed end; however, type II merozoites are round and measured between 0.5 μm to 1 μm in diameter (Borowski *et al.,* 2010). Type I merozoites are either recycled, clustering together to produce more type I meronts and hence amplifying the number of type I merozoites; or give rise to type II meronts.Type II merozoites differentiate into micro- and macrogamonts (Valigurova et al ., 2008) .

2.4.6. Macrogamonts

Large in size typically (4 × 4 – 5 × 4μm), ovular shape, rough surface and possess a large peripheral nucleus, it have been observed adhering to the surface of macrogamonts and have also been reported being visualized within a microgamont, representing the fertilization process (Borowski *et al.,* 2010). Once fertilization is complete, a zygote (or unsporulated oocyst) is formed, which then matures into a sporulated oocyst containing four sporozoites (Caccio and Putignani, 2014).

2.4.7. Microgamonts

The size of microgamonts is (2 × 2 μm) which become multi-nucleate and each nucleus are incorporated into a microgamete. Microgametes appear to bud from the surface of the microgamont and appear as bullet or spherical shape with rough surface and display a jerky, gliding movement, measured 0.1 μm in diameter (Borowski *et al.,* 2010).

**2.5. Life cycle of *Cryptosporidium* in birds**

*Cryptosporidium* is completed Life cycle inside a solitary host (monoxenous cycle) in the gastrointestinal tract or respiratory tract of host and involves both sexual and asexual phases (Caccio and Putignani, 2014).

The fully sporulated oocyst is shed in the feces or with respiratory secretions (coughing, sneezing) of an infected host (Caccio and Putignani, 2014; Mor *et al.*, 2018). oocysts are ingested with food , water and contaminated materials, or oocysts are inhaled by susceptible host, later sporozoites excyst and penetrate to gastrointestinal epithelial cells or respiratory tract (Fayer and Ungar, 1986; Current *et al* 1986). After sporozoites penetration, the life cycle of *C. baileyi* In birds differs slightly from other cryptosporidia infecting mammals because type I meronts have cyclical development, while the invasive merozoit released from type II meronts which then develop into type III meronts, which than later developed to merozoites and into sexual stages macro and microgametes (Current *et al*., 1986).

The fertilized macrogamonts( zygot ) develop into oocysts by gametogony , there are two types of sporulated oocysts (thin- and thick-walled) inside enterocytes , and contain four free sporozoites(banana shape). The thin-walled oocysts are surrounded by single layer membrane which are able to autoinfect the same parasitized host , so small number of ingested oocysts can produce severe infection, the thick-walled oocysts composed from multi-layered,and have ability to resistant environmental factors , it pass out the body to transmit infection in to other hosts (Current *et al*., 1986).*C. baileyi* its stabilization in the epithelium mucosa of neumerious internal organs, whereas *C. meleagridis* is almost located in the small intestine (Bermudez *et al*., 1988; Pavlásek, 1994 ) .

According to Current *et al.*(1986) the prepatent and patent period of *C. baileyi* are ( 2–7 )days and (4–32 )days respectively, while in *C. meleagridis* are ( 3–5) days and ( 6–16 ) days, in turkeys respectively (Bermudez *et al*., 1998 ; Takano *et al*., 1992; Sréter *et al*., 1995 ; Taylor *et al*., 1994) .Conversely with *C. baileyi*, the oocyst

shedding of *C. meleagridis* infected birds is mostly low (Lindsay, 1987 ; Taylor *et al* 1999)

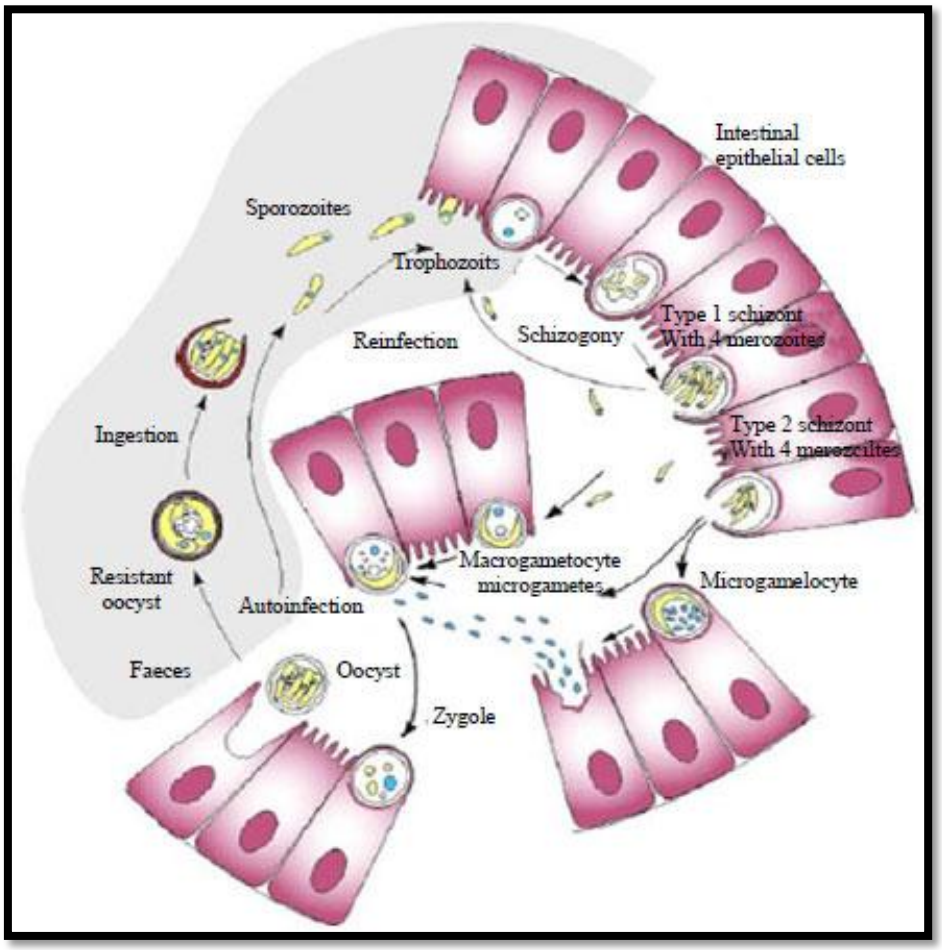



**Figure (2-1) life cycle *Cryptosporidium spp* .( Caccio and Putignani, 2014)**

## 2.6. Epidemiology of *Cryptosporidium* in birds

There are several factors control *Cryptosporidium* epidemiology, where they facilitate spreading and make the eradication is very difficult, It detected in water surface of sewage and in water sources that supplies across North America and

Europe(O'donoghue,1995;Widmer *etal*.1996;Smith and Rose,1989;Dillingham *et al.*,2002 )

The parasite excreted large number of sporulated oocyst from infected hosts(Chappel *et al*., 1996) Although low dosage ( 50 oocysts ) can impact the hosts causes infection (DuPont *et al*., 1995) , direct life cycle not more than 48 – 72 hours, reduced or lack host specificity, more than one host can infected with the same species of *Cryptosporidium* (O'Donoghue, 1995 and Laberge *et al*., 1996 ) The tiny size of oocysts make there filtration difficult from contaminated water (Current and Garcia, 1991), the thick – walled oocysts are highly resistance and stability to environmental condition ,like freezing, dryness,which can remain viable under cool conditions for more than 4 months in water temperatures of rivers .(Caccio and Putignani, 2014). Thin–walled oocysts play important role in autoinfection which can excyst endogenously, help in persistent infection in host that infected with other immunosuppressed individuals (Fayer, 2004) as well as the oocysts flotated on the waste surface and drinking water with low sedimentation speed (Egyed *et al*,.2003)

The distribution of oocysts among poultry flocks, primary occur by drinking water since *C. baileyi* can infected numerous avian hosts , wild birds (Ostriches ) ( Wang *et al* ,. 2010). The rodents (mice, voles and rats) are susceptible to *C.meleagridis* infection and may serve as carriers ( Sréter *et al*., 2000**)**. The families of different flies serve as mechanical vectors which carrying viable *Cryptsporidium* oocysts internally and externally with rate 55.56% of fly exanimated sample (Conn *et al* ., 2007) Oocysts remarkably resistant antimicrobial agents, disinfectants and other chemotherapy e,g water chlorination treatment, in which they cannot damage oocysts also oocysts are remain viable in slurry stores particularly in the top few centimeters therefore, elimination of *Cryptosporidium* parasites from flocks it is intractable by once

established (Fayer and Ellis ,1993; Tzipori and Griffths ,1998 Keidel and Daugschies, 2013).

## 2.7. Prevalence of intestinal protozoa in Ostriches

The total distribution rate of *Eimeria* oocysts in Nigeria Ostriches farms 11.6 % .in chicks, the rate (43.5%) while in adult 17.1% of samples from adults.( Mshelia et al.,2010). Boughton ( 1937) showed that Ostriches not infected with *Eimeria spp* during their study.

A few number of *Eimeria* oocyst will observed in faecal samples of some Ostriches in farm of Garmsar Province for the first time in Iran .but not completed their sporulation (Eslami et al., 2007) .

In South American ratite breeding showed cysts of *Entamoeba struthionis* and *Giardia* spp. which observed in the fecal samples , also the researchers of this study were unable to induce sporulation to coccidian oocysts , and thus the genus remained unknown ( Gallo *et al*.,2018)

The prevalence of intestinal parasites from various species of birds housed in a zoological garden in Egypt , showed that Ostrich infected with *Eimeria* spp. in rate 25% and *Cryptosporidium* spp.in rate 11.1% (El-Shahawy and Abou Elenien,2015)

In Botswana Coccidia oocysts were demonstrated in the rate 34 % apparently healthy Ostrich chicks, young chicks had the highest infestation while those more than 9 weeks old had no oocysts at all in their faeces (Mushi *et al.,* 1998 )

Ostriches and other ratite infected with many intestinal protozoa *Balantidium struthionis, Giardia spp., Trichomonas spp Cryptosporidium spp., Histomonas meleagridis* and *Hexamita spp.,*. which causes gastrointestinal infection and lead to wasting, anorexia, diarrhoea and death for many ratite spp.( Tully and Shane , 1996 ) .

## 2.8. Prevalence of *Cryptosporidium* in Ostriches and other birds

Avian *Cryptosporidium* has been recorded in more than 30 species of domestic and wild birds are *C. baileyi, C. galli* and *C. meleagridis*( Ng *et al.,* 2006)

The prevalence data of Ostriches Cryptosporidiosis in southern Iran and the oocysts morphology will be described, the *Cryptosporidium* oocysts were found in faeces of 21 of 75 examined Ostriches (28%) without effect of Ostriches population in the prevalence of *Cryptosporidium spp*. ( Behzadi *et al* ., 2009)

In china many studies showed that the Cryptosporidium infection are presented in ZhengZhou Ostriches 2.48% (10/404) the pattern of oocysts shedding was different in Ostrich,which has multiple peaks, main respiratory clinical symptom, sneezing , cough , dyspnea were observed in chickens and young ostriches infected with the Cryptosporidium oocysts,while signs in adult ostriches was mild *Cryptosporidium baileyi* from ostrich parasitized in cloaca and bursa of Fabricious , mainly causes thickness of mucous membrane and epithelium cells swelling ( Sun *et al* ., 2007)

another study in the same province, Zhu *et al* ( 2008) found that the total infection rate was 1.7%(14/829). The most positive *Cryptosporidium* isolates come from( 20−40 days) old Ostriches,which enhanced that young ostriches chicks more susceptible to *Cryptosporidium* infection than adult .

The microscopic analysis of Ostriches fecal samples showed rate 10.2%in aged 16-60 days, 1.2% in those aged > 10 years. The genotyped with a restriction fragment length polymorphism analysis and DNA sequence analysis of the small subunit (SSU) rRNA gene. The isolates identified from gerbil was *Cryptosporidium muris*, whereas the isolates from Ostriches were *Cryptosporidium bailey*i.(Qi *et al*.;2014)

The prevalence of *Cryptosporidium* in Ostriches recorded 53 samples positive from 452 fecal samples collected from five farms , zoo and animal rescue center in Zhengzhou, Henan province with rate 11.7 % . The young chicks(4-8 weeks ) show high percentage 16.2% than adult ostriches more than 12 months with rate 7.2 %. (Wang *etal*.,2011)

Microscopic analysis showed that the *Cryptosporidium spp* infection rate was 10.2 % from 303 samples in China , in ostriches aged 16-60 days the infection rate was 27.6% aged 61–180 days rate was 1.2 % the and 20.4% in ostriches aged > 10 years , while the ostriches aged < 15 days not detected any Cryptosporidium oocysts in feces, there are not observed clinical signs in the Cryptosporidium-positive Ostriches during the period of sample collection ( Huang *et al.,* 2018)

The percentage of *Cryptosporidium* avian genotype Ⅱ in Ostriches in the central of Vietnam was 23.7 % , prevalence of *Cryptosporidium* varies widely among age groups of Ostriches 45 days, 45-60 days, 61-90 days, 12 months and more than 12 months was 23.1 % ,33.3% ,35.2% ,0 , and 5.8% respectively .the majority of infection rate in 2- 3 months ostriches chicks, while the lower rate was recorded in one year age adult ostriches( Nguyen *etal*.,2013)

In Brazil , the prevalence of *Cryptosporidium spp* and genotypes of Ostriches in captivity showed rate 14. 6% and the *Cryptosporidium avian* genotype Ⅱ was recorded in Ostriches(*Struthio camelus*) ( Nakamura *et al*., 2009)

Oliveira et al (2008) was observed *Cryptosporidium* spp in 44.4% of the examined Ostriches. However,Oocysts was subspherical in shape with 6.33× 4.31.

In Canada Cryptosporidial oocytes were found in the adult Ostriches feces at rate 8.5% , it appears that this *Cryptosporidium* of Ostriches is distinctive and may be a found a new species, *Cryptosporidium sp* have possibility to present in Ostriches and causes disease in young Ostriches and birds that are immunocompromised or secondary bacterial or viral infection ( Gajadhar ,1993)

The first isolation of *Cryptosporidium* from broiler chickens in Europe specifically from Scotland where recorded infection in rate 18.7% ( Randall, 1982) *Cryptosporidium* reported in broiler chickens in Japan with infection rate 33.3% (Itakura *et al* .,1984). while in the United States Current *et al* ( 1986) recorded infection in rate of 5.9% in layer chickens which has been named *C.baileyi* in Birds.

In American zoo outbreaks and fatalities in pet birds well occure due to the *Cryptosporidium* infection lead to deaths of 72% of quails( Hoerr, 1986) . Cockatoos infected in rate 22% ,infected bird had showed intermittent to protracted diarrhea before death ( Latimer ,1992)

In northern Georgia the overall, 23 of 56 (41%) broiler flocks had infected with *C. baileyi* tracheitis .The parasitism rates among flocks infected ranged from a low rate of 10% to a high rate of 60% (Goodwin *et al.*, 1996)

The total prevalence of *Cryptosporidium* oocysts in wild, local and exotic birds in Zaria, Nigeria was 7.4% , local birds had the highest rate of 9.5% followed by exotic birds 6.6% while the wild birds with rate 5.3% however a significant difference between sexes were noted . (Bamaiyi *et al* ., 2013 )

The Turkey broiler chickens infected with rate 4.4% ( Kucukerden *et al*., 1999). poultry is one of the most hosts where outbreaks of *Cryptosporidium* are occure, the parasite presence in the broiler and layer chickens, young ages are more sensitive to the infection from the first day of life ,also the migratory birds could be a reservoir of the *Cryptosporidium* perhaps that transmits infection to humans and other animals (Sreter *et al*.,2000 )

In China ,the overall infection rate of *Cryptosporidium* was 10.6% . in layer chickens 3.4% in broilers and in Pekin ducks respectively. The highest infection rates were observed in( 31-60 day) old layer chickens (24.6%) and (11-30 day) old Pekin ducks (40.3%). the highest prevalence rate in spring season was 15.6% and the lowest was winter 2% ( Wang *et al*., 2011) .

In Iran Cryptosporidiosis is reported in turkey poults suffering from diarrhea and unthriftness , oocyst shedding was detected in 29% of the histologically positive birds( Gharagozlou *et al* ., 2006) while Shemshadi *et al* .( 2011) recorded the infection in rate 23.75% of broilers, the 10.83% of samples that had infected in the intestine only and 4.58% had an infection only in the trachea, the overall infection rate for both the trachea and intestine was 8.33%

In the Arab world, the parasite recorded in Morocco, the infection rate in chickens was 24% ( Kichaw *et al*., 1996) . while in Tunisia recorded infection in chickens rate 4.5 % ( Soltane *et al*.,2007). In Egypt Shaapan *et al*. (2011) isolation

*Cryptosporidium* from the quail drops and clarify the rate of red quails , brown quails and white quails was 31.9% , 33.3% and 30.8% respectively .

In Iraq, Al-Attar and Abdul Aziz (1985) recorded for the first time the infection of the *Cryptosporidium* in the bursa of Fabricia in broiler chickens in Baghdad at rate 8.8% with no clinical signs appears in the infected chickens .

In Baghdad, the females love birds infected in rate 18%,while children in aged 4 days to 11 years that suffer from symptoms of gastroenteritis the infection rate 14.6% (Al-gailani 1998) . the total of broiler chickens infection was 21.8%. in Abu-grap area was 34.04% which appeared at ages 1-8 weeks ( Al-Bayati2002)

Mohammed (2010) detected the *Cryptosporidium* in duck feces in the Nineveh Province at rate 77% and noted the a significant increase in the incidence of parasite infection in females when compared to males at rate 87.6 % 57.1 % respectively .

In the slaughtered broiler chicken in local markets of Baghdad city the infection rate was 35% with highest rate of infection occurs in April, reached 46% , while the lowest rate of infection in June, reached 20% (AL-Zubaidi *et al*., 2018).

**2.9. Routes of transmission**

*Cryptosporidium* is transmitted between different animals through numerous routes, the most important one is direct contact between them (Current, 1986 ) or as a result of ingestion of parasite oocysts those shed with the feces and contaminated food or water (Shafiq *et al* ., 2015) also carriers of the disease, as well as autoinfection

may happen due to the presence of thin wall oocysts and excyst inside the same host (Leitch and He, 1999).

### 2.9.1. Direct transmission (bird-to-bird contact)

Ostriches and other birds infected from contaminated grown with birds feces that play important role in distribution of *Cryptosporidium* infection in crowding farms that Suffer from poor management, furthermore some of the sporulated oocysts adhere to feathers surrounding the cloacal area that can be transmitted directly to another birds ( Dritch *et al*., 1993**) .**

### 2.9.2. Waterborne transmission

Water act as the most important sources of indirect Cryptosporidial infections. It is a common waterborne parasitic infections in bird and other animals (Zahedi *et al.*, 2018).water is a perfect medium to oocysts survival in the different environmental circumstances, because the water diluted and reduce the effect of increase temperature and drainsis and kept the oocysts a life more than two months , also heavy rain disseminated these oocysts to many areas and contaminated pasture.(Hunter and Nichols, 2002**) (**Baldursson and Karanis,2011) **.**

The un treated sewage drainage from cities and villages to rivers lead to increased water contamination with *Cryptosporidium* oocysts that infected wide range of animales and human through drinking water (Madore *et al*., 1987and Casemore,1990.).

### 2.9.3. Food-borne transmission

The oocysts can persist and survive for long periods of time both in foods and water, *Cryptosporidium* oocysts are immediately infectious upon excretion or shedding from the infected host. As a result, these prtozoal parasites have emerged as public health risks and have become dangerous to the food industry ( Rose and Slifko, 1999 ) . *Cryptosporidium* also appears waterborne more than foodborne transmission, but some time they may be foodborne or transmitted by direct contact suspected foodborne outbreaks have been reported from travelers who visited Mexico, the United Kingdom, and Australia. (Smith ,1993).

### 2.9.4. Airborne transmission

Dust plays an important role in transmission of the oocysts and the birds can be infected by inhalation of dust particles suspended in the air that carrying *Cryptosporidium baileyi* oocysts also worker in the birds farms can transmitted the infection by their clothes and shoes (Okhuysen *et al*., 1999).

### 2.9.5. mechanical transmission

There are three families Calliphoridae, Sarcophagidae, and Muscidae which serve as mechanical vectors of *Cryptosporidium* and *Giardia* among livestock and wildlife (Conn *et al* .,2007) also beetles ,cockroaches ,rodents and stray dogs in birds farms that transmitted oocysts from one place to another (Hurkova *et al*., 2003 and Hajdusek *et al*., 2004) .

## 2.10. Pathological changes

The *Cryptosporidium* spp. infected the small intestine such as *C.baileyi* and *C.meleagridis* in birds , but *C.galli* localized in stomach also the parasite may infect the respiratory tract and conjunctiva, but the pathognomic lesion appear in alimentary

canal causes intestinal cryptosporidiosis (Lindsay *et al*., 1989 ; Clayton *et al*., 1993and Ravich *et al* ., 2014).

The primary clinical singes of *Cryptosporidium* oocysts shedding is mucus in the stool than a yellowish greenish diarrhea without blood or pus (Dobos-Kovács *et al*., 1994). histpathological change occurs in tips end of the affected epithelial cell, loss of enterocytes and blunting, atrophy, fusion and shortening of the intestinal villi, which reduces the intestinal surface, leading to decrease nutrient and water absorption ( Perry *et al* ., 1991 ).

The reduce in villi cells Lactase lead to malabsorption of lactose, which increases the permeability of the intestinal epithelial layer and accumulates fluid in the intestinal cavity (Nappert *et al*.,1993 and Gerba *et al*., 1996 ) . The affected areas of intestinal crypts are dilated and contain tissues debris , neutrophil granulocytes, and a massive mononuclear cell infiltration also were proven in the lamina propria mucosa (Di Genova and Tonelli, 2016). Dehyderation leading to increased blood concentration , increase packed cell volume , electrolyte deficiency such as sodium and a significant increase in urea and creatinine. (Soetan *et al* ., 2010 ).

*Cryptosporidium meleagridis* may infect the intestinal tract, bursa of Fabricius and cloaca of turkeys and chickens but the illness, including diarrhea and moderate mortality in turkeys ( Bermudez *et al* ., 1998 ) *Cryptosporidium baileyi* may infect the respiratory tract (larynx, trachea, primary and secondary bronchi, air sacs), The pathological change in respiratory cryptosporidiosis was excessive mucoid exudate, airsacculitis, deciliation, epithelial hypertrophy and hyperplasia( Lindsay and Blagburn, 1987 ).

Cryptosporidiosis in chickens usually detected as respiratory disease, and occasionally as intestinal or renal disease ( Lindsay *et al* 1989) . The infected birds were always clinically ill, viruses and bacteria or both often accompanied respiratory Cryptosporidium sp. infections, the major symptoms are depression, anorexia, emaciation, coughing, sneezing, dyspnoea. (Goodwin *et al* 1988 ) *C. baileyi* is agents to be considered in the respiratory disease complex so the high carcass condemnation at processing places deuto air sac disease ( Goodwin and Brown , 1989 ).

The most pathophysiological singes in the male ostrich chicks was prolapse of the phallus and cloaca, heavy infection of *Cryptosporidium sp.* lead to cloacal and bursal tissue effected with progressing of prolapse, histopathological included loss of the microvilli borders , epithelial hyperplasia and degeneration, ultrastructurally indicated swelling of organelles, and nuclear changes ( Penrith *et al* .,1994) The appearance of prolapsed tissues was congested and oedematous; however,the chicks were show a good physical condition, postmortem examination of birds were obviously see mild dehydration and emaciation , Congestion of duodenum mucous membrane ,the contents of the duodenums were watery , The caeca were distended with brown watery feces, histopathological examination appear a mild infiltration of the rectum tunica serosa layer , with infiltration of lymphocytes and macrophages (Iordanidis *et al* ., 2003 ) .

Ostriches with poor growth and marked emaciation revealed a pancreatic cryptosporidiosis which lead to pancreatic atrophy. histological examination showed a large numbers of *Cryptosporidium spp*. found in the ductal epithelium causing marked necrosis and lymphoplasmacytic inflammation(Jardine and Verwoerd ,1997).

The investigation of cloacal prolaps in Ostrich chicks hatched and raised on the experimental farm, the condition which occurred in male chicks in rate 14.65% from the total number of chicks hatched. The mortality rate was very high amongst chicks with cloacal prolapse. Although the pathogenesis of the condition is related with *Cryptosporidium* species which present in massive numbers in affected cloacas. (Bezuidenhout *et al.*,1993 ).

## 2.11. Clinical signs in ostriches and other birds

Cryptosporidiosis in birds has a broad varying clinical signs from asymptomatic to serious infection which may lead to death, and that mainly associated with high morbidity and mortality in poultry ( Santin, 2013) .

The most commonly recorded species in birds was *Cryptosporidium baileyi* and main clinical signs including dyspnea, coughing, sneezing and depression ( De-Graa *et al*., 1999) while infection with *C. galli* primarily causes diarrhea, chronic enteritis, weight loss and high mortality ( Ryan *et al* ., 2003) Although, some birds infected with *C. galli* remained free of clinical signs ( Silva *et al* ., 2010).

Adult birds with experimental *Cryptosporidium* infection showed normal appetites , furthermore no animals died during the experiment ( Cui *et al* ., 2018 ).

In chickens *Cryptosporidium meleagridis* is leading to diarrhea , turkeys and birds, diarrhea is yellow to green with foul odor and sometimes contains mucus (Tzipori *et al.*, 1982; Baroudi *et al.*, 2013).

The respiratory Cryptosporidiosis usually lead to severe pathogenicity and sometimes acute infection causes high mortality rate, the disease is accompanied by sneezing, coughing, difficulty to breathing, followed by the extension of the head forward to facilitate breathing with swelling of the head sometimes and the presence of mucous with respiratory secretions (Current, 1997) . Other singes ruffled Feathers, depression , birds not response to external stimulation , with low growth and weight loss, and arithritis , liaminesis and recoumbancy (Sreter, 1998**).**

## 2.12. Diagnosis

The diagnosis of Cryptosporidiosis in Ostriches and other birds is traditionally performed by microscopically examination of a fecal ( drops ) samples , also immunological assays and recently PCR techniques are increasingly applied in Veterinary medicine (Petry, 2000 ; Maggi *et al*.,2000).

### 2.12.1. Conventional methods

#### 2.12.1.1. Clinical Signs

The clinical sign vary depending on *Cryptosporidium* species from watery diarrhea or yellow to green with foul odor and sometimes contains mucus (Radostits *et al*., 1994) dyspnea, coughing, sneezing , extension of the head forward to facilitate breathing due to obstruction with exudate, depression (Current, 1997) .

#### 2.12.1.2. Macroscopic examination:

Grossly examination of drops or feces record its color, consistency and smell (Sreter, 1998).

**2.13.1.3. Microscopic examination:**

The microscopic examination methods widely used for diagnosis of *Cryptosporidium* by detection of sporulated oocysts in fecal smears depending on Light Microscopy (LM), Electron microscopy (EM) and Laser scanning confocal microscopy (LSCM) (Fayer and Xiao, 2008).

**2.12.1.3.1. Direct wet smear method:**

The *Cryptosporidium* oocysts detected by adding Lugol's Iodine stain to the direct smears and examines under the oil immersion lenses, but because the smaller size of the oocysts and its general similarity with yeasts make this method inadequate Therefore, nemuros methods were used for diagnosis as flotation and staining techniques (Crawford and Vermund, 1988).

**2.12.1.3.2. Concentration Methods:**

The important of this methods ,when there are low numbers of oocysts were excreted by an infected host ,and its more commonly used to improve the previously diagnostic techniques. (Bukhari and Smith, 1995; Pacheco *et al.,* 2013).

**2.12.1.3.2.1. Flotation Methods**

1- Sheather's Sugar Solution is one of the most important flotation solution used for oocysts flotation method (Current and Garcia, 1991).

2- Zinc-sulfate flotation solution (Ma and soave ,1983).

3- NaCl flotation solution (Kuczynska and Shelton ,1999).

4- Acid flocculation concentration (AF) method separates oocysts from fibrous fecal material (Wells *et al.*, 2016).

5- Formalin-ethyl acetate (FEA) is one of the most important methods used for flotation (MacPherson and McQueen, 1993).

**2.12.1.3.2.2. Sedimentation Methods:**

The concentration methods can used by simple gravity sedimentation (SGS), formalin-ether and water ether, so the oocysts vitality do not affect during procedure , the sediment deposited in the bottom of the laboratory tube and then examined to investigate the presence of *Cryptosporidium* oocysts **(**Rezende *et al.*, 2015 and Pacheco *et al.*, 2013 ).

**2.12.1.3.3. Staining methods**

There are many staining methods used to determine *Cryptosporidium* by preparing thin fecal smears were prepared and stained by the following stain for determination *Cryptosporidium* oocysts :

1- Modified Ziehl-Neelsen Stain (Current and Garcia, 1991).

2- Fast Zheil Neelsen Stain (Brondson *et al*., 1984).

3- Giemsa Stain (Ogata *et al*., 2009).

4- Auramine phenol Stain (Fleck and Moody, 1988).

5- Acridine Orange Stain (Ignatius *et al*., 1997).

6- Aura mine and Rhodamine stain (Goddard *et al*., 2000).

7- Grams stain (Ogata *et al*., 2009).

8- Sufranin Methylin Blue Stain (Baxby *et al*., 1984).

9- Undiluted carbol fuchsin stain (Heine, 1982)

10- Trichrom Stain (Ignatius *et al*., 1997).

**2.12.1.4.1 Immunological Methods:**

Immunoassay techniques display the increasing both sensitivity and specificity of tests when compared with the other conventional staining methods (Ahmed and Karanis, 2018).

**2.12.1.4.2. Enzyme linked immunosorbent assay (ELISA)**

A Techniques depend on the detection of *Cryptosporidium* antigen in fecal samples.coproantigen detection techniques,Enzyme linked immunosorbent assay (ELISA), enzyme immunoassay (EIA) and immunochromatographic dipstick assay (ICT) are antigen detection tools that it is prepare quick and easy diagnostic tools (Aghamolaie *et al.,* 2016; Ahmed and Karanis, 2018). In the United States routine detection use for cryptosporidiosis diagnostic techniques that have variability of sensitivity and specificity of coproantigen techniques were affected by the quantity of

*Cryptosporidium* oocysts, per gram of stool. ICT assays provide results within 10-15 min and these advantage lead to its expanded use for these techniques (Roellig *et al.*, 2017).

Enzyme immunoassays (ELISA and EIA) a performance higher sensitivity and specificity than traditional microscopic diagnostic methods for *Cryptosporidium* coproantigen with a limited detection of oocysts/ ml. The uses of spectrophotometric reading will exclude subjectivity of microscopical explanation .

A massive numbers of stool samples can be quickly and easily examined with increased accuracy of results (Ghoshal *et al.,* 2018). Longer processing period (1-2 h.) should be considered with enzyme assay. The sensitivity variable (59-100%) and specificity (93-100%) are also reported with this technique (Ryan *et al.,* 2016; Ghoshal *et al.,* 2018)

**2.12.1.4.3. Immunofluorescence techniques:**

The immunofluorescent antibody (IFA) is more sensitive and specific method of diagnosis in fecal samples isolated from both man and animal sources (Rossle and Latif, 2013 ; Ryan *et al.,* 2016 ). Its high sensitivity to the detection of oocysts isolated from fecal samples when compared with other conventional staining methods; auramine and Dimethyl sulfoxide DMSO (Rossle and Latif, 2013)

IFA allow clearlly visualization of the intact oocysts, providing a definitive diagnosis, it has achieved high specificity (98.5-100%) , sensitivity (96-100%), and reliability in the diagnosis of *Cryptosporidium* sp (Mirhashemi *et al.,* 2015).

**2.12.1.5. Histopathological Examination:**

Gastrointestinal biopsy examination of specimens tacken from infected birds with *Cryptosporidium* species shows mild to moderate acute inflammation of the lamina propria and surface epithelial disorder. The rectum tunica serosa, which characterised by the presence of lymphocytes and macrophages (Iordanidis *et al*.,2015) Staining of tissue sections by the Warthin-Starry staining method is a perefect effective diagnostic pathway for the microscopic detection of *Cryptosporidium* and preferable symptomatic abilities over than the haematoxylin and eosin stain (De, 2013).

The lesions was described in Cryptosporidial cloacas infection in other species of birds included loss of the microvillous border and epithelial hyperplasia, and degeneration, which was indicated ultrastructurally by vacuolation of the apicalcytoplasm, swelling of organelles, and nuclear changes. ( Penrith *et al*., 1994).

Pancreatic Cryptosporidiosis with large numbers of *Cryptosporidium sp*. present in the ductal epithelium causing necrosis and lymphoplasmacytic inflammation (Jardine and Verwoerd ,1997 ) .

**2.12.2. Molecular Diagnosis:**

Molecular techniques are wide used for detection of *Cryptosporidium* spp because of the inability to differentiate *Cryptosporidium* species by traditional microscopic methods. These tools of molecular techniques helped to detect and differentiate *Cryptosporidium* species/genotype and subtype if present (Xiao and Ryan, 2004; Caccio *et al.,* 2005). The results of molecular techniques has made significant contributions to our understanding of the prevalence and epidemiology of *Cryptosporidium* species (Xiao and Ryan, 2008).

**2.12.2.1. Polymerase Chain Reaction (PCR):**

The PCR technique is a computerized machine , recycling technique and it includes numerous quantity processing (Ryan *et al.,* 2016; Adeyemo *et al.,* 2018). The benefit of PCR for the detection of *Cryptosporidium* in clinical samples diagnosis are: high sensitivity and specificity, ease of use, ability to speciate (Eliminating false positives encountered with cross reactions of antibodies to non-pathogenic protozoan species) and strain typing potential , ability to analyze large numbers of samples in the same time, (Checkley *et al.,* 2015; Ignatius *et al.,* 2015). A different approaches using a range of varying primers have been described for the clinical samples, and in all comparative trials PCR has been shown to be more sensitive and reliable than other diagnostic techniques for the detection of *Cryptosporidium* in different samples origins (OIE, 2008; Yu *et al.,* 2009) The importance of PCR should be take into consideration in terms of the practicality and cost effectiveness of using such a techniques for many routine diagnosis (Arbabi and Hooshyar, 2009).

A wide range of molecular techniques for the detection and differentiation studies of *Cryptosporidium* have been described recently, the most common techniques used in PCR technique are:

1. **Conventional PCR technique**: the aim of technique is detection of *Cryptosporidium* species using PCR, oligonucleotide primer design targeting specific sequences is decisive. Furthermore, standardization of PCR and nucleic acid protocols can be easily carried out, which permit oocysts that may be present in low numbers for detection limit of other assays to be detected (Girones *et al.,* 2010 ).

2. **Nested PCR**: the most remarkable technique have been used to determine *Cryptosporidium* species in both human and animal fecal samples ( Koehler et al., 2017 and Mirhashemi *et al.,* 2015) . It has confirmed more beneficial than primary

PCR, producing more positive results and elevate the amplification sensitivity of *Cryptosporidium* DNA with fecal samples (Sadek, 2014). Almost two primer sets are used , so if there is nonspecific binding of a DNA template with the 1st primer sets, it is improbable happen with the 2nd primer sets (Ahmed and Karanis, 2018). A few oocyst detection limit even if they are one oocyst has been observed in many nPCR studies, making it one of the most accurate molecular detection techniques for *Cryptosporidium* species from different sample sources (Yu *et al.,* 2009).

In previous establishment of nested PCR technique was compared for their reliability to detect *Cryptosporidium* 18S rRNA in animal fecal samples (Mirhashemi *et al.,* 2015 and Nichols *et al.,* 2010 ).Some samples of PCR protocols within (SSU) produced relatively specific amplification from *Cryptosporidium* DNA isolated from human fecal samples, however, when DNA originates from an animal fecal sample, PCR assays do not obtain high specificity (Ahmed and Karanis, 2018).

3. **Real-time PCR (RT-PCR):** used for sensitive detection of several pathogens and can be used to detection of genetic variability within *Cryptosporidium* isolates (Honsvall and Robertson, 2017). And for this technology, specific fluorogenic dyes will be added to the amplicon during PCR, thus amplicon fluorescence increases as more PCR product can be generated (Rajalakshmi, 2017).The Quantitative real-time PCR (qRT-PCR) methods have been used to recognize different *Cryptosporidium* species by advantage of the genetic polymorphism of the 18S rRNA gene for innovations probes with differing melting temperatures (Fayer and Xiao, 2008 and Hadfield *et al.,* 2011) The increased sensitivity of ( RT-PCR) warranty increased speed of detection and qualitative diagnosis while the quantitative nature of the assay will be precious in estimating levels of contamination (Rajalakshmi, 2017).

**Multiplex real-time PCR assays** are recently used and become available for the identification and detection of gastrointestinal protozoa being highly valuable with mixed parastic infection (Nurminen *et al.,* 2015). There are many panels are used within the system ofmultiplex real time PCR assays for detection *Cryptosporidium* with other protozoa ,these panels sensitivity ranged from (95-100%) and specificity from (99.6-100 %) when used to detection *Cryptosporidium* (Ryan *et al.,* 2017).

4. **Restriction fragment length polymorphism (RFLP)** is one of the most common analysis methods used to examination of nucleic acids for the existence of known sequence variants and for genotyping (Rasmussen, 2012). RFLP is used for identifiection and detection of different species and sub species of *Cryptosporidium* in a sensitive way by using specific restriction enzymes to digest amplicons in fragments of varying size, depending on the *Cryptosporidium spp*., that cause the products to migrate in various distances on the gel (Ghaffari *et al.,* 2014).The segments that is to be check and digested by the suitable restriction enzyme, and then separated by agarose gel electrophoresis (OIE, 2008), In *Cryptosporidium* used PCR-RFLP for small subunit ribosomal RNA (SSU rRNA) (Keshavarz *et al.,* 2008; Feng *et al.,* 2007), *Cryptosporidium* oocyst wall protein ( COWP) and 60 KDa glycoprotein (GP60) (Jex and Gasser, 2010 and Cai *et al.,* 2017)

**2.12.2.2. DNA sequence analysis**

The sequencing or genotyping is more suitable of all methods, is measured by their ability to differentiate or identification of species/genotypes based on their genetic sequence, DNA sequence analysis allow direct characterization and comparison of isolates (inter- and intragenic variation). Furthermore establishes a reference

framework for the phylogenetic characterization of new or relatively obscure *Cryptosporidium* spp genotypes (Ruecker, 2013). A new genotypes and subtypes species are characterized at the DNA sequence level, while most of the molecular technique described above will require routine validation in order to confirm existing pattern of sensitivity and specificity of the assay , many cases, DNA sequencing used as confirmatory test of a method's robustness for specificity (Munshi, 2012).

The sequencing technology of DNA has been applied in many fields of diagnosis , it more helpful in the detection of mutations and leads to the rapid discovery of single nucleotide polymorphisms (SNPs) (Dwivedi *et al.,* 2017). These technology was able to estimate the genomic variation within and among *Cryptosporidium* spp. (Jex and Gasser, 2014). Sequencing techniques of a wide range of Cryptosporidial PCR products has aided the identification of most of the *Cryptosporidium* spp. (Ryan *et al.,* 2017).

The National Center for Biotechnology Information (NCBI) houses the sequence reference database known as Gen-Bank.provides a large ward of online resources for biological information and data , NCBI has a web interface tool known as BLAST , nucleic acid sequence database and the PubMed database of citations and abstracts for published life science journals ( Agarwala *et al*., 2016 ) . Also they well compares DNA sequence for regions of local similarity between the unknown sequence and those in these databases and calculates a level of significance for the match. provides analysis and retrieval resources for the data in GenBank and other biological data made available through NCBI's Web site. NCBI resources include Entrez, the Entrez Programming Utilities (Weeler , 2006 ) . The sequence information stocked in Gen-Bank supports the commonly accepted methods utilizing phylogenetic methods. This begins with a DNA sequence annealing of the unknown sequences to a

overall database of known sequences, followed by phylogenetic analysis appeared the relationship between species and genotypes and result represent this analysis is called a phylogenetic tree (Taha *et al.,* 2017). Molecular antedate estimates derived from various inference methods can be in struggle, and so can the results obtained with different taxon sampling, gene sampling and calibration strategies( Rutschmann *et al* .,2006) .Phylogenetic trees, based on distance, parsimony, maximum-likelihood, and bootstrap analysis have been used to elucidate the evolutionary divergence, as well as the host adapted nature of *Cryptosporidium* spp and genotypes (Lv *et al.,* 2009 ). Phylogenetic relationships are holding truelly across multiple gene targets (18S rRNA, actin and HSP70) and are traditionally used as a tool in the naming of new *Cryptosporidium* spp. (Elwin *et al.,* 2012) .

There are many of commercially viable and free software tools to carry out DNA sequence alignments and phylogenetic analysism, most importantly methods for *Cryptosporidium* have used evolutionary distance to conclude phylogenetic relationships (Ruecker, 2013).

In bird determinate genotypic characterization and phylogenetic analyze based on primers of the SSU rRNA gene of *Cryptosporidium* positive samples (18S rRNA) sequences of *Cryptosporidium spp*. the DNA sequence analysis obtained from the detected *Cryptosporidium baileyi* (Jasim and Marhoon ,2015).

Molecular characterization of poultry *Cryptosporidium* in Brazil , from analysis of 18S rRNA gene of *Cryptosporidium* followed by DNA sequencing of PCR products revealed the occurrence of *C. baileyi and C. meleagridis* (Da Cunhaa *et al.,* 2018)

Czech Republic was genotyped *Cryptosporidium* by PCR and DNA sequence analysis at the 18S rRNA and actin gene loci. Sequence and phylogenetic analysis

identified four genetically distinct genotypes, avian genotypes I to IV, from different avian hosts. furthermore, the host range for *Cryptosporidium galli* isolate( Ng *et al* ., 2006 )

### 2.13. Control of Cryptosporidiosis in Ostriches and other birds

Control of *Cryptosporidium* infection birds is so difficult due to the tiny size of oocysts make easily escape from filter pors that used to purification of water sources for ostrich fields (Current and Garcia, 1991) , oocyst highly stable and survive for long periods of time in cool, moist conditions, infected birds shed large numbers of infective oocysts, as well as the small infection dose can pass through a big group of susceptible hosts very quickly (Fayer *et al.,* 2010 ). moreover resistance of oocyst to many disinfectants, Antimicrobial agents,and other chemotherapy . treatment options of infected animals are limited (Mmbaga and Houpt, 2017).

#### 2.13.1. Treatment

Oocysts of *Cryptosporidium* parasite are resistant to environmental conditions and sterilizers compounds,and there is uneffective chemotherapy against *Cryptosporidium* parasite in birds ( Ryan *et al* ., 2018). Treatment of Cryptosporidiosis is unknown and difficult which is often based on supportive drugs and immuno Enhancer drugs because of their importance in healing infected animals based on supportive therapy which it is importance in healing infected ( Arsenopoulos *et al*., 2017). the available drugs are not very effective it can only diminish the duration period of shedding and have little effect on immunocompromised individuals (Thompson, 2016 and Mmbaga and Houpt, 2017).

### 2.13.2. Vaccines

The immunological methods against Cryptosporidiosis are currently exist, many observations suggest that vaccination against this disease should be practicable in experimental and commercial uses. The celluler immunity forms are predominant protective immune response against coccidiosis, and these made a major progress to development vaccines against *Eimeria spp*. By analogy, the similarity between them lead to develop vaccines against *Cryptosporidium* infection ( Fayer and Ellis,1993) , but in reality this may not be the case, which depending on rehydration therapy and there are no available vaccines to prevent the disease (Thomson, 2016 ).

Although the chickens developed vigorous immunity to *C. baileyi* and became resistant to reinfection with *Cryptosporidium* oocysts (Rhee et al., 1996 ) probably *Cryptosporidium* with increasing prevalence of antigenic variants in the poultry ,It became necessary to use of attenuated strains from local isolates for vaccine development, in another word, the coccidian prtozoal infection must be identified, isolated, attenuated, incorporated into vaccines and then used , while the host immune response should be monitored carefully (Lillehoj et al., 2000).

### 2.13.3. Disinfectants

Oocysts resistant to many traditional sterilizers used in sterilizing poultry farms, but in vitro the use of formalin 10% or minors 70% disinfect to the floor and wall of poultry farms they will killed most oocysts ,also lose their vitality when exposed to ammonia 5% for 20 minutes , Hydrogen peroxide 3% for 10 minutes and chloride dioxide 0.4% for 15 minutes (O'Donoghue, 1995 and Naciri *et al.*, 2011). They are not sufficiently compelling to depend on for the successful elimination of *Cryptosporidium* oocysts on the farms (Keidel and Daugschies, 2013).

### 2.13.4. Farm management

Management, biosecurity measures and hygiene practices are necessary to reduce the environmental contamination and controlling Cryptosporidiosis because oocysts are very difficult to eliminate ; The support therapy is essential to improvement of infected animals, as well as to prevent continued infection in one farms or among other breeding farms (Thompson, 2016 and Shahiduzzaman and Daugschies, 2012 ) .

The immunosuppression factors should be removed during cryptosporidiosis treatment (Graczyk et al .,1996). Overcrowding in breeding is an immunosuppressant, the presence of large numbers of birds in the same farms facilitated incidence of this infection , therefore the distribution of chicks in the sheds should be considered. Although ,temperature , moisture , diets and water contaminated with faces and other important management should be considered . (Morgan et al., 2001 and Akram et al., 2018) .

# 3. Materials and Methods

## 3.1. Materials

### 3.1.1. Equipment's and Instruments recorded in (Table 3-1):

**Table (3-1): Equipment's and Instruments**

| No. | equipment | Company / Country |
|---|---|---|
| 1 | High speed Cold Centrifuge | Eppendorf/ Germany |
| 2 | Incubator | Memmert (Germany) |
| 3 | Nanodrop | Thermo Scientific/ UK |
| 4 | Vortex | CYAN/ Belgium |
| 5 | Micropipettes (different volumes) | Eppendorf / Germany |
| 6 | Exispin vortex centrifuge | Bioneer/ Korea |
| 7 | Refrigerator | Concord/ lebanon |
| 8 | Gel electrophoresis | Bioneer/ Korea |
| 9 | U.V transilluminator | Wised/Korea |
| 10 | Digital Camera | Nikon/Japan |
| 11 | T100 Thermal cycler PCR | Bio-Rad/ USA |
| 12 | Sensitive Balance | Ohaus /USA |

| No. | Instruments | Company |
|---|---|---|
| 1 | Disposable syringe | China |
| 2 | Disposable gloves | China |
| 3 | Eppedorf tube | Bioneer/ Korea |
| 4 | Filter paper | China |
| 5 | Gauze | China |
| 6 | Plastic containers | China |
| 7 | Pasteur pipette | China |
| 8 | Slides and cover slides | China |
| 9 | Sterile test tube | Superestar/ India |
| 10 | Eppendorf tubes | Biobasic/ Canada |
| 11 | Water bath | Kottermann (Germany) |

### 3.1.2. kits

Table (3-2): Kits used in the study with their companies and countries of origin:

| No. | Kit | Company | Country |
|---|---|---|---|
| **1** | **AccuPrep® stool DNA Extraction Kit** | **Bioneer** | **Korea** |
| | Proteinase K | | |
| | Stool Lysis buffer (SL) | | |
| | Binding buffer (ST) | | |
| | Washing buffer 1 (W1) | | |
| | Washing buffer 2 (W2) | | |
| | Elution buffer (E) | | |

| | | | |
|---|---|---|---|
| | GD column | | |
| | Collection tube 2ml | | |
| **2** | **Maxime PCR PreMix** | **iNtRON** | **Korea** |
| | Tap DNA polymerase | | |
| | dNTPs (dATP, dCTP, dGTP, dTTP) | | |
| | Tris-HCl pH 9.0, KCl, $MgCl_2$ | | |
| | Stabilizer and Tracking dye | | |

### 3.1.3. Primers

The PCR primers used for detection *Cryptosporidium* spp. based small subunit ribosomal RNA (18SrRNA gene) were design in this study based on NCBI-Genbank *Cryptosporidium* sp. Small subunit ribosomal gene sequence (DQ002931.1) and primer 3 plus design. These primers was provided from Macrogen company, Korea as following (Table 3-3):

Table (3-3)**:** The Nested PCR primers used for detection *Cryptosporidium* spp.

| **Primers** | **Sequence 5'-3'** | | **Amplicon** |
|---|---|---|---|
| 18SrRNA gene primary primer | **F** | **CGGGTAACGGGGAATTAGGG** | **616bp** |
| | **R** | **TCGTCTTCGATCCCCTAACT** | |
| 18SrRNA gene secondary primer | **F** | **CCTGAGAAACGGCTACCACA** | **459bp** |
| | **R** | **GCCCCCAACTGTCCCTATTAA** | |

### 3.1.4. Chemicals(Table 3-4):

Table (3-4): Chemicals and their company and origin

| No. | Chemical | Company and Origin |
|---|---|---|
| 1 | Absolute Ethanol | BDH (England) |
| 2 | Agarose | BioBasic (Canada) |
| 3 | Basic carbol fuchsin | BDH (England) |
| 4 | Disodium phosphate $Na_2HPO_4$ | Local market |
| 5 | Ehidium Bromide | BioBasic (Canada) |
| 6 | Free nuclease water | Biolab/ USA |
| 7 | Hydrochloric acid HCl 100% | BDH (England) |
| 8 | Iodine | BDH (England) |
| 9 | Isopropanol | GCC (England) |
| 10 | Methanol 100% | BDH (England) |
| 11 | Methylene Blue | BDH (England) |
| 12 | Oil immersion | BDH (England) |
| 13 | Phenol | BDH (England) |
| 14 | Potassium dichromate 2.5 % $K_2Cr_2O_7$ | BDH (England) |
| 15 | Potassium dihydrogen phosphate $KH_2PO_4$ | BDH (England) |
| 16 | Potassium chloride KCL | BDH (England) |
| 17 | TBE buffer | BioBasic (Canada) |
| 18 | Xylol | BDH (England) |
| 19 | Zinc sulfate $ZnSo_4$ | BDH (England) |

### 3.1.5. Solutions

1- Sheather's Sugar Solution:

For preparation Sheather's sugar solution 500 gram sugar dissolved in 320 ml distilled water, with 6.5 gram phenol as preservative (Chermette and Boufassa ,1988).

2- Saturated salt solution

Saturated NaCl solution prepared by mixing an excess amount of salt into distilled water, according to Dryden et al (2005). The solubility of NaCl in $H_2O$ is approximately 35g/100ml at 20℃, so 100g of NaCl into 200ml of $H_2O$ should be sufficient. Transfer enough of the saturated solution into a Petri dish to cover the surface of the dish.

3- Potassium Dichromate 2.5%:

Potassium Dichromate 2.5% was prepared by dissolved 25 gram of potassium dichromate in 1 liter of distilled water (Ma and Soave, 1983).

4- Acid Alcohol:

Acid Alcohol was prepared by adding 3 ml of concentrated HCL to 97 ml of ethanol 95 % (Coles, 1986).

5- Phosphate Buffer Saline (PBS):

Phosphate Buffer Saline was prepared by dissolved: 0.2 gram $KH_2PO_4$, 0.9 gram $Na_2HPO_4$, 8 gram NaCl and 0.2 gram KCl in 1 liter of distilled water (Coles, 1986).

6. Agarose:

Agarose 1% (1gm /100 mg) and 1X TBE buffer 30 ml. Agarose was dissolved in 1X TBE buffer and heated in microwave for 1 min. This was allowed to cool (<60 °C) and 3 µl of working solution of ethidium bromide was added (Venu, 2010).

### 3.1.6. Stains

#### 3.1.6.1. Modified Ziehl-Neelsen Stain:

Components:-

- Basic carbol fuchsin (4 grams).

- Phenol (8 grams).

- Ethanol 95 % (20 ml).

- Distilled water (100 ml).

Preparation:-

- Solution A:

prepared by dissolved basic carbol fuchsine stain (4 gram) in 20 ml of ethanol 95 %.

- Solution B:

Phenol (8 gram) was added to 100 ml distilled water with continuous mixing until completely dissolved, then solution B was added to solution A and mixed well (Beaver and Jung, 1985)(Brondson,1984)

#### 3.1.6.2. Methylene Blue Stain (1%):

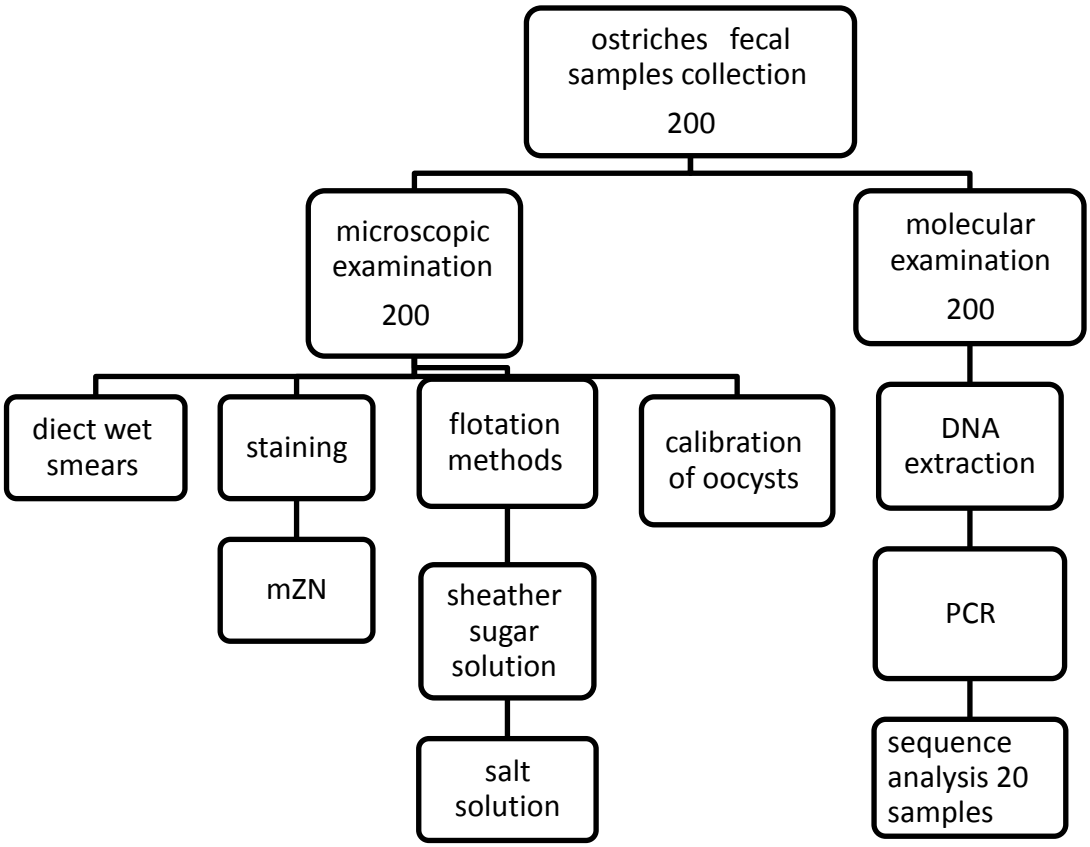
It was prepared by dissolving 1 gram methylene blue in 100 ml distilled water (Levine, 1961).

## 3.2. Methods

### 3.2.1. fecal samples collection

A 5-20 grams of fecal samples were collected from 200 Ostriches (24 male,70 female and 106 young chicks ) with age groups ranging from less than 1 month to 4 years old, during the period from the beginning of December 2018, to end of September 2019, from different provinces in the central and south parts of Iraq (Baghdad , Babylon, Waist, Qadisiyyah , Diyala and Najaf ) . Faecal samples were collected from fresh faces after Ostriches (Defecation) dropping immediately, and were collected in a clean plastic container and given sequential numbers, age, sex, date of sampling also included , protective measures was taken such as wearing disposable gloves. The samples were transported in cool box to parasitology laboratory and divided into two parts for traditional and molecular examination at College of Veterinary Medicine-University of Baghdad.

**Diagram of study design**



### 3.3. Morphological characterization of oocysts

#### 3.3.1.Laboratory Tests of fecal samples

#### 3.3.2. Microscopic Examination:

Four tests used for fecal samples examination :

#### 3.3.3. Direct wet smear:

Direct smears were prepared and examined after using Lugol's Iodine stain according to (Levine, 1961).

#### 3.3.4 Staining Methods:

Smears were prepared and stained by Modified Ziehl-Neelsen (mZN) stains to investigate *Cryptosporidium* oocysts according to (Beaver and Jung, 1985).

#### 3.3.5. Flotation Methods:

Sheather's solution were used to investigate intestinal protozoa oocysts according to (Chermette and Boufassa, 1988).

### 3.4. Calibration of oocysts by using Ocular Micrometer according to Zeibig

( 1997) as following :

1- Ocular eye piece was replaced with one containing an ocular micrometer.

2- Stage micrometer was placed on the stage microscope.

3- Using the 10x objective , it was focused in on the stage of micrometer and was arranged so that the left edge of the micrometer stage lines up with the left edge of the ocular micrometer, successful completion of the zero points of each calibration device and then the numbers are superimpose.

4- A point farthest to the right of the zero was located where both devices again super imposed. The number of microns equal to each unit on the ocular micrometer .

The process was repeated for each objective and the microns equivalent to each ocular unit was calculated by using the following:

$$\frac{\text{Number of stage micrometer units} \times 1000}{\text{Number of ocular micrometer units.}}$$

### 3.5.1.Nested PCR (nPCR)

Two hundred fecal samples were collected selectively from farming Ostriches were used for nPCR screening**.**

The nPCR technique was performed for detection *Cryptosporidium* spp. based small subunit ribosomal RNA gene from Ostriches fecal samples. This method was carried out according to Yu *et al* ( 2009) and Ruecker *et al* ( 2013) as following steps:

### 3.5.2. Genomic DNA Extraction

Genomic DNA were extracted from fecal samples by using AccuPrep® stool DNA Extraction Kit , Bioneer. Korea, and done according to company instructions as following steps:

1. A 200 mg of fecal sample was transferred to sterile 1.5ml microcentrifuge tube, and then 20µl of proteinase K and 400 µl Stool lysis buffer (SL) were added mixed by vortex. And incubated at 60℃ for 10 minutes.
2. After 10 mins, the tube placed in centrifuge at 12,000rpm for 5 mins.

3. The supernatant was transferred in to a new tube and 200µl Binding buffer was added to each tubes.

4. The tubes were incubated again for 10 min at 60℃.

5. 100 µl isopropanol was added and the samples mixed by lightly vortex for about 5 seconds, then spin down for 10 seconds to down the liquid clinging to the walls and lid of the tube.

6. DNA filter column was placed in a 2 ml collection tube and transferred all of the mixture (including any precipitate) to column. Then centrifuged at 8000rpm for 5 minutes. And the 2 ml collection tube containing the flow-through were discarded and placed the column in a new 2 ml collection tube.

7. 500µl W1 buffer were added to the DNA filter column, then centrifuge at 10000rpm for 30 seconds. The flow-through was discarded and placed the column back in the 2 ml collection tube.

8. 500µl W2 Buffer (ethanol) was added to each column. Then centrifuged at 8000rpm for 30 seconds. The flow-through was discarded and placed the column back in the 2 ml collection tube.

9. All the tubes were centrifuged again for 1 minutes at 12000 rpm to dry the column matrix.

10. The dried DNA filter column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of pre-heated elution buffer were added to the center of the column matrix.

11. The tubes were let stand for at least 5 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 30 seconds to elute the purified DNA.

### 3.5.3.Genomic DNA estimation

The extracted genomic DNA from fecal samples was checked by using Nanodrop spectrophotometer (THERMO. USA), that checked and measured the purity of DNA through reading the absorbance in at (260 /280 nm) as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).
2. A dry wipe was taken and cleaned the measurement pedestals several times. Then carefully pipette 2µl of free nuclease water onto the surface of the lower measurement pedestals for blank the system.
3. The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of DNA was added to measurement.

### 3.5.3.1.Primary PCR master mix preparation

PCR master mix was prepared by using (**Maxime PCR PreMix Kit**) and this master mix done according to company instructions as following (Table 3-5) :

**Table (3-5) Primary PCR Master Mix.**

| PCR Master mix | Volume |
|---|---|
| DNA template<br>5-50ng | 5µL |
| 18SrRNA Forward primary primer (10pmol) | 1µL |
| 18SrRNA Reverse primary primer (10pmol) | 1µL |
| PCR water | 13 µL |
| Total volume | 20µL |

The PCR master mix component that mentioned in table (3-5 ) placed in standard **Maxime PCR PreMix** that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl,

$MgCl_2$,stabilizer, and tracking dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. before placed in PCR Thermocycler.

### 3.5.3.2. PCR Thermocycler Conditions

PCR thermocycler conditions by using convential PCR thermocycler system as following ( Table 3-6 ):

**Table (3-6) PCR thermocycler conditions**

| PCR step | Temp. | Time | repeat |
|---|---|---|---|
| Initial Denaturation | 95ºC | 5min. | 1 |
| Denaturation | 95 ºC | 30sec. | 30 cycle |
| Annealing | 58 ºC | 30sec | |
| Extension | 72 ºC | 1min. | |
| Final extension | 72 ºC | 5min. | 1 |
| Hold | 4 ºC | Forever | - |

### 3.5.3.3. Secondary Nested PCR master mix preparation

Nestsed PCR master mix was prepared by using (**Maxime PCR PreMix Kit**) and this master mix done according to company instructions as following ( Table 3-7)

**Table (3-7) Secondary Nested PCR Master mix**

| Nested PCR Master mix | Volume |
|---|---|
| DNA template | 2.5µL |
| 18SrRNA Forward secondary primer (10pmol) | 1µL |
| 18SrRNA Reverse secondary primer (10pmol) | 1µL |
| PCR water | 15.5 µL |
| Total volume | 20µL |

After that, nPCR master mix component that mentioned in table (3-7) placed in standard **Maxime PCR PreMix** that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,stabilizer, and tracking dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler.

### 3.5.3.4. PCR Thermocycler Conditions

PCR thermocycler conditions by using convential PCR thermocycler system as following table:

**Table (3-8) nPCR thermocycler conditions**

| PCR step | Temp. | Time | repeat |
|---|---|---|---|
| Initial Denaturation | 95ºC | 5min. | 1 |
| Denaturation | 95 ºC | 30sec. | 30 cycle |
| Annealing | 58 ºC | 30sec | |

| | | | |
|---|---|---|---|
| Extension | 72 ºC | 1min. | |
| Final extension | 72 ºC | 5min. | 1 |
| Hold | 4 ºC | Forever | - |

**3.5.3.5. PCR product analysis**

The PCR products was analyzed by agarose gel electrophoresis as following steps:

1- 1% Agarose gel (1gm /100 mg) was prepared and used 1X TBE (30 ml ) and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50°C.

2- Then 3µl 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 10µl of PCR product were added in to each comb well and 5ul of (100bp Ladder) in one well.

4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 AM for 1hour.

5- PCR products 459bp were visualized by using UV Transilluminator.

**3.6.DNA sequencing method**

DNA sequencing method was performed for species typing of some positive local *Cryptosporidium* isolates as following step:

1- The PCR product of small subunit ribosomal RNA genes were sent to Macrogen Company in Korea in ice bag by DHL for performed the DNA sequencing by AB DNA sequencing system.

2- The DNA sequencing analysis (Phylogenetic tree analysis) was conducted by using Molecular Evolutionary Genetics Analysis version 6.0. (Mega 6.0) and Multiple sequence alignment analysis based ClustalW alignment analysis and The evolutionary distances were computed using the Maximum Composite Likelihood method by phylogenetic tree UPGMA method.

3- The *Cryptosporidium* typing analysis was done by phylogenetic tree analysis between local *Cryptosporidium* isolates and NCBI-Blast known *Cryptosporidium* .( Tamura *et al.*, 2013).

4- Finally identified *Cryptosporidium* species isolates were submitted into of NCBI-GenBank to get Genbank accession number .

**3.7. Statistical Analysis**:

The Chi-square ($X^2$) test was used for comparison between the results. Differences were considered statistically significant at $P \leq 0.05$ (Petrie and Watson, 2006).

**4. Results**

**First part**

**4.1.Total infection rate of *Cryptosporidium spp*. in Ostriches by using conventional methods ( microscopic examination).**

A total of 200 Ostriches fecal samples were examined by conventional methods ( flotation , staining by modified Ziel-Neelsen ( mZN) stain ) for detection the prevalence of *Cryptosporidium spp*. and revealed the overall infection rate of *Cryptosporidium spp*.in Ostriches in central and south parts of Iraq 11% (22/200) .

**4.1.1. Prevalence rate of *Cryptosporidium spp*. infection in relation to sex.**

The results showed that male and female Ostriches recorded 8.33 % ( 2/24) and

10 % (7/70) respectively rate of infection with *Cryptosporidium spp.* without significant differences (P<0.05).

### 4.1.2. Prevalence rate of *Cryptosporidium spp*. infection in relation to age groups.

No significant difference (P<0.05) were recorded in the prevalence rates between age groups,the highest infection rate 12.26% (13/106) was recorded in Young (chicks) (< 9 Months),while the lowest infection rate 9.57% (9/94) was recorded in the adult(> 9 Months).

### 4.1.3. Prevalence of *Cryptosporidium spp*. in relation to areas of study.

Twenty tow positive fecal samples fromOstriches were examined microscopically from seven provinces in the central and south parts of Iraq , including: Wasit , Baghdad , Babylon , Diyala , Karbala , Al-Najaf and Al-Qadisyiah and recorded 0% , 4.17% , 15.79% , 3.70% , 11.54% , 23.53% and 19.23%, respectively . there were significant differences (P<0.05) in the prevalence among these different areas of study, Al-Najaf showed highest prevalence rate (23.53%) while Wasit showed the lowest rate ( 0 %) .(fig.4-1)

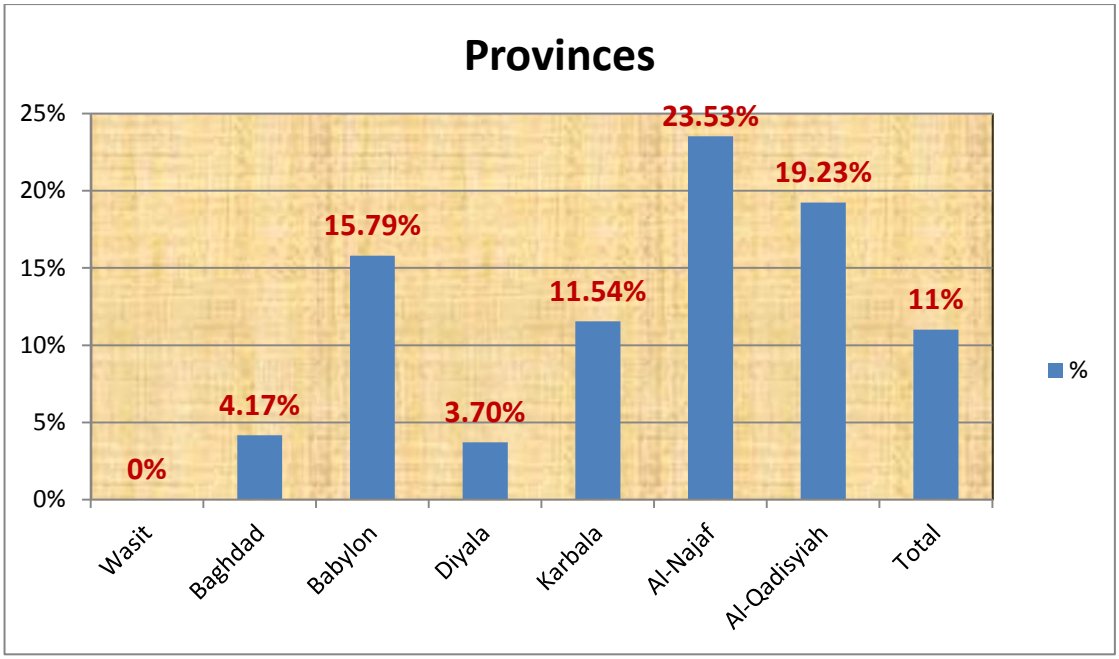



**Figure ( 4-1) Prevalence of *Cryptosporidium* spp. in relation to areas of study**

### 4.1 . Prevalence of *Cryptosporidium spp*. in relation to months of study

The result showed that the highest prevalence was recorded in March 50% (9/18) while the results were convergent during the months of February and April, 41.67%(5/12) and 27.27% (3/11) , respectively . the lowest prevalence rate recorded during August 0% ( 0/29) ,with significant difference (P<0.05) .(fig.4-2).

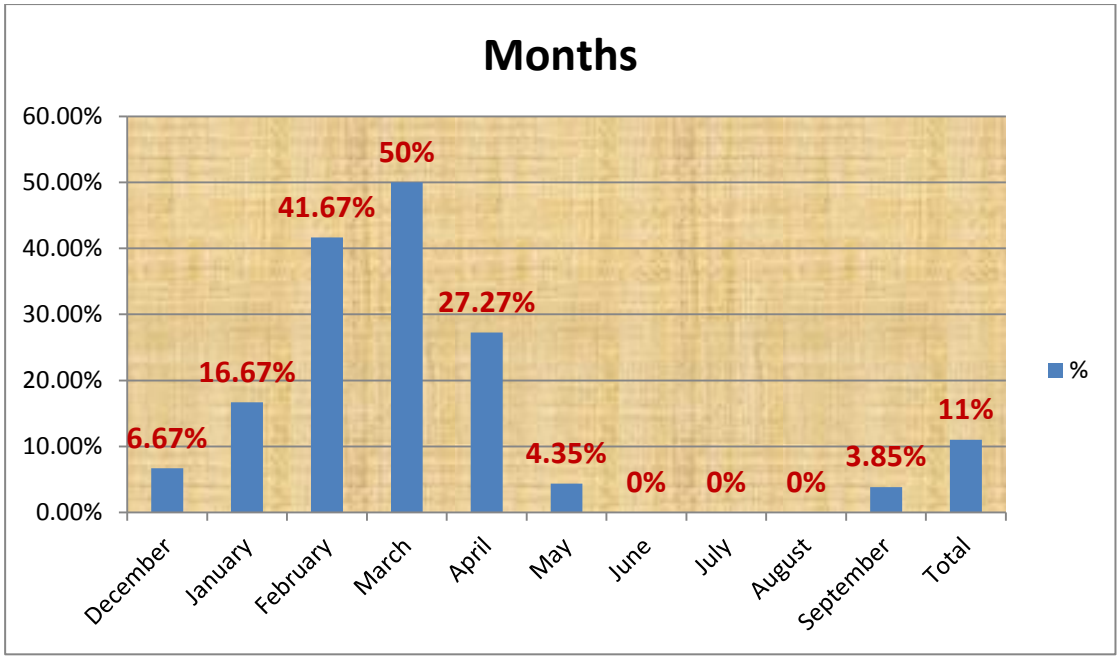
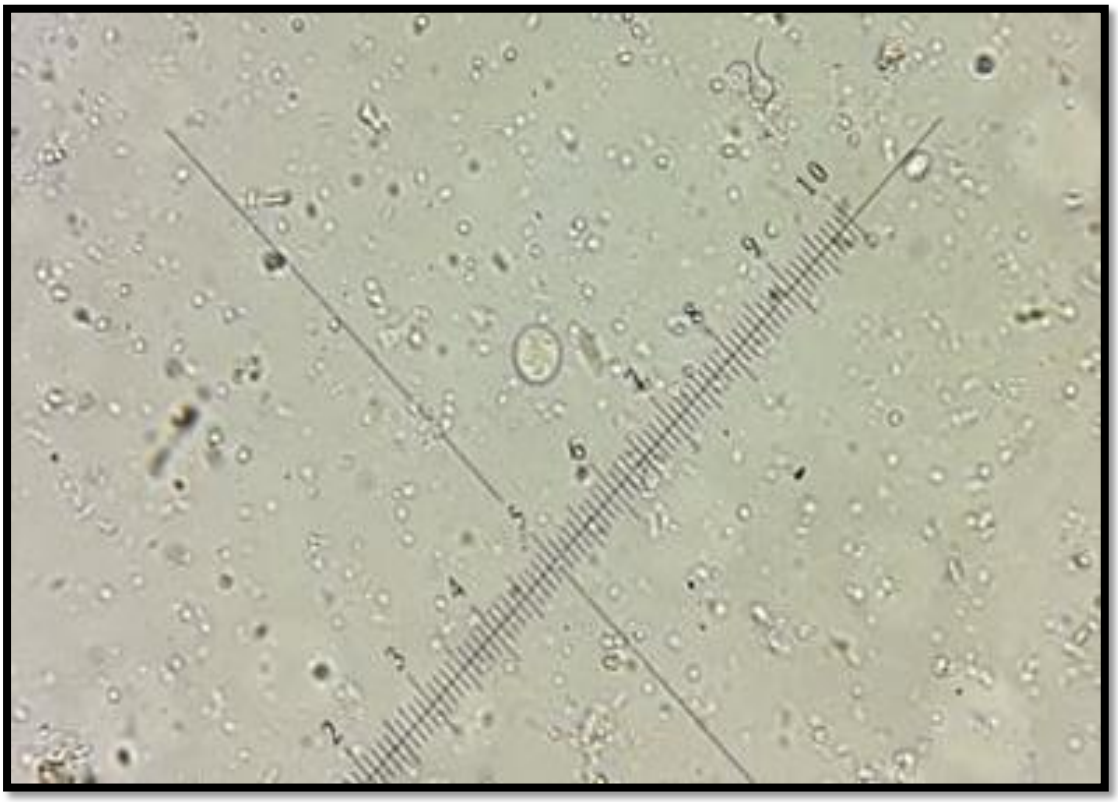



**Figure ( 4-2) Prevalence of *Cryptosporidium* spp. in relation to months of study**

### 4.1.5. Morphological characterization and measurements of *Cryptosporidium* spp. oocysts

The microscopic examination *Cryptosporidium parvum* oocysts by flotation with salt and sheathers solution revealed that oocysts appeared spherical or rounded with a thin membrane , the four sporozoit look as black bodies inside the oocysts ( fig. 4-3). the oocysts of *C.parvum* while oocysts stained with Modified Ziehl-Neelsen ( m ZN) appeared spherical dark red in color with a clear halo around the oocysts , .the average length and width measurements of *C.parvum* oocysts had almost ( 4.3× 4.9 ± 0.9**)** µm. Measurement of oocysts was done by using ocular micrometer ( fig. 4-4 ). oocysts of *C. baileyi* have ovoid shape and more larger with average size (6.0×4.6).( fig.4-5) oocysts of *C. meleagridis* are spherical with average measurement ( 5.2×4.6) µm( fig.4-7) ,while *C. galli* oocysts are larger with average measurement(8.3 × 6.3 ) µm.( fig.4-6)



**Figure(4-3): C. *parvum* oocysts, flotation with sheathers solution, measured 4.3 x 4.9 µm ( 100 x )**

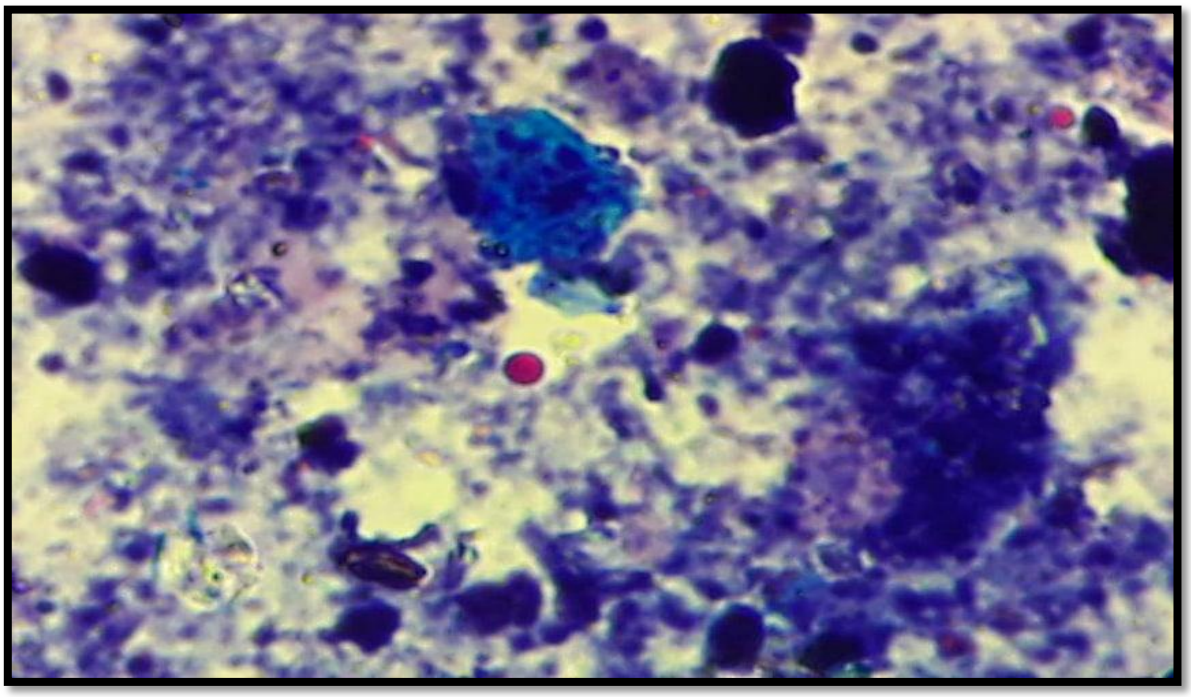


**Figure (4-4):C.*parvum* oocysts stained with Modified Ziehl- Neelsen ( 100 x )**

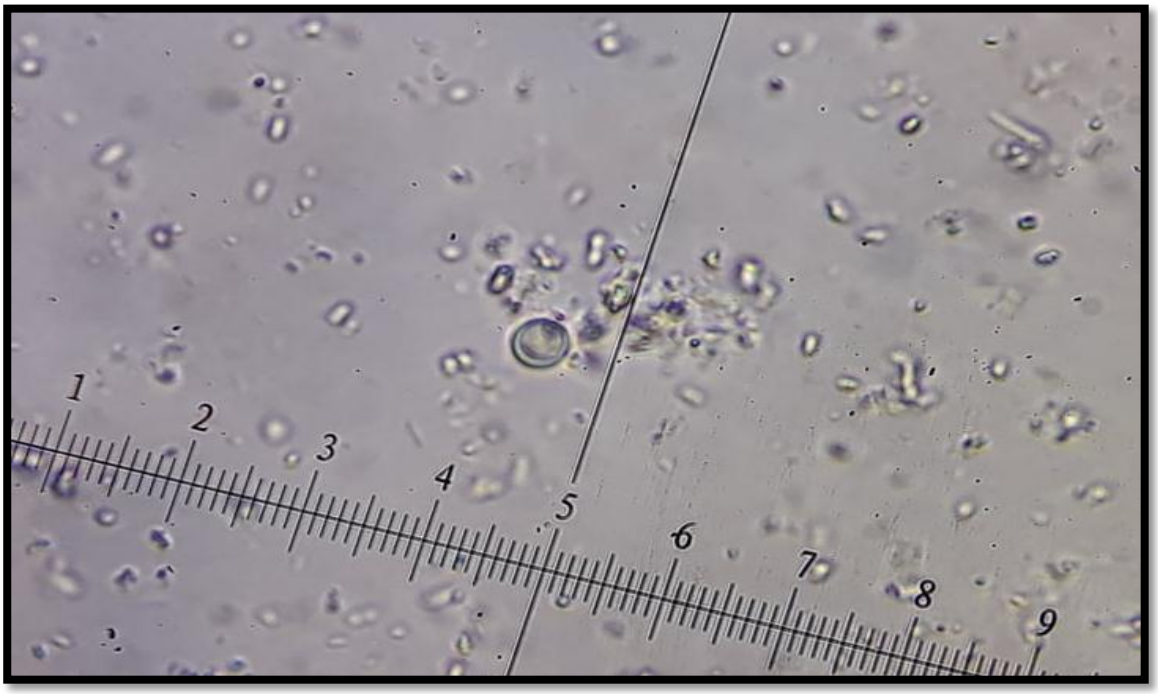



**Figure (4-5):C. *baileyi* oocysts, flotation with salts solution measured 6.0×4.6 (100 x )**

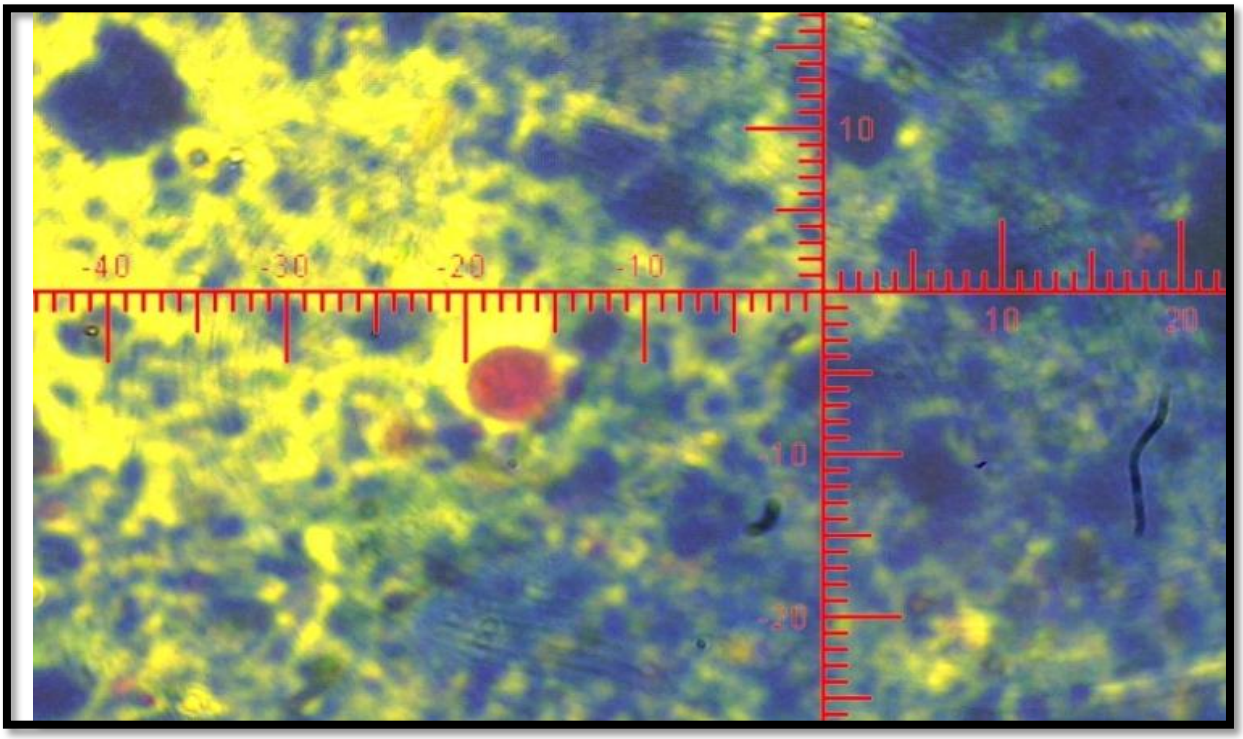



**Figure (4-6):C.*galli* oocysts stained with Modified Ziehl- Neelsen ( 100 x )**

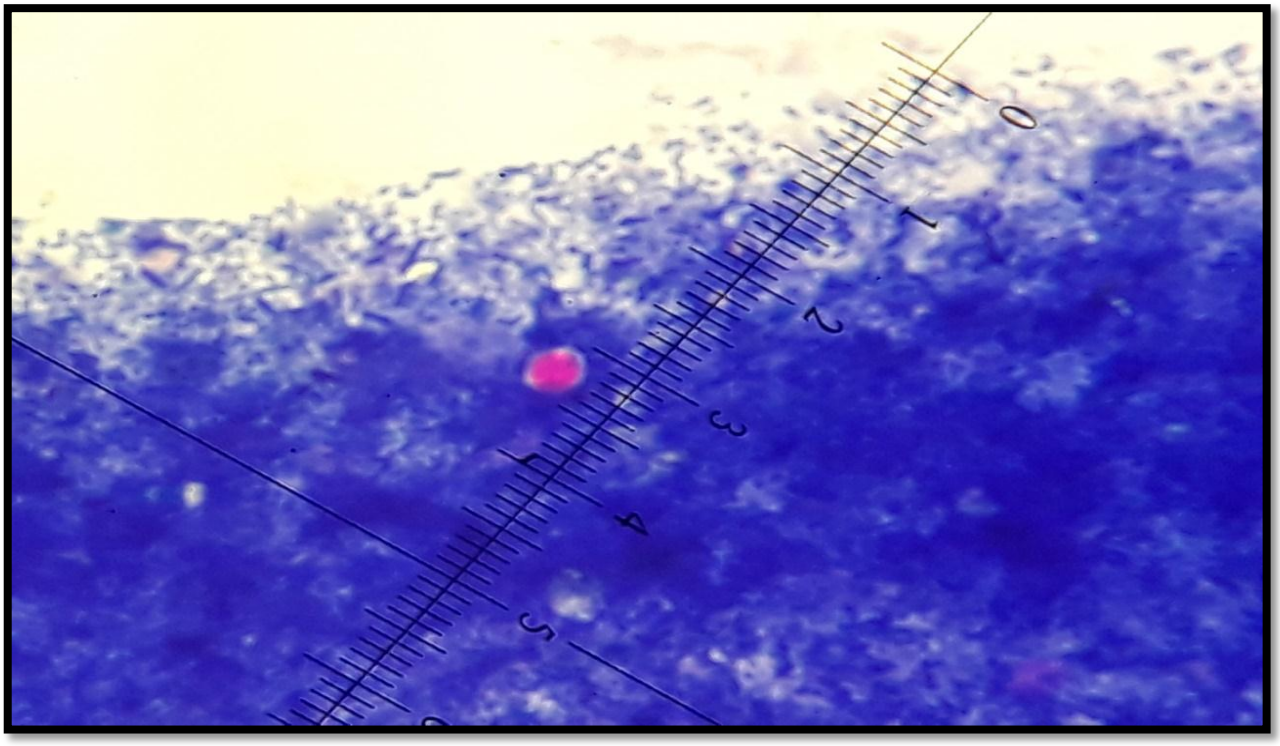


**Figure (4-7):C. *meleagridis* oocysts stained with Modified Ziehl- Neelsen ( 100 x )**

**Second part**

**4.2. molecular study Nested PCR.**

**4.2.1. Prevalence of Cryptosporidium spp.in Ostriches by Nested PCR**

Nested PCR revealed total infection rate with *Cryptosporidium* spp.in Ostriches 26.5% ( 53/200), statistical analysis showed significant differences between conventional and molecular techniques and its relation with sensitivity and specificity of each diagnostic test (P<0.05) ( Table 4-1).

**Table ( 4-1): Total prevalence of Cryptosporidium infection by molecular techniques ( Nested PCR) in Ostriches**

| Host | No. of samples examined | No. of Positive | % |
|---|---|---|---|
| **Ostriches** | **200** | **53** | **26.5** |

### 4.2.2. Prevalence of *Cryptosporidium* infection by Nested PCR in relation to age groups

The results showed a significant difference (P<0.05) in the prevalence among age groups . the highest rate recorded in Young's Ostriches < 9 Months 30.19% ( 32/106) , while the lowest 22.34% ( 21/94) recorded in the adult > 9 Months (Table 4-2) . there was age-related distribution of *Cryptosporidium spp* among the Ostriches , *C. baileyi* and *C. parvum* was dominant species in young Ostriches 40% (8/20),15% ( 3/20) respectively, while *C. meleagridis* was dominant species in adult Ostriches > 9 Months age 15% (3/20) although *C. galli* was occurred in adult less than 2 years 10%(2/20) .

**Table ( 4-2) : Prevalence of *Cryptosporidium* infection by nested PCR in relation to age groups**

| Age groups | | No. of samples examined | No. of Positive | % |
|---|---|---|---|---|
| Young's | < 9 Months | 106 | 32 | 30.19 * |
| Adult | > 9 Months | 94 | 21 | 22.34 |
| Total | | 200 | 53 | 26.5 |

**Significance * (P<0.05)**

### 4.2.3. Prevalence of *Cryptosporidium* infection by nested PCR in relation to sex of Ostriches

Depending on the nested PCR results, 5 Ostriches male were found infected with *Cryptosporidium* out of 24 with rate 20.83 % (5/24) while Ostriches female recorded rate 22.86% (16/70). without significant differences.(P<0.05)(Table 4-3).

**Table ( 4-3): Prevalence of *Cryptosporidium* infection by nested PCR in relation to sex of Ostriches**

| Sex of Ostriches | No. of samples examined | No. of Positive | % |
|---|---|---|---|
| **Male** | **24** | **5** | **20.83** |
| **Female** | **70** | **16** | **22.86** |
| **Total** | **94** | **21** | **22.34** |

**Not Significance (P<0.05)**

### 4.2.4. Prevalence of *Cryptosporidium* infection by nested PCR in relation to areas

Molecular study by Nested PCR in seven provinces in central and south parts of Iraq including : Wasit , Baghdad , Babylon , Diyala , Karbala , Al-Najaf and Al-Qadisyiah revealed prevalence 30%(6/20), 16.67%(8/48), 31.58%(6/19),11.11%(3/27), 30.77%(8/26) , 38.24%(13/34) and 34.62%(9/26) respectively. Al-Najaf province showed the highest prevalence rate(38.24%) while Diyala province showed the lowest rate (11.11%).with significant differences among provinces (P<0.05) (Table 4-4).

**Table( 4-4): Prevalence of *Cryptosporidium* infection by nested PCR in relation to areas**

| Province | No. of samples examined | No. of Positive | % |
|---|---|---|---|
| **Wasit** | **20** | **6** | **30** |
| **Baghdad** | **48** | **8** | **16.67** |
| **Babylon** | **19** | **6** | **31.58** |
| **Diyala** | **27** | **3** | **11.11** |
| **Karbala** | **26** | **8** | **30.77** |
| **Al-Najaf** | **34** | **13** | **38.24 **** |
| **Al-Qadisyiah** | **26** | **9** | **34.62 *** |
| **Total** | **200** | **53** | **26.5** |

**Significance * (P<0.05)**

### 4.2.5. Prevalence of *Cryptosporidium* infection by nested PCR in relation to months of study.

Nested PCR revealed significant difference (P<0.05) between prevalence of *Cryptosporidium* infection in relation to months of study. April recorded highest rate of infection72.73% (8/11) while the lowest rate 7.14% ( 2/28) recorded in July. (Table 4-5).

**Table( 4-5):Prevalence of *Cryptosporidium* infection by nested PCR in relation to months**

| Year | Months | No. of samples examined | No. of Positive | % |
|---|---|---|---|---|
| 2018 | December | 15 | 3 | 20 |
| 2019 | January | 12 | 4 | 33.33 |
| | February | 12 | 6 | 50 |
| | March | 18 | 13 | 72.22 * |
| | April | 11 | 8 | 72.73 ** |
| | May | 23 | 7 | 30.43 |
| | June | 26 | 4 | 15.38 |
| | July | 28 | 2 | 7.14 |
| | August | 29 | 3 | 10.34 |
| | September | 26 | 3 | 11.54 |
| Total | | 200 | 53 | 26.5 |

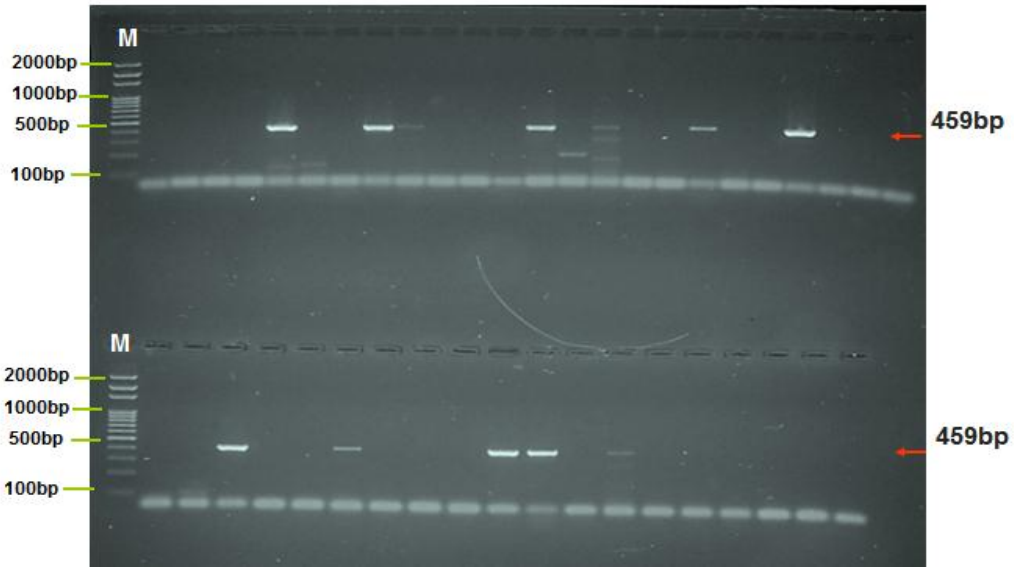
**Significance * (P<0.05)**

### 4.3.Molecular detection of *Cryptosporidium* spp.in Ostriches by Nested PCR

Genomic DNA was obtained from Ostriches fecal samples were subjected to molecular analysis by nested PCR using small subunit ribosomal RNA gene specific primers to identify the species of *Cryptosporidium*. nested PCR of all 200 samples will employed band at (459bp) .Nested PCR product size on agarose gel confirming of

presence *Cryptosporidium* spp.in Ostriches( fig.4-8) *Cryptosporidium* DNA was found in 53 of 200 Ostriches fecal samples with percentage 26.5% ( Table 4-1).

**Figure (4-8) : Agarose gel electrophoresis image showed the PCR product analysis of small subunit ribosomal RNA gene in *Cryptosporidium* sp. from Ostriches fecal samples. Where M: marker (2000-100bp) , lanes showed some positive *Cryptosporidium* sp. at (459bp) PCR product .**




4

The sequences analysis of nested PCR products using NCBI BLAST tool, The results revealed the presence of four *Cryptosporidium* species in Ostriches at central and south provinces of Iraq, namely *C.parvum*, *C. baileyi*, *C. meleagridis* and *C. galli* ( Table 4-6) . *C. baileyi* was the most prevalent species that detected in Ostriches ( 9/20) , following *C. meleagridis* ( 5/20) , *C.parvum* (4/20) and finally *C. galli* (2/20).

The sequence analysis confirmed the identification of C. *baileyi* 100% homology was observed with their respective species sequences reported on Gen Bank on accession numbers (MN410723.1) in China , while, *C. parvum* identity was 99.76 % on accession number (KM870602.1 ) in Thailand. *C.meleagridis* 99.53 % homology sequence identity on accession number (MN410718.1) in China and homology sequence of *C.galli* identity was 99.28 % on accession number (GU816045.1) in Brazil ( Table 4- 6).

**4.5.Phylogenic analysis :**

Phylogenetic tree was constructed using Unweight Pair Group method with Arithmetic Mean (UPGMA tree) in MEGA .X version for all 20 local *Cryptosporidium* isolate PCR products to assess the genetic relationship among various *Cryptosporidium* species in Ostriches . All positive PCR products were deposited in GenBank database under accession no. ( Table 4-6) which recorded five isolates of *Cryptosporidium meleagridis* have been consigned in the GenBank database under accession no. MN410718.1.Sex isolates of *Cryptosporidium baileyi* under accessision no MN410723.1.three isolate of *Cryptosporidium parvum* under accession no. KM870602.1 and tow isolate of *Cryptosporidium galli* under accession no. GU816045. To construct the relationship between *Cryptosporidium* spp. were aligned with the NCBI BLAST *Cryptosporidium spp*. sequences of Ostriches obtain from GenBank at total genetic changes (0.010-0.060%).

The first used of phylogenetic analysis is to help resolve the controversy of the taxonomy of the genus *Cryptosporidium*. 20 sequenced isolates of *Cryptosporidium* species from Genomic DNA of Ostriches were describe in phylogenetic tree with respective reference sequenced retrieved from GenBank . phylogenetic tree was

constructed for sequences of C. *meleagridis*, *C.parvum* ,C. *baileyi and C.galli* isolated separately to highlight the differences between these four species DNA STAR in Ostriches ( fig.4-9)  (fig.4-10) .

**Table (4-6) The NCBI-BLAST Homology Sequence identity (%) between local *Cryptosporidium* spp. Ostriches isolates and NCBI-BLAST submitted *Cryptosporidium* spp. isolates**

| Local Ostriches *Cryptosporidium* sp. No. | Gen –Bank accession No. | NCBI BLAST Homology sequence identity | | |
|---|---|---|---|---|
| | | NCBI BLAST *Cryptosporidium* sp. | Gen –Bank accession No. | Identity (%) |
| 1 | MN515110 | *C. meleagridis* | MN410718.1 | 98.82 |
| 2 | MN515111 | *C. baileyi* | MN410723.1 | 99.53 |
| 3 | MN515112 | *C. meleagridis* | MN410718.1 | 99.29 |
| 4 | MN515113 | *C. parvum* | KM870602.1 | 99.07 |
| 5 | MN515114 | *C. baileyi* | MN410723.1 | 99.52 |
| 6 | MN515115 | *C.meleagridis* | MN410718.1 | 99.53 |
| 7 | MN515116 | *C. baileyi* | MN410723.1 | 98.80 |
| 8 | MN515117 | *C. galli* | GU816045.1 | 99.27 |
| 9 | MN515118 | *C. baileyi* | MN410723.1 | 99.28 |
| 10 | MN515119 | *C. baileyi* | MN410723.1 | 99.28 |
| 11 | MN515120 | *C. baileyi* | MN410723.1 | 100 |
| 12 | MN515121 | *C.baileyi* | MN410723.1 | 99.52 |
| 13 | MN515122 | *C. baileyi* | MN410723.1 | 99.26 |
| 14 | MN515123 | *C. baileyi* | MN410723.1 | 99.29 |
| 15 | MN515124 | *C. parvum* | KM870602.1 | 99.29 |
| 16 | MN515125 | *C.meleagridis* | MN410718.1 | 98.80 |
| 17 | MN515126 | *C.galli* | GU816045.1 | 99.28 |
| 18 | MN515127 | *C.meleagridis* | MN410718.1 | 99.53 |
| 19 | MN515128 | *C. parvum* | KM870602.1 | 99.76 |
| 20 | MN515129 | *C.baileyi* | MN410723.1 | 99.52 |


**Figure (4-9): Multiple sequence alignment analysis of small subunit ribosomal RNA gene in local *Cryptosporidium* spp. Ostriche isolates and NCBI-Genbank *Cryptosporidium spp*. isolates. The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA 6.0 version). That showed the nucleotide alignment similarity as (*) and substitution mutations in small subunit ribosomal RNA gene.**

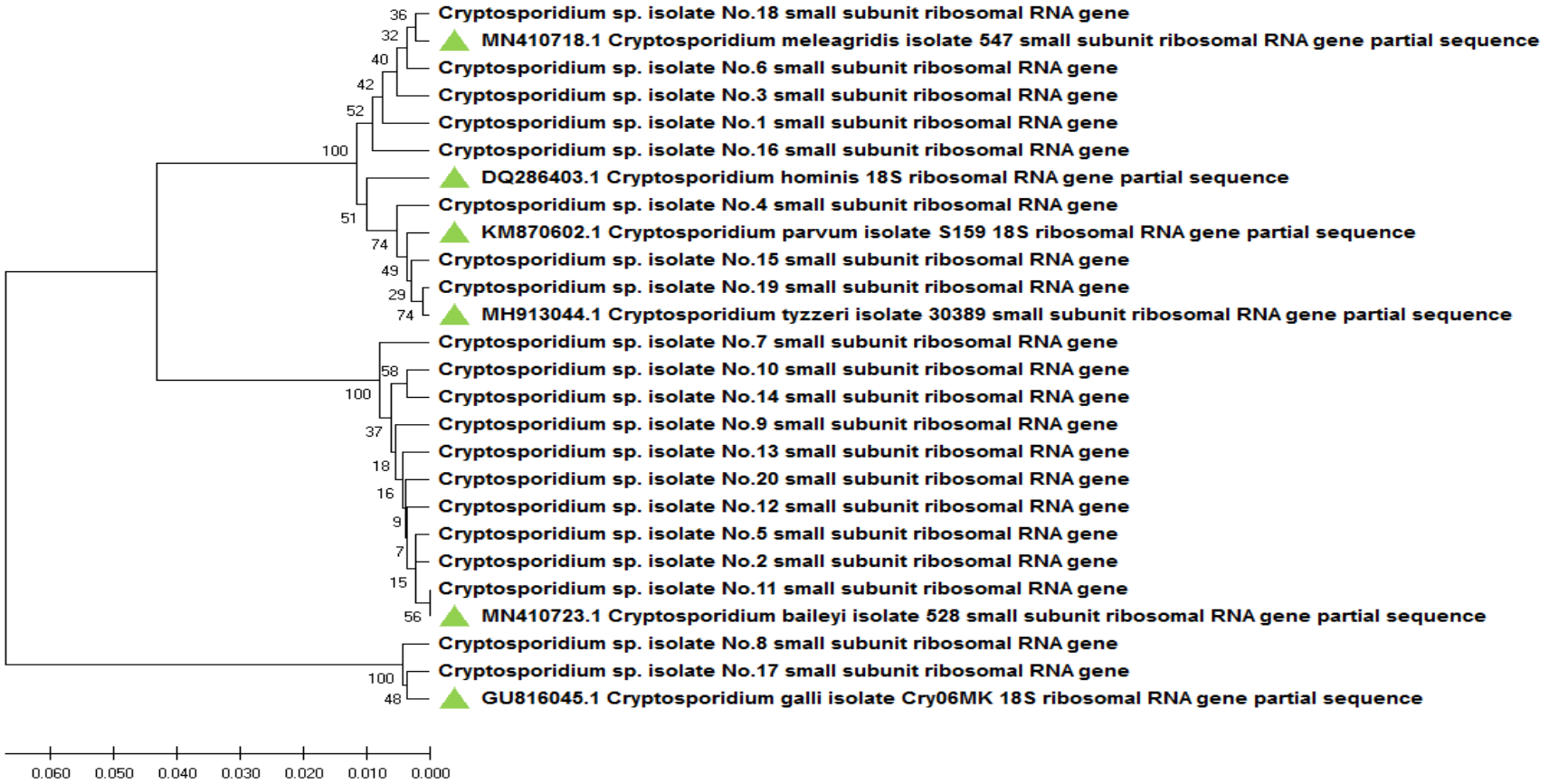



**Figure (4-10): Phylogenetic tree analysis based on small subunit ribosomal RNA gene partial sequence in local *Cryptosporidium spp*. Ostriches isolates that used for genetic *Cryptosporidium* species identification . The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA .X version). The local *Cryptosporidium* isolate IQ.Ostriche (No.1 No.3 No.6 No.16 and No.18) were showed closed related to NCBI-BLAST *Cryptosporidium meleagridis* (MN410718.1). The local *Cryptosporidium* isolate IQ. Ostriches (No.2 No.5 No.7 No.9-No.14 and No.20) were showed closed related to NCBI-BLAST *Cryptosporidium baileyi* (MN410723.1). The local *Cryptosporidium* isolate IQ.Ostriche (No.4, No.15 and No.19) were showed closed related to NCBI-BLAST *Cryptosporidium parvum* (KM870602.1). The local *Cryptosporidium* isolate IQ.Ostriche (No.8 and No.17) were showed closed related to NCBI-BLAST *Cryptosporidium galli* (GU816045.1) at total genetic changes (0.010-0.060%).**

## 5. Discussion:

**First part**

### 5.1. Conventional microscopic examination of Ostriches fecal samples

The total prevalence of *Cryptosporidium* spp. infection in central and south parts of Iraq using modified Ziehl-Neewlsen (mZN) and flotation methods 11%.This result was agreement with the result of Wang *etal*.(2011) in China whom recorded *Cryptosporidium* in Ostriches by conventional methods was 11.7% , also Qi *et al*.(2014) in China whom recorded infection rate 10.2%. Huang *et al.*(2018) recorded rate 10.2% in China

These differences in prevalence rate of *Cryptosporidium* spp. infection in Ostriches that recorded by several workers were due to difference in areas of study and samples collection. While own result was disagree with the high rate 28% that recorded in south Iran by Behzadi *et al* .( 2009) , and which showed that the prevalence of *Cryptosporidium* oocysts in south of Iran was 28% , and Nguyen *et al*.(2013) whom reported 23.7 % in central of Vietnam .this differences were due to stressing conditions leading to immunosuppression or poor husbandry practices related to feed, water or hygiene while the lowest prevalence rate 2.48% recorded by Sun *et al* .(2007) in ZhengZhou Ostriches at China, Zhu *et al* ( 2008) whom recorded 1.7% in Brazil, Nakamura *et al*.(2009) in Brazil recorded prevalence rate 4.86%.these attributed to differences of samples number at limited areas, methods of rearing and methods of diagnosis.no significant differences was found between sex of Ostriches that infected with *Cryptosporidium* , which was accordance with previous study by (Bamaiyi *et al* ., 2013 ) in Nigeria.

### 5.1.1. Prevalence rate of *Cryptosporidium* spp. infection in relation to age groups

The higher rate of infection 12.26% was recorded in Young (chicks) (< 9 Months),while the lower infection rate 9.57%was recorded in the adult(> 9 Months).without significant difference. These results were accordance with those found previous studies whom showed that the infection was more prevalent at age group( 20-40 days) more than adult Ostriches(Zhu *et al*., 2008), also Qi *et al*.(2014) in China recorded 10.2% rate in Ostriches aged 16-60 days and 1.2% in those aged > 10 years , Wang *et al*.(2011) in China reported that the young chicks(4-8 weeks ) showed high percentage of infection 16.2% than adult more than 12 months with rate 7.2 %. Huang *et al*.(2018) showed that Ostriches with age 16-60 days recorded 27.6% , while with > 10 years old recorded 20.4% rate of infection. Nguyen *et al*.(2013) in Vietnam found the majority of infection rate in 2- 3 months Ostriches chicks, while the lower rate was recorded in one year age adult Ostriches.

### 5.1.2. Prevalence of *Cryptosporidium spp*. in relation to areas of study

Microscopic examination of Ostriches fecal samples from seven provinces in central and south parts of Iraq , including: Wasit , Baghdad , Babylon , Diyala , Karbala , Al-Najaf and Al-Qadisyiah recorded 0% , 4.17% , 15.79% , 3.70% , 23.53%. Al-Najaf showed highest prevalence rate (23.53%) due to poor husbandry practices, contaminated of water sources, drinking water and Breeding other domestic birds near the Ostriches farms.

### 5.1.3. Prevalence of *Cryptosporidium spp*. in relation to months of study

The result showed that the highest prevalence 50% was recorded in March while. the lowest prevalence rate 0%. recorded during August the result agreement with the Wang *et al*.(2011) in China whom reported highest prevalence rate 15.6% in spring season was. Goodwin (1989) in Georgia and Muchiri *et al*(2009) recorded high prevalence rate 9% in spring and lowest rate in winter 3.5%, the peak of prevalence occurred in March and April( 19%) for each months for two year of study and the apparent seasonal period peak of detection at the end of the rainy season.

### 5.1.4. Morphology and measurement of *Cryptosporidium* spp. oocysts

Morphologically *Cryptosporidium parvum* oocysts appeared by flotation with salt and sheathers solution spherical or rounded with a thin membrane , with four sporozoit look as black bodies inside the oocysts, Oocysts with Modified Ziehl-Neelsen stain appeared spherical stained dark red with clear halo around the oocysts , against a blue background of the methylene blue stain. The average length and width measurements of *C.parvum* oocysts had almost ( 4.3× 4.9 ± 0.9) µm. These results were comparable to the results of Khalil (2010) who recorded that the average length and width measurement of *Cryptosporidium parvum* were (5.2 x 4.2 ± 0.3) µm, Also Al-alousi and Mahmood (2012) whom recorded that the diameter of *Cryptosporidium* oocyst was 5.2 x 4.4 µm and, Thigeel (2016) who recorded the measurements of *C. parvum* had a mean size of 5.01 x 4.56 µm Al-Zubaidi (2012) in Iraq, who recorded the measurements of *C. parvum* oocysts were 5.2± 0.41 µm in length and 4.1± 0.21 µm in width.

*C. baileyi* oocysts have ovoid shape and more larger with average size 6.0×4.6 µm or 6.3×5.2 µm. These results were compatible with the results of Current *et al.*( 1986) whom recorded the oocyst measured 6.2 × 4.6 \ 5.6 – 6.3 × 4.5 – 4.8 µm and *Hajdusek et al* ( 2004) in Czech Republic recorded average size of *baileyi* oocysts 5.2 ± 0.2 × 4.9 ± 0.2 µm, Gajadhar ( 1993) in Canada reported oocysts measurment 4.0-6.1 x 3.3-5.0 µm , Egyed et al.(2003) who recorded that the measurements of *C. baileyi* were 5.6–7.5 ×4.0–5.7 µm shape index 1.35 µm. in Brazil Meireles and Figueiredo (1992) whom recognized *C. baileyi* oocysts have an ovoid shape and measure 6.0×4.6 µm; shape index 1.31.Santos *et al* (2005) reported measurement of oocysts 6.0×4.8 µm with shape index 1.31 similar to C. *baileyi*.

*C. meleagridis* are spherical with average measurement 5.2×4.6 µm, Our results were in agreement with Slavin (1955) who recorded 5.2 × 4.6 \ 4.5 – 6 × 4.2 – 5.3 µm , It is not possible to differentiate between C. *baileyi* and C.*melegridis* but *C. melegridis* are spherical or sub spherical and measured 5.2×4.6µm with shape index 1.31 ( Lindsay et al.,1989). Egyed *et al*.(2003) recorded *C. meleagridis* measurment 4.5–6.0× 4.2–5.3 with shape index 1.14.Sreter and Vagra (2000)**,** Upton and Current (1985) recorded measured 5.0 (4.5–6.0)× 4.4 (4.2–5.3) µm with shape index 1.1 while *C. galli* oocysts are larger with average measurement 8.3 × 6.3 µm. These results were comparable to the results of Santos *et al* (2005) measured 8.5×6.3µm have shape index ( length/ width) 1.30 , Pavlasek( 1999) and Ryan etal.(2003) whom recorded 8.3 × 6.3 \ 8 – 8.5 × 6.2 – 6.4 µm.

**Second part**

## 5.2. Molecular detection:

Polymerase chain reaction is high sensitivity because of the nested protocol and multi-copy nature of one gene. PCR procedures will amplified of defined DNA sequences has been gain considerable promise for the development of highly sensitive and specific diagnostic tests. Although the benefits of PCR technique are its ability to batching, ease of interpretation and the potential to further differentiate to the species and genotype/sub genotype level (Geurden *et al.*, 2006). molecular techniques is used for confirmation *Cryptosporidium* spp detected, is also important since they cannot be identified by morphology (Robinson *et al.,* 2006). The molecular detection methods are also included in the present study for diagnosis and identification *Cryptosporidium* spp .infection.

### 5.2.1. Nested PCR (nPCR)

These the first molecular study by Nested PCR of *Cryptosporidium* spp infected Ostriches in Iraq .There are many species of *Cryptosporidium* were detected by nested PCR protocol targeting the 18S rRNA gene which has been shown highly sensitive and having successfully amplified the DNA from just one oocyst (Xiao *et al*.,1999 ;Santin *et al.*, 2008; Karanis *et al*., 2010; Thigeel, 2016). The nPCR more sensitive as a compared to modified Ziehl-Neelsen (mZN) staining technique (Uppal *et al* 2014). Therefore the 18S rRNA gene fragment was targeted in the current study to detected *Cryptosporidium* spp.

### 5.2.2. Prevalence of *Cryptosporidium* spp.in Ostriches by Nested PCR

Statistical analysis showed significant differences between conventional and molecular techniques and its relation with sensitivity and specificity of each diagnostic test, Nested PCR revealed total infection rate with *Cryptosporidium* spp.in Ostriches was 26.5%.This finding was comparable with the results of Nguyen etal.(2013) whom recorded infection rate 23.7 % , Silva *et al*( 2010) reported 24.5% from adult birds in Brazil . However, Oliveira *et al* (2008) recorded 44.4% prevalence rate *Cryptosporidium spp* in examined Ostriches. On the other hand, the infection rate was lower in study of Wang *et al* ( 2011) in China which recorded overall prevalence 11.7%, Ng *et al.* (2006) in Australia recorded infection rate in (6.3%). Nakamura *et al*.( 2009) reported infection rate 4.86% in captive birds in Brazil.

These differences were attributed to husbandry practices, stressing condition ,poor type of feed , water or hygiene management, area of sampling (farming / captive) Ostriches , environmental conditions, the sampling method and samples size(.(Caccio and Putignani, 2014).

### 5.2.3. Prevalence of *Cryptosporidium* spp. in Ostriches by Nested PCR in relation to age groups

The prevalence showed a significant differences among age groups The higher rate recorded in Young's Ostriches < 9 Months 30.19% while the lower infection rate 22.34% recorded in the adult > 9 Months age group. These results were agreement with those found in previous studies which showed that young Ostriches chicks more susceptible to *Cryptosporidium* infection than adult from the first day of life (.Zhu *et al*., 2008;Sreter *et al*.,2000).

Qi *et al*.(2014) whom recorded infection10.2% in aged 16-60 days chicks, 1.2% in those aged > 10 years adult .Wang *etal*.(2011) recorded in young chicks(4-8 weeks ) show high percentage 16.2% than adult Ostriches more than 12 months with rate 7.2 %. Nguyen *et al*.(2013) recorded prevalence of Ostriches *Cryptosporidium* in Vietnam as <45 days-90 days 23.5 % while in >12 months was 5.8% .

There was age-related distribution of *Cryptosporidium spp* among the Ostriches , *C. baileyi* and *C. parvum* was dominant species in young Ostriches 40% (8/20),15% ( 3/20) respectively, while *C. meleagridis* was dominant species in adult ostriches > 9 Months age 15% (3/20) although *C. galli* was occurred in adult less than 2 years 10% (2/20) .

These results were accordance with(Meireles and Figueiredo 1992; Pavlasek, 1993 : Sreter *et al*.,1995 ;Sreter and Varga, 2000; Morgan et al., 2001; ) whom recorded that *Cryptosporidium baileyi* is a dominant parasite of various species of young birds, including chickens, turkeys, ducks, cockatiels, a brown quail, gulls and Ostrich. Quah *et al*( 2011) reported *C.parvum* infection in adult birds 10% Although C. *parvum* has not been reported to cause infection in birds,. Laatamna *et al* (2017) explained that *C.meleagridi*s more prevalence in small aged Farmed turkeys than ostriches .Santos *etal.(*2005) showed that C. *galli* have light infection rate in adult birds and less pathogenic than other *Cryptosporidium* spp in bird . Jasim and Marhoon(2015) identified *Cryptosporidium parvum* in all birds age , *Cryptosporidium baileyi* more common in domestic young chickens while *C. galli* only recorded from domestic adult chickens.( Ryan et al.,2003).

In relation to sex nested PCR results showed that Ostriches male were found infected with *Cryptosporidium* at rate 20.83 %while Ostriches female recorded rate

22.86% without significant differences. which was accordance with many previous studies (Mohammed,2010; Bamaiyi *et al* ., 2013 ) .

### 5.2.4. Prevalence of *Cryptosporidium* infection by nested PCR in relation relation to areas

The prevalence in seven provinces at central and south parts of Iraq including : Wasit , Baghdad , Babylon , Diyala , Karbala , Al-Najaf and Al-Qadisyiah revealed prevalence 30% 16.67%, 31.58% ,11.11% , 30.77% , 38.24% and 34.62% respectively. Al-Najaf province showed the highest prevalence rate(38.24%) while Diyala province showed the lowest rate (11.11%). with significant differences among provinces. The highest results in Al-Najaf was due to many reasons including poor management systems, density of breeding farms, contaminated of water sources as compared to Diyala province**,** Behzadi *et al* .(2009) were recorded Ostrich farms infection at rate 28% in Shiraz province southern Iran. Sun *etal* .(2007) in ZhengZhou province recorded 2.48% . In Henan Province, Zhu *et al* ( 2008) whom showed the infection rate with *Cryptosporidium* spp 1.7%.anther study in Henan province Wang *etal*.(2011)recorded infection rate 11.7 %. in Khanh Hoa province in central Vietnam, Nguyen *etal*.(2013) recorded prevalence of *Cryptosporidium* was 23.7 % . Nakamura *et al*., (2009) showed total infection rate of three provinces (Brazilian states of Goias , Parana and Sao Paulo) was recorded 14.6%. Oliveira et al (2008) whom reported prevalence rate 44.4% in Rio de Janeiro province. Brazil.

The differences could be attributed to the variation between regions , breeding conditions , climatic nature, distribution of Ostriches farms, poor husbandry practices, environmental conditions , Age of birds ,sampling method and size and stress condition.( Godro *et al.*, 2002 ) .

### 5.2.5. Prevalence of *Cryptosporidium* infection by nested PCR in relation to months of study

Nested PCR revealed significant difference between prevalence of *Cryptosporidium* infection in relation to months of study. April recorded highest rate of infection72.73% while the lowest rate 7.14% recorded in July. the result agreement with the Wang *et al*.(2011) which clarify that the extremely shedding of oocysts are the most responsible for high prevalence of *Cryptosporidium* spp. particularly during spring season, and they recorded highest prevalence rate in spring season was 15.6% Goodwin (1989) recorded high prevalence rate 9% in spring with seasonal period peak of detection at the rainy season and lowest rate in winter 3.5%, Muchiri *et al*(2009) showed the peak of prevalence occurred in March and April( 19 % and 19 %) respectively of each two year of study.the differences of low infection in summer it may be the all examined Ostriches were adult and have high resistant to infection as compare with young chicks .

### 5.2.6. Sequence analysis of *Cryptosporidium* spp. in Ostrich

DNA sequencing analysis has the most commonly used approach for typing *Cryptosporidium* spp. isolates from different geographical areas recently (Xiao *et al.*, 1999). DNA sequencing based on small subunit ribosomal RNA gene which followed in the present study was identical to many previous studies(Nakamura *et al*., 2009 ;Wang *et al,*. 2011; Jasim and Marhoon, 2015).

Results of DNA sequencing revealed the presence of four *Cryptosporidium* species in Ostriches at central and south provinces of Iraq, *C.parvum, C. baileyi, C. meleagridis* and *C. galli*. Our results were in agreement with results of Xiao and Ryan,(2004) in Korea , Behzadi et al.,2009) in Iran whom recorded the species infecting birds are *C. baileyi, C. galli* and *C. meleagridis*. Current *et al.*(1986 ) recorded the validity of

*Cryptosporidium meleagridis* and *C. baileyi* as distinct species in Ostriches, Santos et al., 2005 in Brazil reported the species infected bird are *C. baileyi*, *C. meleagridis* and *C. galli*. (Ng et al., 2006; Xiao and Fayer, 2008; Qi et al., 2011) in China whom showed that *C. baileyi*, *C. meleagridis*, and *C. galli* are the commonest *Cryptosporidium* species and have been identified in many avian hosts. In brazilain Ostrich, Meireles et al.( 2006) detected *C. baileyi* ,*C. parvum*, *C. meleagridis* . In China Wang et al (2011) reported *C. baileyi* only in five farms, zoo, and an animal rescue center in Zhengzhou, Henan Province.

The result of phylogenic tree analysis indicated a high genetic variation among 20 local *Cryptosporidium* spp. isolates and other NCBI BLAST *Cryptosporidium spp*. sequences of Ostriches obtain from GenBank with (0.010-0.060%) as total genetic changes and recorded that dominant Ostriches species, *C. baileyi* 45% ( 9/20) was detected in different age groups of Ostriches , following *C. meleagridis* 25% (5/20) , *C.parvum* 20% ( 4/20) and finally *C. galli* 10% (2/20). The results were accordance with( Meireles and Figueiredo 1992; Pavlasek, 1993; Sreter *et al*.,1995; Sreter and Varga, 2000; Morgan et al., 2001 ) whom recorded that *Cryptosporidium baileyi* is a dominant parasite of various species of young birds. Wang et al (2011) recorded that the most common *Cryptosporidium* of Ostriches in China is *C. baileyi*. Zhu et al (2008) recorded *C.baileyi* in Ostrich from many areas in Henan Province, China .

## 6.1.Conclusions:

According to the results obtained from the study, following conclusions recorded:

1. The overall infection rate of *Cryptosporidium* spp. in Ostrich was 11% (22/200) with significant differences recorded between different provinces and months of study.

2.Molecular prevalence recorded 26.5% infection rate for 200 Ostriches samples analyzed by nPCR .

3. Molecular analysis showed a significant differences between age groups the highest rate recorded in Young's Ostriches < 9 Months, while sex recorded percentage without significant differences among male and female Ostriches. Al-Najaf province showed the highest *Cryptosporidium* prevalence rate than other provinces in central and south parts of Iraq .

4. *Cryptosporidium baileyi* and *C. parvum* were dominant species in young Ostriches while *C. meleagridis* and *C. galli* are dominant species in adult .

5. First time in Iraq, phylogenic analysis revealed the presence of four *Cryptosporidium* species in Ostriches : *C.parvum*, *C. baileyi*, *C. meleagridis* and *C. galli*.

## 6.2. Recommendation :

1. Identification of *Cryptosporidium* infection in north parts of Iraq

2. Study prevalence of other intestinal protozoa infected Ostriches .

3. Identification , genotyping of *Eimeria* spp in farming Ostriches in Iraq

4. Experimental study with *Cryptosporidium parvum* isolated from human sources in infected Ostriches chicks.

5. Study the relation between Cryptosporidial infection and other intestinal causative agent in Ostriches.

## References


**Abbas G; Zahid O.; Khan M. S. A.; Sajid M.; Asif M. and Saeed H. (2018**) Future of Ostrich Farming in Pakistan.Adv. in Zool. and Bot. 6(2): 55-65.

**Abeywardena H.; Jex A. R.;Koehler A. V.; Rajapakse R.P.V.; Udayawarna K.; Haydon S. R.; Stevens M. A.and Gasser R. B (2014)** First molecular characterization of *Cryptosporidium* and *Giardia* from bovines(Bos taurus and Bubalus bubalis) in Srilanka:unexpected absence of C. parvum from pre-weaned calves. J. of Parasites and Vectors 7(75):1-10.

**Adeyemo F.E.; Singh G.; Reddy P. and Stenström T.A. (2018).** Methods for the detection of *Cryptosporidium* and *Giardia* From microscopy to DNA based tools in clinical and environmental regimes. Acta. tropica.18:415-28.

**Aghamolaie S; Rostami A., Fallahi S; Biderouni F.T.; Haghighi A. andSalehi, N. (2016).** Evaluation of modified Ziehl – Neelsen, direct fluorescent- antibody and PCR assay for detection of *Cryptosporidium* spp. in children faecal specimens. J. Parasit. Dis. 40, 958–963.

**Agarwal A. (2016 )** Database resources of the National Center forBiotechnology Information. Nucleic Acids Res.44 :7– 19.

**Ahmed SA. and Karanis P. (2018).** Comparison of current methods used to detect *Cryptosporidium* oocysts in stools. Inter.J.of hyg.and envir. Heath.221(5):743-763**.**

**Akram M. Z.; Zaman M. A.; Jalal H.; Yousaf S.; Khan A. Y. , Farooq M. Z. , Rehaman T.; Sakandar A.; Qamar M. F. and Bowman D.D.(2018)** Prevalence of Gastrointestinal Parasites of Captive Birds in Punjab, Pakistan. Pak. Vet. J.123:1-3.

**Al-alousi T.I. and Mahmood O.I. (2012).** Detection of *Cryptosporidium* oocysts in calves and children in Mosul, Iraq. Proceeding of the Eleventh Veterinary Scientific Conference, 280 -285.

**Al - Bayati, Harith Mouaed. (2002).** Study of Cryptosporidiosis spp. In chicken fields and brioler chickens abbitiors and its relationship with workers. Msc.Thesis.College of Veterinary Medicine. Baghdad University.

**Al-Attar A. and Abdul aziz T. A. (1985).** Cryptosporidiosis of the bursa of Fabricius in broiler chickens. Iraq. J. Vet. Med. 9:49-53.

**Aldeyarbi, H.M.and Karanis (2015)** Electron microscopic observation of the early stages of *Cryptosporidium parvum* asexual multiplication and development in in vitro axenic culture Euro. J. of Protistol.15:1-26.

**Al-gailani, Ban Abdu-wahab ( 1998).** Epidemiological study of Cryptosporidiosis Baghadad .M.sc. thesis.collage of veterinary medicen ,university of Baghadad. P. 104.

**Al-Mahmood S. S.(2011)** Experimental histopathological study of chicks infected with *Cryptosporidium baileyi* isolated from wild pigeons in Mosul. Iraqi J.of Vet. Sci.25(1):43-49.

**Al-zubaidi, M. Th. S. (2012).** Prevalence of some *Cryptosporidium* species in cattle in Baghdad, Iraq. Al-quadisiya. J. Vet. Med. Sci. 11(2): 177–182.

**AL-Zubaidi M. Th. S.; Kadhim L. I.; Ibrahim Z. I.; Al-Rikabi A. Sh.(2018)** incidence and experimental infection of *Cryptosporidium baileyi* in chicken. Iraqi J.of Agri.Sci.49(2):269- 278.

**Anderson, B. C. (1981).** Patterns of shedding of Cryptosporidial oocysts in Idaho calves. J. Am. Vet. Med. Asso. 178: 982-984 .

**Arbabi M. and Hooshyar H. (2009).** Gastrointestinal parasites of stray cats in Kashan, Iran.Trop. Biomed.26(1):16-22.

**Arrowood M. J. (2002)** In Vitro Cultivation of *Cryptosporidium* Species. Clinical microbial. Rev..15(3):390–400.

**Arsenopoulos K.; Theodoridis A. and Papadopoulos E. (2017).** Effect of colostrum quantity and quality on neonatal calf diarrhoea due to Cryptosporidium spp. infection. Comparative immunology, micro.and infec. Dis. 53:50-55

**Baldursson S.and Karanis P.(2011)** Waterborne transmission of protozoan parasites: Review of worldwide outbreaks . Water Res. 45:6603-6614.

**Bamaiyi P.H. ; Umoh J.U.; Abdu P. A. and Lawal I. A. (2013)** The Prevalence of Cryptosporidium Oocysts in Birds in Zaria, Nigeria.. Borneo J. Resour.Sci.Tech. 2(2): 52.

**Baroudi D.; Khelef D.; Goucema R.; Adjoub K. T.; Adamuc H.; Zhangc H. and Xiao L. (2013)** Common occurrence of zoonotic pathogen *Cryptosporidium meleagridis* in broiler chickens and turkeys in Algeria.Vet. Parasitol.10:1-6.

**Baxby D.; Blundell N. and Hart C. A. (1984)** The development and performance of a simple, sensitive method for the detection of *Cryptosporidium* oocysts in faeces. J. llyg. Camb.92: 317-323.

**Beaver P.C. and Jung R.C. (1985).** Animal Agents and Vectors of Human Disease 5th ed. Lea and Febiger , Philadelphia , p 249.

**Behzadi M. A.; Razavi S. M; Yazdanpoor H., Mirzaei A.; Tamadon A. and Gandomani M. J.( 2009)** Epidemiology of *Cryptosporidium* infection in Ostriches (*Struthio camelus*) in Iran. Bulg. J. of Vet. Med.12( 1): 55−61.

**Bermudez A.J.; Ley D. H.;Levy M.G., Ficken M. D.; Guy J. S., and Gerig T. M.(1998**) Intestinal and Bursal Cryptosporidiosis in Turkeys Following Inoculation with Cryptosporidium sp. Isolated from Commercial Poults. Avi. Dis.32:445-450.

**Bezuidenhout A.J,;Penrith M.L.;Burger W.P.(1993)** Prolapse of the phallus and sewer in the Ostrich ( Strthuio camelus) .J.of south Afr.Vet.Asso.64:156-168.

**Borowski H.; Thompson ; Armstrong T. and Clode P. L.(2010)** Morphological characterization of *Cryptosporidium parvum* life-cycle stages in an in vitro model system . Parasitol.J. 137:13–26.

**Boughton D. C.(1937)** Notes on Avian Coccidiosis. Auk.Oct.J.45:500-509.

**Brondson Z. ( 1984)** rapid dimethyl sulfoxide - modified acid fast stain of *Cryptosporidium* oocysts in stool specimens .J.Clin.Microbiol.19:952-953 **.**

**Bukhari Z. and Smith H. V.(1995)** Effect of Three Concentration Techniques on Viability of Cryptosporidium *parvum* Oocysts Recovered from Bovine Feces J. of Clin.Micro.33(10): 2592–2595.

**Caccio S. M. and Putignani L.( 2014 )** Epidemiology of Human Cryptosporidiosis S.M. Caccio` and G. Widmer (eds.), *Cryptosporidium*: parasite and disease DOI 10.1007/978-3-7091-1562-6-2.

**Caccio S.M.; Thompson R.C.; McLauchlin J. and Smith H.V. (2005).** Unravelling *Cryptosporidium* and *Giardia* epidemiology. Trends Parasitol 21, 430-437.

**Cai M.; Guo Y.; Pan B.; Li N.; Wang X.; Tang C.; Feng Y. and Xiao L. (2017).** Longitudinal monitoring of *Cryptosporidium* species in pre-weaned dairy calves on five farms in Shanghai, China. Vet. Parasitol. 241, 14–19.

**Casemore D. P.(1990)**Epidemiological aspects of human cryptosporidiosis. Epidemiol. Infect.104:1-28.

**Chappell C. L.; Okhuysen P. C.; Sterling C. R.and DuPont H. L.( 1996)** *Cryptosporidium parvum:* Intensity of Infection and Oocyst ExcretiPattern in Healthy Volunteers. J. of Infec.Dis. 173:232-236.

**Checkley W.; White A.C.; Jaganath D.; Arrowood M.J.; Chalmers R.M.; Chen XM. and Fayer R. *et al.* (2015).** A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for *Cryptosporidium*. Lancet.Infect. Dis. 15, 85–94.

**Chermette R. and Boufassa Q.S. (1988**). Cryptosporidiosis a Cosmopital Disease in Animals and Man , 2nd ed. Office International Epizooties . France.

**Clayton F.; Heller T. and Kotler D.(1993)** Variation in the Enteric Distribution of Cryptosporidia in Acquired Immunodeficiency Syndrome Anat. Patho.J. 102(4):420-425.

**Coles E.H. (1986).** Veterinary clinical pathology (4th ed.) WB Saunders company Philadelphia. P. 374–453.

**Conn D. B.; Weaver J.; Tamang L.and Graczyk T. K.(2007**) Synanthropic Fliesas Vectors of *Cryptosporidium* and *Giardia* among Livestock and Wildlife in aMultispecies Agricultural Complex. Vector-Borne and Zoonotic Diseases. 7(4):643-651.

**Cooper R. G.; Mahrose Kh. M. A.; Horbańczuk J.O.; Villegas-Vizcaíno R.; Kennou Sebei S. and Mohammed A. E.F.(2009)** The wild Ostrich (*Struthio camelus)*: A review. Trop. Anim.Health .Prod. 41:1669–1678.

**Crawford F. G.; Vermund S. H. ( 1988)** Human Cryptosporidiosis . Crit. Rev. in Microbiol.16(2):113-158.

**Cui Z.; Song D.; Qi M.; Zhang S.; Wang R.; Jian F.; Ning C.and Zhang L.(2018)** Revisiting the infectivity and pathogenicityof *Cryptosporidium avium* provides new information on parasitic sites within the host. Parasi.andVect.11(514 ):2-7.

**Current W. L.; Upton S. J., and Haynjw T. B. (1986)** The Life Cycle of *Cryptosporidium baileyi* (Apicomplexa, Cryptosporidiidae) Infecting Chickens. *J. Protozool*.33(2):289-296.

**Current, W. L. (1997**). Cryptosporidiosis. In: Diseases of Poultry, Tenth Edition, Calnek, B. W.; Barnes, H. J.; Beard, C. W.; McDougald, L. R. and Saif, Y. M. eds. Mosby-Woolfe, Mosby International, Lynton House,7-12Tavistock Square, London WC1H 9LB, UK. 883-890 .

**Current, W. L. and Garcia, L. S. (1991**). Cryptosporidiosis. Clin.Microbiol. Rev. 4(3): 325-358 .

**DaCunha M. J. R.; CurybM. C.and Santína M. (2018)** Molecular characterization of *Cryptosporidium* spp. in poultry from Brazil. Vet. Sci. 118: 331–335 .

**De A. (2013).** Current laboratory diagnosis of opportunistic enteric parasites in human immunodeficiency virus-infected patients.Trop. Parasitol. 3(1):7-16.

**De Graaf D.C.; Vanopdenboscha E.;. Ortega-Mora L. M; Abbassi H.; Peeters J. E.(1999)** A review of the importance of Cryptosporidiosis in farm Animals. Inter. J. for Parasitol.29:1269-1287.

**Dillingham R. A.;Lima A. A. and Guerrant R. L.(2002)** Cryptosporidiosis: epidemiology and impact. Microbes and Infection 4:1059–1066.

**Ditrich O.;Kopacek P.and Kucerova Z.(1993)** Antigenic characterization of human isolates of Cryptosporidia .Folia.Parasitologyica.40:301-305.

**Dryden M. W.; Payne P. A.; Ridley R. and Smith V. (2015)** Comparison of Common Fecal FlotationTechniques for the Recovery of Parasite Eggs and Oocysts . Vet. Therap. 6(1):15-28.

**Dobos-Kovács M.; Varga I.; Békési L.; Drén C. N.;Németh I. and Farkas T. (1994)** Concurrent cryptosporidiosis and chicken anaemia virus infection in broiler chickens. Avi. Patho. 23:365-368.

**Dupont H. M.D.; Cynthia L.; Chappell PH.D.; Charler. S.; Pabloc PH.D.; Okhuysen M.D.; Rose J. PH.D and Jakubowski W.(1995**) The Infectivity Of *Cyptosporidium Parvum* In Healthy Volunteers. New Engl.J. of med.332 (13):855-859.

**Duszynski D. W. (1969)** Two New Coccidia (Protozoa: Eimeriidae) from Costa Scan Lizards with a Review of the *Eimria* from Lizards. J. *Protozool.* 16(3): 581-589.

**Dwivedi S.; Purohit P.; Misra R.; Pareek P.; Goel A.; Khattri S.; Pant K.K.; Misra S. and Sharma P. (2017).** Diseases and molecular diagnostics: A step closer to precision medicine. Indian J. Clin. Biochem. 32, 374–398.

**Egyed Z.; Sreter T.; Szell Z and Varga I.(2003)** Characterization of *Cryptosporidium* spp.—recent developments and future needs.Vet. Parasitol.J. 111:103–114.

**El-Shahawy, I.S.and Abou Elenien F (2015)** Enteric parasites of Egyptian captive birds: A general coprological survey with new records of the species Tropical Biomedicine 32(4): 650–658 .

**El-safty .S.A.El.( 2015)** Ostrich's eggs: The productivity, quality and hatchability. World's Poultry Science Journal 48: 1-17.

**Elwin K.; Hadfield S.J.; Robinson G.; Crouch N.D. and Chalmers R.M. (2012).** *Cryptosporidium viatorum* n. sp (Apicomplexa: Cryptosporidiidae) among travellers returning to Great Britain from the Indian subcontinent, 2007-2011. International Journal for Parasitology 42, 675-682.

**Eslami A.;Rahmat H.;Meshgi B.and Ranjbar-Bahador S.(2007)** Gastrointestinal parasites of ostrich(*Struthio camelus domesticus*) raised in Iran. Iranian J. of Vet. Res.8(1):80-82.

**Faraj A. A.(2014)** Distribution of *cryptosporidium spp* infection in wild pigeons in Baghdad city –Iraq. Bas.J.Vet.Res. 1(2 ):48-53.

**Fayer R.(2004)** *Cryptosporidium*: a water-borne zoonotic parasite.Vet. Parasitol. 126: 37–56.

**Fayer R. and Ungar B. L. P.(1986)***Cryptosporidium* spp. and Cryptosporidiosis. Micro. Rev. 50(4):458-483.

**Fayer R.(2004)** *Cryptosporidium*: a water-borne zoonotic parasite.Vet. Parasitol. 126:37–56.

**Fayer R.; Santín M. and Macarisin D.(2010).***Cryptosporidium* sp. in animals and humans. Vet. Parasitol. 172(1-2): 23-32.

**Fayer R.and Ellis W.(1993)** Paromomycin is effective as prophylaxis for Cryptosporidiosis in dairy calves.J.Parasitol.79(5):771-774.

**Fayer R.and Xiao L. (2008)** *Cryptosporidium* and cryptosporidiosis spp. 2nd .edn .CRC press, Boca Raton , UK.pp.1-560.

**Feng Y.; Alderisio K.A.; Yang W.; Blancero L.A.; Kuhne W.G.; Nadareski C.A.; Reid M. and Xiao L. (2007).** *Cryptosporidium* genotypes in wildlife from a new york watershed. Appl. Environ. Microbiol. 73:6475-6483.

**Feng Y.and Xiao L.(2017)** Molecular epidemiology of Cryptosporidiosis in China. Fron. in Micro. J.8:1-11.

**Fleck S.L.and Moody A.H. (1988)** Diagnosis Techniques in Medical Parasitology .Butterworth- Heinemann Ltd ., London, UK.

**Galloa S. F. ;Teixeiraa C. R.; Ederlib C. S.;and Oliveira N. B ( 2018)** Gastrointestinal parasites of a population of emus (*Dromaius novaehollandiae)* in Brazil. Brazilian J. of B.https://doi.org/10.1590/1519-6984.

**Gajadhar A. A.(1993)***Cryptosporidium* species in imported Ostriches and consideration of possible implications forbirds inCanada.Can.Vet.J.34:115-116.

**Geurden T.; Goma F.Y.; Siwila J.; Phiri I.G.; Mwanza A.M.; Gabriel S.; Claerebout E. and Vercruysse J. (2006).** Prevalence and genotyping of *Cryptosporidium* in three cattle husbandry systems in Zambia. Vet Parasitol 138(3-4), 217-222.

**Ghaffari S. and Kalantari N. (2014).** Recognition of *Cryptosporidium* oocysts in fresh and old stool samples: comparison of four techniques. Asian Pacific J.of Trop.Biomed.4:570-S574.

**Gharagozlou M. J.; Dezfoulian O.; Rahbari S.; Bokaie S.; Jahanzad I. and Razavi A. N. E. (2006)** Intestinal Cryptosporidiosis in Turkeys in Iran.J.of Vet.Med.53:282-285.

**Ghoshal U.; Jain V.; Dey A. and Ranjan P. (2018).** Evaluation of enzyme linked immunosorbent assay for stool antigen detection for the diagnosis of Cryptosporidiosis among HIV negative immunocompromised patients in atertiary care hospital of northern India. J. Infect. Public Health 11, 115–119.

**Girones R.; Ferrus M.A.; Alonsoc J.L.; Rodriguez-Manzano J.; Calgua B.; de Abreu Correa A.; Hundesa. A.; Carratalaa A. and Bofill-Mas S. (2010).** Molecular detection of pathogens in water-The pros and cons of molecular techniques. Water Res. 44(15): 4325-4339.

**Goodwin M. A.; Latimer K. S. ; . Brown J; W. L., Steffens P. W.; Martin P.W.; Resurreccion R. S.; Smeltzer M. A. and Dickson T. G. (1988)** Respiratory Cryptosporidiosis in Chickens. Poult. Sci. .67:1684-1693.

**Goodwin M. A.and Brown J.(1996 )**Respiratory Coccidiosis *Cryptosporidium baileyi* Among Northern Georgia Broilers in One Company Avi. dis.J.40:572-575 .

**Goodwin M.A.and Brown J.(1989)** Intestinal Cryptosporidiosis in Chickens. Avi. Dis. 33:770-777.

**Gordo F. P. ; Herrera S.; Castro A.T.; Durán B. G.and Diaz R.A. M.(2002)** Parasites from farmed ostriches (*Struthio camelus*)and rheas (*Rhea americana*) in Europe. Vet. Parasitol. 107: 137–160.

**Graczyk T.K.; Fayer R.and Cranfield M.R.(1997)** Zoonotic transmission of *Cryptosporidium parvum*: Implications for water-borne cryptosporidiosis. Parasitology Today.13:348-351.

**Hadfield S.J.; Robinson G.; Elwin K. and Chalmers R.M. (2011).** Detection and differentiation of *Cryptosporidium* spp. in human clinical samples by use of real-time PCR. J Clin. Microbiol 49, 918-924.

**Harris J. R.;Adrian M.and Petry F.(2004)** Amylopectin: a major component of the residual body in *Cryptosporidium parvum* oocysts. Parasitol.J.128:269–282..

**Hassan S. M.; Siam A. A.; Mady M. E. and Cartwright A. L.(2004**) Incubation Temperature for Ostrich (*Struthio camelus*) Eggs Poul. Sci. 83:495–499.

**Hajdušek O.; Ditrich O.and Šlapeta J.(2004)** Molecular identification of *Cryptosporidium* spp. in animal and human hosts from the Czech Republic.Vet. Parasitol.122:183–192.

**Heine V.J. (1982**) Eine einfache Nachweis methode fur Kryptosporidien im Kot. Zbl. Vet. Med.J.29:324-327.

**Hoerr F. J.;Current W. L.and Haynes T. B.(1986)** Fatal Cryptosporidiosis in Quail. Avi.Dis.30(2):421-425.

**Honsvall B.K. and Robertson L.J. (2017).** Real-time nucleic acid sequence-based amplification (NASBA) assay targeting MIC1 for detection of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts.Experimental Parasitology 172: 61-67 .

**Huang J.;Zhang Z.; Zhang Y.; Yang Y.; Zhao J.; Wang R.; Jian F.; Ning C.; Zhang W.and Zhang L.(2018)** Prevalence and molecular characterization of *Cryptosporidium* spp. and Giardia duodenalis in deer in Henan and Jilin, China. Paras. and Vect. 11(239):3-10.

**Hunter P. R. and Nichols G. (2002)** Epidemiology and Clinical Features of *Cryptosporidium* Infection in Immunocompromised Patients. Clin. Microbiol. Rev.15(1):145-154.

**Hurkova L.; Hajdusek O. and D. Modry ( 2003)** Natural Infection of *Cryptosporidium muris* (Apicomplexa: Cryptosporiidae) in Siberian Chipmunks . J. of Wildlife Dis. 39(2):441–444.

**Ignatius R.; Klemm T.; Zander S.; Gahutu J. B.; Kimmig P.; Mockenhaupt F. P. and Regnath T.(1997)** Highly specific detection of *Cryptosporidium* spp. oocysts in human stool samples by undemanding and inexpensive phase contrast microscopy. Parasitol. Res.3:48-59.

**Iordanidis M. G.; Papazahariadou G. K.;Georgiades N. Gand Papaioannou S. E. Frydas(2003)**Cloacal prolapse in Ostrich chicks with histomoniosis.Vet. Rec.153: 434-435.

**Itakura, C, Goryo, M. and Umemura, T. (1984).** Cryptosporidial infection in chickens. Avi. Patho.13: 487-499.

**Jardine J. E.and Verwoerd D. J.( 1997)** Pancreatic Cryptosporidiosis in Ostriches Avian Pathology 26:665-670 .

**Jasim G.A. and Marhoon I.A. ( 2015)** Prevalence and Molecular Analysis of *Cryptosporidium* Spp.Isolated From Wild and Domestic Birds. Act .Parasitologica Globalis 6 (2): 65-70.

**Jex A.R. and Gasser R.B. (2010).** Genetic richness and diversity in *Cryptosporidium hominis* and *C. parvum* reveals major knowledge gaps and a need for the application of "next generation" technologies - Research review.Biotech.Adv.28:17-26.

**Kar S.; Gawlowskab S.; Daugschiesb A and Bangourab B.(2011)** Quantitative comparison of different purification and detection methods for *Cryptosporidium parvum* oocysts. Vet. Parasitol.177: 366–370.

**Karanis P.; Eiji T.; Palomino L.; Boonrod K.; Plutzer J.; Ongerth J. and Igarashi I. (2010).** First description of *Cryptosporidium bovis* in Japan and diagnosis and genotyping of *Cryptosporidium* spp. in diarrheic pre-weaned calves in Hokkaido. Vet. Parasitol., 169: 387-390.

**Keidel J. and Daugschies A. (2013).** Integration of halofuginone lactate treatment and disinfection with p-chloro-m-cresol to control natural cryptosporidiosis in calves. Vet Parasitol 196:321-326.

**Keshavarz A.; Athari A.; Haghighi A.; Kazami B.; Abadi A.; Nazemalhoseini E.N. and Kashi L. (2008).** Genetic Characterization of *Cryptosporidium* spp. among Children with Diarrhea in Tehran and Qazvin Provinces, Iran. Iranian J.Parasitol.3(3):30-36.

**Khan A.; Jahangheer S.; Shaik M and Grigg E. (2018)** Genomics and molecular epidemiology of *Cryptosporidium* species. Acta Tropica 184 :1–14.

**Khalil M.M. (2010).** Diagnostic parasitic study of cryptosporidiosis in pregnant cows in neonatal calves, and study the effect of pregnancy and parturition on oocysts shedding pattern in cows. M.Sc. Thesis submitted to the university of Baghdad-college of veterinary medicine.

**Kichou F.;Saghir F.and El Hamidi M.(1996)** Natural *Cryptosporidium* sp. infectionin broiler chickens in Morocco. Avi. Patho. 25: 103-111.

**Koehler A.V; Korhonen P.K.; Hall R.S.; Young N.D.; Wang T.; Haydon S.R. and Gasser R.B. (2017).** Use of a bioinformatic-assisted primer design strategy to establish a new nested PCRbased method for *Cryptosporidium*.Parasit. Vect. 10, 509.

**Kucukerden, N.; Ozturk, G. and Angin, M. (1999).** The prevelance of Cryptosporidiosis in broilers at Elazig district. Turkish J. Vet. Ani. Sci. 21:153-156

**Kuczynska E. and Shelton D. R.(1999)** Method for Detection and Enumeration of Cryptosporidium *parvum* Oocysts in Feces, Manures, and Soils. Appl. and environ. Microbial.J.65(7): 2820–2826.

**Laatamna A.E.; Holubova N.; Sak B. and M. Kvac (2017**) *Cryptosporidium* meleagridis and *C. baileyi* (Apicomplexa) indomestic and wild birds in Algeria. Folia Parasitologica. 64:2-7.

**Laberge I.;Griffiths M.W. and Griffiths M.W.(1996)** Prevalence, detection and control of *Cryptosporidium parvum* in food. Inter.J.of Food Microbiol.31:l-26.

**Latimer, K. S. (1992) .** Cryptosporidiosis in four cockatoos with psittactine beak and feather disease. J. Am. Vet. Med. Asso. 200: 707-710.

**Leitch GJ and He Q. (1999)** Reactive nitrogen and oxygen species ameliorate experimental cryptosporidiosis in the neonatal BALB/c mouse model. Infect Immun.67:5885–5891.

**Levine N.D. (1961).** Protozoan Parasites of Domestic Animals and Man. Burgress Publishing Company. Minnesota, USA. pp. 118–122.

**Lillehoj; C.H. Yun and H. S. Lillehoj (2000)** Vaccines against the avian enteropathogens *Eimeria*,*Cryptosporidium* and *Salmonella* . Ani.Hlth.Res. Rev. 1(1):47–65.

**Lindsay D. S.; Blagburn B. L.; Sundermann C. A. and Hoerr F. J.(1989)** Experimental Infections in Domestic Ducks with *Cryptosporidium baileyi* Isolated from Chickens .Avi. Dis. 33:69-73.

**Lindsay D. S.; Blagburn B. L.; Sundermann C. A.; Hoerr F. J.;and Giambrone J. J.(1987**).*Cryptosporidium baileyi*: Effects of Intra-abdominal and Intravenous Inoculation of Oocysts on Infectivity and Site of Development in Broiler Chickens. Avi.Dis .31(4 ):841-843.

**Lv C.; Zhang L.; Wang R.; Jian F.; Zhang S.; Ning C.; Wang H.; Feng C.; Wang X.; Ren X.; Qi M. and Xiao L. (2009).** *Cryptosporidium* spp. in wild, laboratory, and pet rodents in china: prevalence and molecular characterization. Appl. Environ. Microbiol. 75:7692-7699.

**Ma P.; and Soave R. (1983).** Three-step stool examination for cryptosporidiosis in 10 homosexual men with protracted watery diarrhea. Journal of Infectious Diseases, 147(5), 824-828.

**Macpherson D. W.and Mcqueen R.(1993)**Cryptosporidiosis: Multiattribute Evaluation of SixDiagnostic Methods J.of Clin. Micr .31(2): 198-202.

**Madore M. S.; Rose J. B.; Gerba C. P.; Arrowood M. J. and Sterling C. R.(1987)** occurrence of *Cryptosporidium* oocysts in sewage effluents and selected surface waters. J. Parasit.73(4):702-705.

**Maggi, P.; Larocca, A.; Quarto, M.; Serio, G.; Brandnisio, O.; Angarano, G. andPastore, G. (2000).** Effect of antiretroviral therapy on cryptosporidiosis and microsporidiosis in patient infected with human immunodiciency virus type 1. Europ. J. Clin. Microbiol. Infect. Dis. 19: 213-217.

**Magige F.and Roskaft E.(2017)** Medicinal and commercial uses of ostrich products in Tanzania. J. of Ethno.bio.and Ethno.med.13(48):2-7.

**Meireles M.V.; Soares R. M.;Santos M.M.A.B and Gennari S.M.(2006)** Biological studies and molecular characterization of A *Cryptosporidium* isolate from Ostriches (*Struthio camelus*). *J. Parasitol.*92(3):623–626.

**Meireles, M. V. and Figueiredo P. C.(1992)** Isolamento e identificação do Cryptosporidium baileyi, Current et al., (Apicomplexa:Cryptosporidiidae) em frangosde corte. Revi. Brasil.Parasitol.Vet. 12:125−130.

**Mirhashemi M.; Zintl A.; Grant T.; Lucy F.E.; Mulcahy G. and De Waal T. (2015).** Comparison of diagnostic techniques for the detection of *Cryptosporidium* oocysts in animal samples. Exp. Parasitol. 151–152, 14–20.

**Mmbaga B.T. and Houpt E.R. (2017).** *Cryptosporidium* and *Giardia* infections in children. Pediatr .Clin. Am. (64):837–850.

**Mohammed R.G .(2010)** Epidemiological study of Cryptosporidiosis in (Anser anser domestics geese) in Nineveh Governorate.J. of Thi-qar Sci.2(1):39-44.

**Mor S. M.; Ascolillo L. R.; Nakato R.; Ndeezi G.; Tumwine J. K.; Okwera A.;Sponseller J. K.; Tzipori S.and Griffiths J.K. (2018)** Expectoration of *Cryptosporidium* Parasites in Sputum of HumanImmunodeficiency Virus–Positive and –Negative Adults. Am. J. Trop. Med. Hyg.98(4):1086–1090.

**Morgan U. M.; Monis P. T.; Xiao L.; Limor J.; Sulaiman I.; Raidal S.; O'Donoghue P.; Gasser R.; Murray A.; Fayer R.; Blagburn B. L.;Lal A. A. and Thompson R.C. A. (2001)** Molecular and phylogenetic characterisation of Cryptosporidium from birds. Inter. J. for Parasitol.31: 289-296.

**Msheliaa W.Ph.; Abdua P.A.; Abdussamad A. M.and Wakawa A. M.(2010)** Prevalence of endoparasites in ostriches (*Struthio camelus*) raised in selected states of northern Nigeria. Conf. on Inter. Res. on Food Sec., Natural Resource Management and Rural Development. ETH Zurich.

**Muchiri J. M.; Ascolillo L.; Mugambi M.; Mutwiri T.; Ward H. D.; Naumova E. N.; Egorov A. I.; Cohen S.; Else J. G. and Griffiths J. K.( 2009)** Seasonality of *Cryptosporidium* oocyst detection in surface waters of Meru, Kenya as determined by two isolation methods followed by PCR. J .Water Health. 7(1): 67–75**.**

**Munshi A. (2012).** DNA Sequencing – Methods and Applications (Book). First published by InTech Janeza Trdine 9, 51000 Rijeka, Croatia. pp. 184.

**Mushi E.Z.; Isa J.F.W.; Chabo R.G.; Binta M.G.; Kapaata R.W; Ndebele R.T.andChakalisa K.C.(1998)**Coccidia oocysts in the faeces of farmed Ostrich Ostrich*(Struthio* camelus) chicks in Botswana. Onder.J. of Vet.Res.65:281-284.

**Mushia E. Z.; Bintab M. G.; Chaboa R.G. and Toto P. A. S.( 2003)** A note on endoparasites of wild ostriches (Struthio camelus ) in the Mokolodi Nature Reserve, Gaborone, Botswana. Tydskr.S.Afr.vet.Ver. 74(1): 22–23.

**Naciri M.; Mancassola R.; Fort G.; Danneels B. and Verhaeghe J. (2011).** Efficacy of amine-based disinfectant kenocox on the infectivity of *Cryptosporidium parvum* oocysts. Vet. Parasitol. 179:43-49.

**Nakamura A. A.and Meireles M.V.(2015)** *Cryptosporidium* infections in birds - A review. Braz. J. Vet. Parasitol.24(3):253-267.

**Nakamura A.A.; Simo˜es D.C.; Antunes R. G.; Da Silva D. C.and Meireles M. V. (2009)** Molecular characterization of *Cryptosporidium* spp. from fecal samples of birds kept in captivity in Brazil.Vet.Parasitol.166:47–51.

**Nemejc K.and Lukesova D.(2012)** Parasite Fauna of Ostriches, Emus and Rheas Agr.Trop.Et.Subtrop.45(1):45-50.

**Ng J.; Pavlasek I. and Ryan U.(2006)** Identification of Novel *Cryptosporidium* Genotypes from Avian Hosts. Appl. and Environ. Microbiol. 72(12):7548–7553.

**Nguyen S. T.; Fukuda Y.; Tada C.; Huynh V. V.; Nguyen D. T.and Nakai Y.(2013)** Prevalence and molecular characterization of *Cryptosporidium* in Ostriches(*Struthio camelus*) on a farm in central Vietnam. Exper.Parasitol. 133:8–11.

**Nichols R.A.B.; Connelly L.; Sullivan C.B. and Smith H.V. (2010**). Identification of *Cryptosporidium* species and genotypes in Scottish raw and drinking waters during a oneyear monitoring period. Appl. Environ. Microbiol. 76:5977–5986.

**Nurminen N. Juuti R.; Oikarinen S.; Fan Y.M.; Lehto K.M.; Mangani C.; Maleta K.; Ashorn P. and Hyoty H. (2015**). High-throughput *Cryptosporidium* species detection in stool samples. Am. J. Trop. Med. Hyg. 92:1222–1226.

**Gharagozlou S. ; Rahbari S. ; Bokaie E.I. ; Jahanzad O. ; Dezfoulian O. (2006)** Intestinal Cryptosporidiosis in Turkeys in Iran.J .Vet.Med.Ser. 64(4):156-158.

**O'donoghue P. J. (1995**) Cryptosporidium and Cryptosporidiosis in Man and Animals Inter. J. for Parasitol. 25( 2):139-195.

**Ogata S.; Suganuma T.; Okada C.; Inoue K.; Kinoshita A.and Sato K.(2009**) A Case of Sporadic Intestinal Cryptosporidiosis Diagnosed by Endoscopic Biopsy. Acta Medica Okayama.. 63(9):287-291.

**OIE (2008).** Chapter 2. 9. 4. Cryptosporidiosis, OIE, terrestrial manual, OIE.:1192 - 1215.pp.

**Okhuysen P. C.; Chappell C. L.; Crabb J. H.; Sterling C. R.;and DuPont H. L.(1999)** Virulence of Three Distinct *Cryptosporidium parvum* Isolates for Healthy Adults. J. of Infec. Dis. 180:1275–1281.

**Oliveira F.C.;Ederli N.B.; Ederli B.B.; Albuquerque M.C. and Dos Santos M.D.(2008)**Occurrence of *Cryptosporidium* spp. oocysts (Apicomplexa, Cryptosporidiidae) in ostriches,Struthio camelus L., 1758 (Aves, Struthionidae) reared in North and Lowered Coastline regions of the state of Rio de Janeiro. Brazil. Rev. Bras. Parasitol.Vet. 1:322-325.

**Pacheco F.T.F.; Silva R.K.N.R.; Martins A.S.; Oliveira R.R.; Alcantara Neves N.M.; Soares N.M.; and Teixeira M.C.A.(2013).** Differences in the detection of *Cryptosporidium* and isospora ( Cystoisospora ) oocysts according to the fecal concentration or staining method used in a clinical labrotoray .J.Parasitol . 99, 1002-1008.

**Pavlasek I. (1999).** Kryptosporidie: biologie, diagnostika, hostitelské spektrum, specificita a vztah kživotnímu prostředí (Cryptosporidia: biology, diagnosis, host spectrum, specificity and the environment). Remedia Klin. Microbiol. 3(9): 290-301 .

**Paviasek, I. (1993).** Black headed Gull (*Larus ridibundus .L*.) A new host of *Cryptosporidium baileyi*. Vet. Med. 38: 629-630.

**Penrith M.L. ; Benzuidenhout A.J. ;Burger W.P.and Putterill J .F.( 1994)** Evidence for Cryptosporidial infection as a cause of prolapse of the phallus and cloaca in Ostriches chicks ( *Struthio camelus* ). Onderstepoort J.of Vet.Res.61:283-289.

**Penrith M.L.;Bezuidenhuout A.J.;Burger W.P.and Putterill J.F.(1994)**Evidence of Cryptosporidial infection as a cause of prolapse of phallus and cloaca in Ostrich chicks ( Struthio cameuls).Onder. J.of Vet. Res.61:283-289.

**Perry R. W.; Rowland G. N. and Glisson J. R.(1991)** Poult Malabsorption Syndrome. I. Malabsorption in Poult Enteritis. Avi. Dis. 35:685-693.

**Petrie A. and Watson P. (2006).** Statistics for Veterinary and Animal Science, Second Edition. Ames: Blackwell Publishing, Pp: 24-112.

**Petry F. (2000)** laboratory diagnosis of *C. parvum* infection . In Cryptosporidiosis and Microsporidiosis , F. Petry , ed ( Basel :S.Karger) , pp.33-49 .

**Qi M.; Huang L.; Wang R.; Xiao L.; Xu L.; Li J. and Zhang L.(2014)** Natural infection of *Cryptosporidium muris* in Ostriches(*Struthio camelus*).Vet. Parasitol.6(35):1-19.

**Quah J.X. ;Ambu S.; Lim Y.A.;Mahdy M.A.and Mak J.W.( 2011)**Molecular identification of *Cryptosporidium parvum* from avian hosts. Parasitol.138(5):573-577.

**Rajalakshmi S. (2017).** Different types of PCR techniques and its applications. IJPCBS, 7(3), 285-292.

**Randall C.J. (1982)**Cryptosporidiosis of the bursa of fabricius and trachea in broilers. Avi. Patho.11: 95-102.

**Rasmussen, H.B. (2012).** Gel Electrophoresis - Principles and Basics. Intech. P. 316-317.

**Ravich M. L.; Reavill D.R.; Hess L.; Childress A. L. and Wellehan J.(2014)** Gastrointestinal Cryptosporidiosis in Captive Psittacine Birds in the United States: A Case Review. J. of Avi. Med. and Surg. 28(4):297–303.

**Rezende H.H. A.; Avelar J.B.; Storchilo H. R.; Vinaud M.C.and De Castro A. M.( 2015)** Evaluation of the accuracy of parasitological techniques for the diagnosis of intestinal parasites in cats.Braz. J.Vet. Parasitol. 24(4):471-474.

**RheeJ.K. ;Kim H.C and Park B.K.(1996)** Chronologic change of serum IgG antibodyresponse in chickens reinfected with *Cryptosporidium bailey*.Kar.J.of Parasitol.34(4):255-258.

**Roellig D.M.; Yoder J.S.; Madison-Antenucci S.; Robinson T.J.; Van T.T.; Collier S.A.; Boxrud D.; Monson T.; Bates L.A.; Blackstock A.J.; SheaS.; Larson K.; Xiao L. and Beach M. (2017).** Community laboratory testing for *Cryptosporidium*: multicenter Study retesting public health surveillance stool samples positive for *Cryptosporidium* by rapid cartridge assay withdirect fluorescent antibody testing. PLoS One 12, e0169915.

**Robinson G.; Thomas A.L.; Daniel R.G.; Hadfield S.J.; Elwin K. and Chalmer R.M. (2006).** Sample prevalence and molecular characterization of *Cryptosporidium andersoni* within a dairy herd in the United Kingdom. Vet. Parasitol., 142: 163-167.

**Rose J. B. and Slifko T. R. (1999)** *Giardia, Cryptosporidium*, and *Cyclospora* and Their Impact on Foods: A Review. J. of Food Prot. 62( 9):1059–1070

**Rossle N.F. and Latif B. (2013).** Cryptosporidiosis as threatening health problem: a review. Asian Pac. J. Trop. Biomed. 3, 916–924.

**Ruecker N.J.; Matsune J.C.; Lapen D.R.; Topp E.; Edge T. and Neumann N.F. (2013).** The detection of *Cryptosporidium* and the resolution of mixtures of species and genotypes from water. Infect Genet Evol. 15: 3-9.

**Rutschmann F. (2006)** Molecular dating of phylogenetic trees: a brief review of current methods that estimate divergence times. Diversity Distrib.12:35–48.

**Ryan U.(2010)** Cryptosporidium in birds, fish and amphibians. Exper.Parasitol .124: 113–120.

**Ryan U.; Lawler S. and Reid S. (2017**). Limiting swimming pool outbreaks of 549 cryptosporidiosis - the roles of regulations, staff, patrons and research. J. 550 Water Health. 15, 1–16.

**Ryan U.; Nawal Hijjawi N. and Xiao L. (2018).** Foodborne Cryptosporidiosis. International Journal for Parasitology N. 48, p.: 1–12.

**Ryan U.; Xiao L.; Read C.; Zhou L.; Lal A.A.and Pavlasek I. (2003)** Identification of Novel *Cryptosporidium* Genotypes from theCzech Republic. Appl. and Environ. Micro. 69(7):4302–4307.

**Ryan U.; Zahedi A. and Paparini A. (2016).** *Cryptosporidium* in humans and animals-a one health approach to prophylaxis. Parasite Immunol. 38, 535–547.

**Sadek G. (2014).** Use of nested PCR-RFLP for genotyping of *Cryptosporidium* parasites isolated from calves and children suffering from diarrhea.Parasitol.United J.7:129–137.

**Santin M.( 2013)** Clinical and subclinical infections with *Cryptosporidium* in animals.New Zealand Vet. J. 61(1): 1–10.

**Santin M.; Trout J.M. and Fayer R. (2008).** A longitudinal study of cryptosporidiosis in dairy cattle from birth to 2 years of age. Vet. Parasitol. 155, 15–23.

**Santos M.M.A.B.;Periro J.R.and Meireles M.V.(2005)** *Cryptosporidium* infection in Ostriches ( *Struthio camelus*)in Brazil: clinical ,morphological and molecular studies 7(2):113-117.

**Seva A. D.; Funadab M. R.; Richtzenhaina L.;Guimaraesc M. B.; Souzaa S. D., Allegrett L.; Sinhorinie J. A.; Duartef V. V.and Soaresa R. M.(2011)** Genotyping of *Cryptosporidium* spp. from free-living wild birds from Brazil. Vet. Parasitol. 175:27–32 .

**Sréter T. and Varga I.(2000)** Cryptosporidiosis in birds — A review. Vet. Parasitol. 87:261–279.

**Shaapan R.M.;Khalil F.A.M;Nadia;Abu-elezz M.T.(2011)** Cryptosporidiosis and Toxoplasmosis in Native Quails of Egypt.Res.J. of Vet.Sci.4(2):30-36.

**Shafiq M. A.; Maqbool A.;Khan U. J.;Lateef M. and Ijaz M.(2015)** Prevalence, Water Borne Transmission and Chemotherapy of Cryptosporidiosis in Small Ruminants. Pakistan J. Zool.47(6): 1715-1721.

**Shahiduzzaman M.D. and Daugschies A.(2012)** Therapy and prevention of Cryptosporidiosis in animals.Vet. Parasitol.188: 203– 214.

**Shemshadi B.; Bahadori S.R. and Mozafari A.(2011)** Study on cryptosporidiosis incidence in broilers in Garmsar region,Iran. Comp. Clin. Pathol.20:143–149.

**Shanawany M.M. and Dingle J.(1999)** Ostrich production systems.FAO.Animal production and health paper.pp241.

**Silva D.C.D; Homem C.G; Nakamura A.A;Teixeira W.F.P; Perri S.H.Vand Meireles M.V (2010).** Physical, epidemiological, and molecular evaluation of infection by *Cryptosporidium galli* in Passeriformes. Parasitol Res.107:271–7.

**Slavin D.(1955) *Cryptosporidium meleagridis***.J.Comp. Path.65:262-266.

**Smith H. V.and Corcoran G.D.(2004 )**New drugs and treatment for Cryptosporidiosis.Curr. Opin. Infect. Dis. 17:557–564.

**Smith H.V.and Rose J.B.(1989**) Waterborne Cryptosporidiosis:Current Status. Parasitol.Today.14( 1):14-22.

**Smith J. L.(1993)** *Cryptosporidium* and *Giardia* as Agents of Foodbome Disease. J. of Food Protection.56( 5) 451-461.

**Soetan K. O.; Olaiya C. O. and Oyewole O. E.(2010)** The importance of mineral elements for humans,domestic animals and plants: A review. Afr. J.of Food Sci.4(5): 200-222.

**Soltane R.; Guyot K.; Dei-cas E.and Ayadi A.(2007)** Prevalence of *Cryptosporidium* spp.Eucoccidiorida: Cryptosporiidae in seven species of farm animals in Tunisia. Parasite.J.14:335-338

**Sotiraki S. T.; Georgiades G.; Sotiriadou K. A.and Himonas C. A.(2001)** Gastrointestinal parasites in ostriches(*Struthio camelus*).Vet.Rec148:84-86.

**Sreter T.; Kovacs G.; Da silva A. J.; Pieniazek N. J.; Szell Z.; Dobos-kovacs Marialigeti K. and Varga I.(2000)** Morphologic, Host Specificity, and Molecular Characterization of a Hungarian *Cryptosporidium meleagridis* Isolate.Appl.and Environ. Micro. 66(2): 735–738.

**Sreter T.; Varga I.and Bekesi L.(1995) Age-Dependent Resistance to** *Cryptosporidium baileyi* Infection in Chickens. J. Parasitol.81(5): 827-829

**Sreter, T. (1998).** Cryptosporidiosis in birds .J . Prstozoal . Res. 8:44-57.

**Sun M.F.;Zhang L.X.;Ning C.S.;Liang H.D.;Jian F.and Zhang M.(2007)** Identification of Cryptosporida Isolate from Ostrich in China and Biological Characters Studies. Scientia Agricultura Sinica.7:1-2.

**Taha S.; Elmalik K.; Bangoura B.; Lendner M.; Mossaad E. and Daugschies A. (2017).** Molecular characterization of bovine *Cryptosporidium* isolated from diarrheic calves in the Sudan. Parasitology research, 116(11), 2971-2979.

**Takano H.;Inamoto T.;Ogimoto K.and Nakai Y.(1992**) Developmental process of *Cryptosporidium* in the intestine and bursa of fabricius of chickens. J.Vet.Med.Sci.54(2):289-292.

**Tamura K.; Stecher G.; Peterson D.; Filipski A. and Kumar S. (2013).** MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution 30: 2725-2729.

**Taylor M.A. ; Catchpole J.; Norton C.C. and Green J.A.( 1994**) Variations in oocyst output associated with *Cryptosporidium baileyi* infections in chickens. Vet. Parasitol. 53:7-14.

**Taylor M.A.; Marshall R.N.;Green J.A.and Catchpole J.(1999)** The pathogenesis of experimental infections of *Cryptosporidium muris* (strain RN 66) in outbred nude mice. Vet. Parasitol. 86: 41–48.

**Thigeel, H.(2016)** A study of factors associated with *giardia* and *Cryptosporidium* infections in humans, dogs and cats in the USA. Ph.D thesis University of Colorado State.299 pp.

**Thomson S. (2016).** Cryptosporidiosis in Farm Livestock. PhD thesis. University of Glasgow.

**Tully T.N. and Shane S.M.(1996)** Husbandry practices as related to infectious and parasitic diseases of farmed ratites. Rev. sci. tech. Off. int. Epiz15 (1):73-8.

**Tyzzer E. E.(1907)** A sporozoan found in the peptic glands of the common mouse . Soc.Exp.Biol.Med.5:12-13.

**Tyzzer E.E. (1910).** An extracellular Coccidium, *Cryptosporidium Muris* (Gen. Et Sp. Nov.), of the gastric Glands of the Common Mouse. J Med Res 23, 487-509.

**Tyzzer E.E. (1912).** *Cryptosporidium parvum* (sp. nov.), a coccidium found in the small intestine of the common mouse. Arch Protistenkunde 26, 294-412.

**Tzipori S.(1982)** Cryptosporidiosis in Perspective. Advan.in Parasitol.27:63-129.

**Tzipori S.and Griffiths J.K .(1998)** Natural History and Biology of *Cryptosporidium parvum* .Advan. in Parasitol .40:5-36.

**Uppal B.; Singh O.; Chadha S. and A. K. Jha (2014)** A Comparison of Nested PCR Assay with ConventionalTechniques for Diagnosis of Intestinal Cryptosporidiosis inAIDS Cases from Northern India. J. of Parasitol.Res.1:1-6

**Upton S. J. and Current W. L.(1985)** The species of *Cryptosporidium* (Apicomplexa: Cryptosporidiidae) infecting mammals. J. Parasitol. 71:625–629.

**Valigurova A.; Jirku M.; Koudela B; Gelnar M.; Modry D.and Slapeta J.(2008)** Cryptosporidia: Epicellular parasites embracedby the host cell membrane. Inter. J. for Parasitol. 38:913–922 .

**Venu R. (2010).** Molecular detection and typing of *Cryptosporidium* in dairy calves. PhD. Thesis, Vet. Parasitology, Madras vet. College tamil nadu veterinary and animal sciences University Chennai- 600- 007.

**Wang R.; Jian F.; Sun Y.; Hu Q.; Zhu J.; Wang F.; Ning C.; Zhang L.and Xiao L.(2010)** Large-scale survey of *Cryptosporidium* spp. in chickens and Pekin ducks (Anas platyrhynchos) in Henan, China:prevalence and molecular characterization. Avi. Patho. 39(6):447-451.

**Wang R.; Qia M.; Jingjing Z.; Sunb D.; Ninga C.; Zhaoa J.; Zhanga L. and Xiaoc L. (2011)** Prevalence of *Cryptosporidium baileyi* in ostriches (Struthio camelus) in Zhengzhou, China.Vet. Parasitol. 175:151–154.

**Wells B.; Thomson S.; Ensor H.; Innes E.A.and Katzer F.(2016)** Development of a sensitive method to extract and detect low numbers of *Cryptosporidium* oocysts from adult cattle faecal samples.Vet. Parasitol. 227:26–29.

**Wheeler D. and Bhagwat. M. (2007)** Example-Driven Web-Based BLAST Tutorial. Comparative Genomics.1:1734.

**Widmer G.; Carraway M.and Tzipori S.( 1996)** water –born *Cryptosporidium* : Aperspective from the USA.Parasitol.Today. 12(7):286-290.

**Wheeler D. and Bhagwat. M. (2007)** Example-Driven Web-Based BLAST Tutorial. Comparative Genomics.1:1734.

**Xiao L and Ryan U. (2004).** Cryptosporidiosis: An update in molecular epidemiology. Curr. Opin. Infect. Dis., 17: 483-490.

**Xiao L. and Ryan U. (2008).** Molecular epidemiology, p. 119-163. In R. Fayer and L. Xiao (eds.), *Cryptosporidium* and cryptosporidiosis. CRC Press, Boca Raton.

**Xiao L.; Escalante L.;Yang C.;Sulaiman I.; Escalante A. A.; Montali R. J.; Fayer R. and Lal A.A.(1999)** Phylogenetic Analysis of *Cryptosporidium*.Parasites

Based on theSmall-Subunit rRNA Gene Locus. Appl. and Environ. Micro.65(4):1578-1583

**Xiao L.; Fayer R.; Ryan U. and Upton S. J.(2004**) *Cryptosporidium* Taxonomy: Recent Advances and Implications for Public Health. Clin. Microbiol.Rev.17(1):72-97.

**Xiao L.; Sulaimana I. M.; Ryanb U.M.; Zhoua L.; Atwillc E.R.; Tischlerd M.L.; Yu J.; Lee S. and Park W. (2009).** Comparative Sensitivity of PCR Primer Detection of *Cryptosporidium parvum*. Korean J.Parasitol.47 (2):293-297.

**Yu J.; Lee S. and Park W. (2009).** Comparative Sensitivity of PCR Primer Sets for Detection of *Cryptosporidium parvum*. Korean J.Parasitol.47 (2):293-297.

**Zahedi A. ; Monis P.;Alexander W. ; Gofton A.;Charlotte L.; Oskam A and Ball A .; Bath A.; Bartkow M.; Robertson I. and Ryan U.( 2018**) *Cryptosporidium* species and subtypes in animals inhabiting drinking water catchments in three states across Australia. Water Research 134:327-34

**Zeibig E.A. (1997).** Clinical Parasitology, W.B Saunders Company; Philadelphia, pp.320.

**Zhu J.,Hu Q.;Fang W.,Ning C.,Jian .,Zhang L.(2008)** Epidemiological investigation of Ostrich *Cryptosporidium* in Henan province. Chin. J. of Vet.Med.9:1-6.