Detection of Genotyping Diversity of *Cryptosporidium parvum* isolated from different birds

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Abstract

The current study included the molecular identification of *C. parvum* from different birds (domestic chicken, domestic pigeon, turkey and ostrich) Based on primers specific to the 18S rRNA ribosomal diagnostic gene, positive samples were amplified by microscopic examination and electrophoretically transmitted by Nested PCR technique to obtain DNA bundles of up to 504 base pairs Then the Nested PCR reaction was conducted again using primers specific for the differential gene gp60 and by amplifying the samples and electrophoresis up to 424 base pairs, The results were that all samples of *C. parvum* from different birds contain the glycoprotein gp60 gene, The DNA sequencing method was performed in the genotyping of *C. parvum* parasite based on the samples of the interfering glycoprotein gp60 gene, Nested PCR products were sent to Macrogen of Korea in an ice bag by DHL for DNA sequencing by AB DNA sequencing system Then, the molecular evolutionary DNA sequence analysis was carried out in (version Mega 6.0) and the multiple sequence alignment analysis was carried out using the alignment tool (ClustalW). The alignment analysis revealed the emergence of 3 genotypes They are IIa, IId, and IIe. The phylogenetic tree was determined for each host, and genotypes were compared in each host, between hosts, and with global isolates recorded in NCBI. Where the results showed a high affinity and match ratio (99.69 -99%), and the isolates were sent to Cryptosporidium parvum from local bird hosts to the NCBI-Genbank database to obtain accession numbers in Genbank.

Keywords: **(Cryptosporidium parvum**,**Nested PCR, phylogenetic tree, genotypes)**

the introduction

The infection with intestinal parasites, including the *Cryptosporidium* parasite, is a source of concern and of great importance to the health of society due to its increased prevalence rate and its severe consequences for the health of individuals, as it is a common disease that causes diarrhea among children and patients with immunodeficiency (Marcos & Gotuzz ,2013)Other clinical symptoms include headache, abdominal cramps, and weight loss, which may be accompanied by fatigue and dehydration, as well as poor absorption, allergy, food poisoning, and bloating (Chalmers *et al*., 2019) Cryptosporidium parasites are responsible for severe diarrhea and can be responsible for developmental defects and mental impairment in children in developing countries and are strongly associated with colon cancer (Pinto & vinayak, 2021)The prevalence of mild-spore infection is high in developing countries, where many people still lack a basic and healthy level of drinking water and sanitation (Bouzid *et al*., 2018)Swimming, contact with people and animals with diarrhea, and international travel are the main factors that contribute to the spread of the parasite (Costa *et al*., 2020).

Molecular characterization of the *C. parvum* is essential to accurately identify genotypes and to assess transmission of infection, whether animal or human, which in turn contributes to the development of successful plans to reduce transmission between hosts (Haileeyesus *et al*., 2014).

The method

Examination of Fecal Samples :

Bird feces samples were collected from the zoo in the city and from animal breeders in the province of Babylon and the districts and districts affiliated to it. It collected 30 samples from domestic chickens, 25 samples from domestic pigeons, 19 samples from turkeys and 12 samples from ostriches, The name of the host, the date of collection, and the place of collection were written on it. The samples were placed in clean and sterile plastic containers with tight lids in order for the samples to remain moist and to prevent them from drying out for the purpose of conducting the microscopic examination using the Ziehl-Neelsen method. The microscopically positive samples were kept at a temperature of -20 degrees Celsius until the molecular examination was performed.

Molecular study :

Nested PCR primers were designed to detect the cryptosporidium parasite *C.parvum* based on the diagnostic gene small ribosomal DNA subunit 18S rRNA and genotyping bases 60 -kDa based on the glycoprotein gp60 gene. In this study NCBI-Genbank was used. (AF308600) (MN192415.1) and primer design 3+ These primers were provided by Scientific Resercher Co.Ltd in Iraq, and as shown in the following table:

Primers		Sequence 5'-3'	Product size
PCR rRNA gene	F	AGACGGTAGGGTATTGGCCT	610bp
	R	TCCTTGGCAAATGCTTTCGC	
Nested PCR rRNA gene	F	CGGGTAACGGGGAATTAGGG	504bp
	R	ATGCCCCCAACTGTCCCTAT	
Primers		Sequence 5'-3'	Product size
PCR Gp60 gene	F	GCTTCCCAACCCACTACTCC	541bp
	R	AGACACCATTGGTAGTGCCG	
Nested PCR Gp60 gene	F	GAAGGCGCAACTACCGAAAC	424bp
	R	CTTCGACGTTTGGTACAGCC	

Results

The results of the polymerase chain reaction (PCR) were shown to amplify 42 samples of extracted DNA based on the primer of the 18S ribosomal diagnostic gene. For C. parvum type, and when migrating on the agarose gel, the results showed that the DNA bundles of 20 samples were amplified up to 504 base pairs, Figure (1)

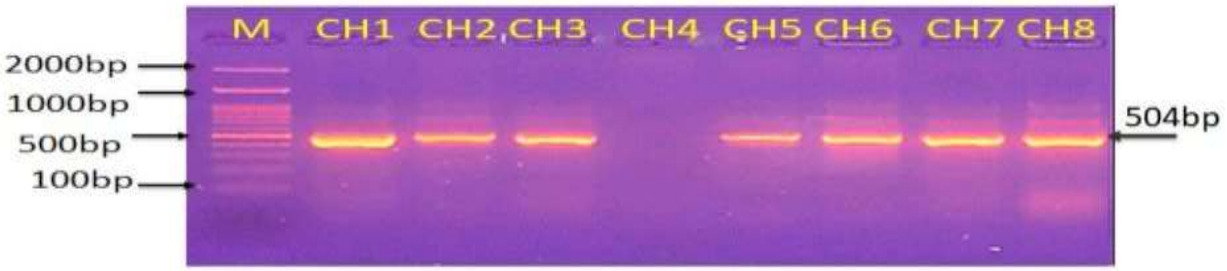


Figure 1: Agarose gel electrophoresis image that showed the Nested PCR product analysis of conserved region small subunit ribosomal RNA gene in *C.parvum* from animals samples. Where M: marker (2000-100bp). Lanes (CH1-CH8) showed some Positive *C.parvum* from chicken samples at (504bp) Nested PCR product.

Among the results of the study in bird hosts, the total infection rate of C. parvum was lower than the total infection rate in mammals, reaching 47.61% (20/42), and the highest infection rate was in turkeys 62.5% (5/8), and the lowest percentage was in A. for domestic chickens, as it reached 36.8% (7/19), while the percentage in domestic pigeons and ostriches was 50% (4/8) and 57.14% (4/7), respectively. The results of the statistical analysis also showed that at the level of significance of 5%, there is no significant difference between the rates of infection in the hosts of birds. Table (1)

Table (1) Percentages of parasite infection in bird hosts.

	host	Examined samples	Positive samples	The ratio
birds	Domestic chicken	19	7	36.84
	Domestic pigeon	8	4	50
	Turkey	8	5	62.5
	Ostriches	7	4	57.14
	Total	42	20	47.61
Chi-square value				1.86
Calculated p value				0.600*

There are no significant differences at the 5% level of significance.

The results of the amplification of 10 DNA samples extracted using the nested PCR technique, based on the primer of the gp60 glycoprotein gene, and when carrying out the migration process on the agarose gel, it was found that the DNA bundles of all samples were amplified to 424 base pairs Figure (2)

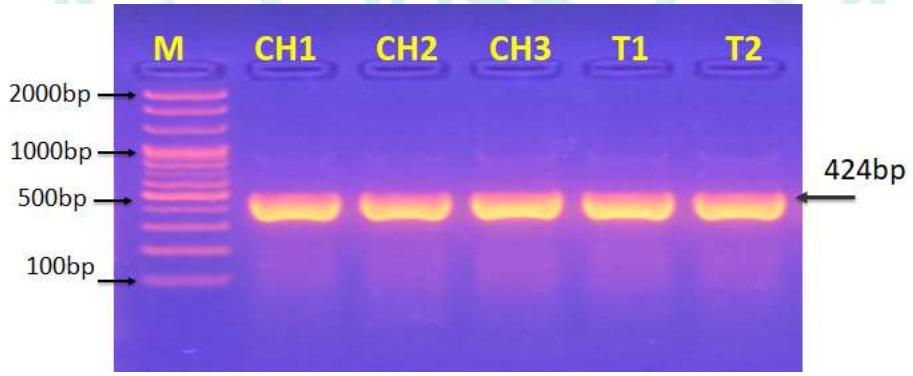


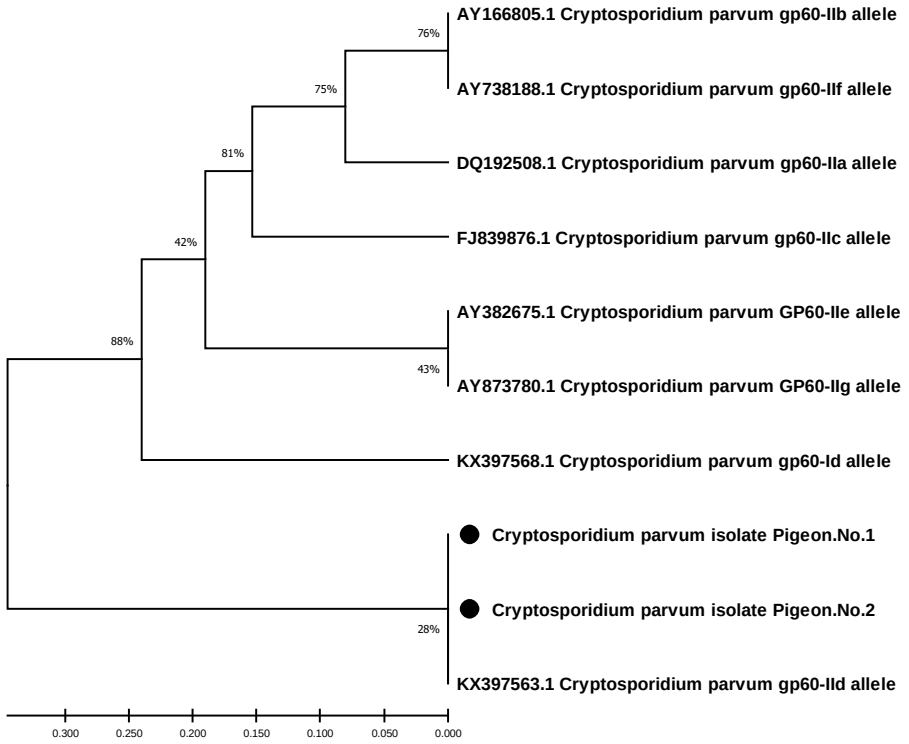
Figure2: Agarose gel electrophoresis image that showed the Nested PCR product analysis of conserved region in 60-kDa glycoprotein gene (gp60) gene in *C.parvum* from animals samples. Where M: marker (2000-100bp). Lanes (CH1-CH3) showed

Positive *C.parvum* from chicken samples and Lanes (T1-T2) showed Positive *C.parvum* from Turkey samples, at (424bp) Nested PCR product.

Phylogenetic tree

Multiple sequence alignment analysis of gp60 protein gene in local *C.parvum* and bird isolates and NCBI-Genbank *C.parvum* genotypes isolates. The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA 6.0 version). That showed the nucleotide alignment similarity as (*) with substitution mutations in of GP60 protein gene.

Figure (3): Phylogenetic tree analysis based on gp60 protein gene partial sequence in *C.parvum* ostrich isolates that used for genetic genotyping identification . The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local *C.parvum* ostrich No.3 isolates were showed closed related to NCBI-BLAST *C.parvum* gp60IIa allele genotype and The local *C.parvum* ostrich No.1 and No.2 isolates were showed closed related to NCBI-BLAST *C.parvum* gp60IIe allele genotype isolate at total genetic changes (0.200-0.050%).



Figure(4): Phylogenetic tree analysis based on gp60 protein gene partial sequence in C.parvum Pigeon isolates that used for genetic genotyping identification . The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local *C.parvum* Pigeon No.1 and No.2 isolates were showed closed related to NCBI-BLAST *C.parvum* gp60IId allele genotype isolate at total genetic changes (0.300-0.050%).

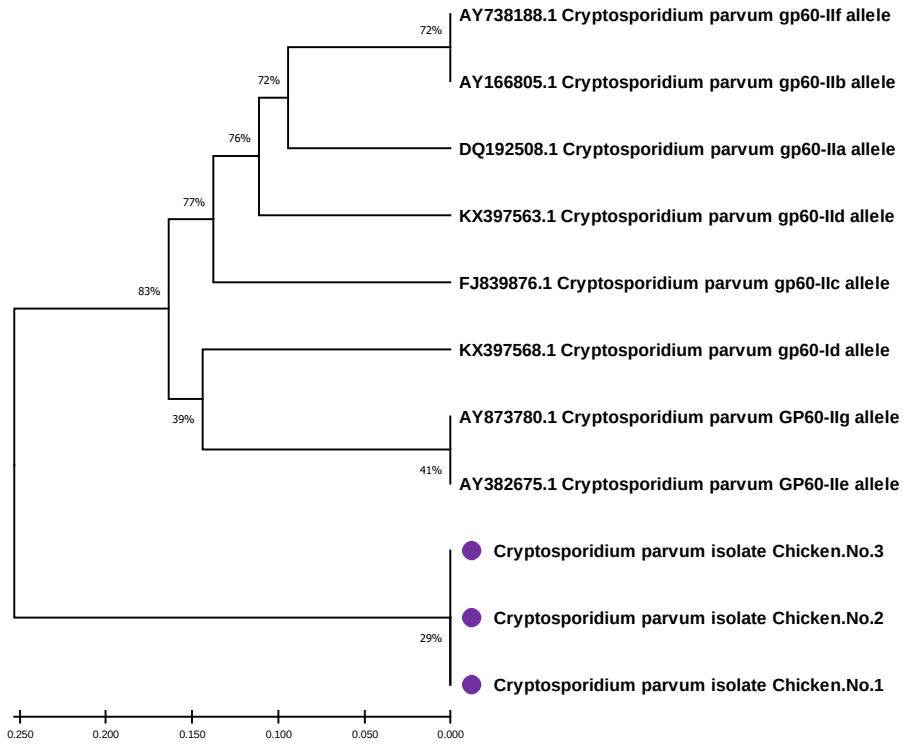


Figure (5): Phylogenetic tree analysis based on gp60 protein gene partial sequence in *C.parvum* Chicken isolates that used for genetic genotyping identification . The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local *C.parvum* Chicken No.1 - No.3 isolates were showed closed related to NCBI- BLAST *C.parvum* gp60IIe allele genotype isolate at total genetic changes (0.250-0.050%).

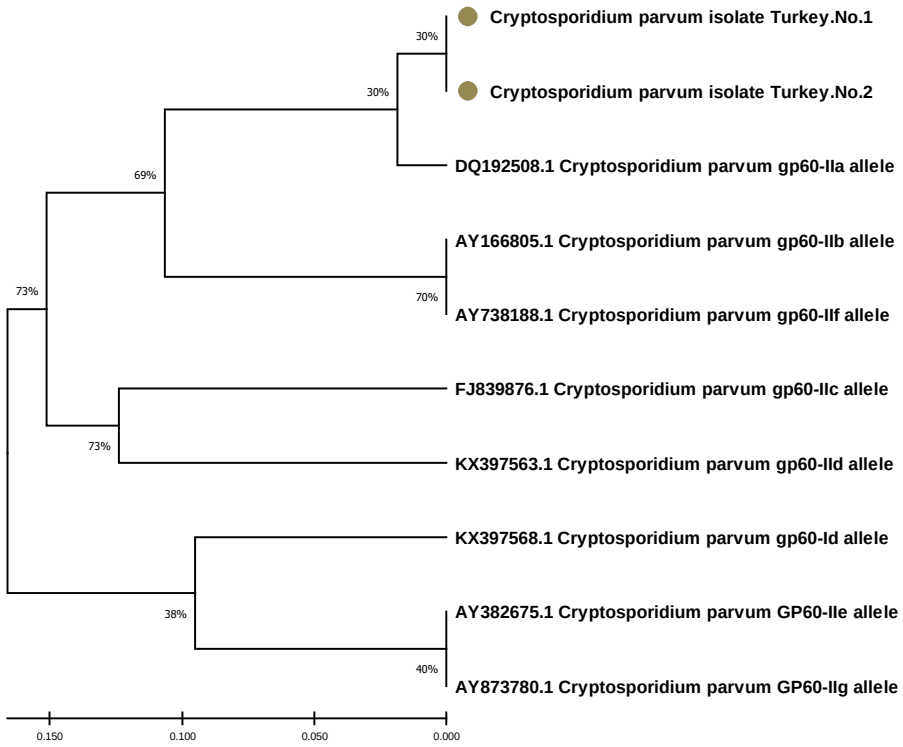


Figure (6): Phylogenetic tree analysis based on gp60 protein gene partial sequence in *C.parvum* Turkey isolates that used for genetic genotyping identification . The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local *C.parvum* Turkey No.1 and No.2 isolates were showed closed related to NCBI-BLAST *C.parvum* gp60IIa allele genotype isolate at total genetic changes (0.150-0.050%).

Comparing the genotypes of the study samples with the genotypes recorded in NCBI

When comparing the genotypes of the study samples with the genotypes of the samples registered in NCBI, it showed that the genotypes in this study matched the genotypes registered in the National Center for Biotechnology NCBI, with a percentage ranging between (99-99.69%) Table (2)

Table (2) the NCBI-BLAST Homology Sequence identity (%) between local *C.parvum* bird isolates and NCBI-BLAST submitted *C.parvum* genotype isolates:

Local C. parvum isolate	Genbank Accession number	NCBI-BLAST Homology Sequence identity (%)		
		Identical genotypes	Genbank Accession number	Identity (%)
C. parvum ostrich isolate No.1	OQ737817	Genotype IIe	AY382675.1	99.69%
C. parvum ostrich isolate No.2	OQ737818	Genotype IIe	AY382675.1	99.67%
C. parvum ostrich isolate No.3	OQ737819	Genotype IIa	DQ192508.1	99.00%
C. parvum Pigeon isolate No.1	OQ737820	Genotype IId	KX397563.1	99.67%
C. parvum Pigeon isolate No.2	OQ737821	Genotype IId	KX397563.1	99.69%
C. parvum Chicken isolate No.1	OQ737822	Genotype IIe	AY382675.1	99.67%
C. parvum Chicken isolate No.2	OQ737823	Genotype IIe	AY382675.1	99.69%
C. parvum Chicken isolate No.3	OQ737824	Genotype IIe	AY382675.1	99.67%
C. parvum Turkey isolate No.1	OQ737825	Genotype IIa	DQ192508.1	99.00%

C. parvum Turkey isolate No.2	OQ737826	Genotype IIa	DQ192508.1	99.00%

The proportions of genotypes in each host

The results of sequencing analysis of the genetic bases of Nested-PCR products of the gp60 gene of *C. parvum* species indicated the presence of three genotypes: IIa, IId, IIe .

The results of the analysis of the sequence of the genetic bases of the products of Nested - PCR assay for the gp60 gene of C. parvum type indicated that there are four genotypes: IIa IId, IIe, the IIa pattern appeared in turkeys by 100% (2/2) and in ostriches 33.33% (1/3) As for type IId, it was found in domestic pigeons 100% (2/2), and as for domestic chickens, type IIe was found in it by 100% (3/3), and the pattern was also found in ostriches by 33.33% (1/3). Type IIe is more visible in samples sent to the gene bank, and the results of statistical analysis showed a significant difference in the proportions of genotypes in bird hosts Table (3)

Table (3) the proportions of genotypes in each host

genotype	Domestic chicken	Domestic pigeon	Turkey	Ostriches	Chi-square value	Calculated p value
Gp60IIa	0(0)	0(0)	2(100)	1(33.33)	15.07**	0.058
Gp60IIb	0(0)	0(0)	0(0)	0(0)	-	-
Gp60IIc	0(0)	0(0)	0(0)	0(0)	15.74	0.046
Gp60IId	0(0)	2(100)	0(0)	0(0)	14.005*	0.082
Gp60IIe	3(100)	0(0)	0(0)	2(66.66)	20.83**	0.008
Gp60IIf	0(0)	0(0)	0(0)	0(0))	0*	1
Gp60IIg	0(0)	0(0)	0(0)	0(0))	0*	1
Gp60Id	0(0)	0(0)	0(0)	0(0)	-	-
The total	3	2	2	3		
Chi-square value	24**	16**	16**	11.81*		
Calculated p value	0.001	0.025	0.025	0.107		

There is no significant difference at the 5% probability level.

. There is a significant difference at the 5% probability level**

Discussion

Infection rates by molecular examination method

Cryptosporidium is a waterborne pathogen that infects a wide range of hosts, including livestock, birds and other animals, thus posing a threat to public health (Gao *et al*., 2021). It may cause slow growth in diseased animals, severe weight loss, low disease resistance, and severe losses in livestock production (Zhang *et al*., 2020) However, there were only a few studies that examined the genetic diversity of the parasite in birds. Birds transmit the parasite by ingestion or inhalation of egg sacs in contaminated materials, feces, water and dust. Poor sanitary conditions were associated with an increased prevalence of disease in poultry flocks (Helmy et al., 2013). The results of a study that included four types of birds (domestic chickens, domestic pigeons, turkeys and ostriches) showed that the total infection rate was 47.61, so the infection rate in domestic chickens was 36.84%, and the result was close to the result of a study in Al-Qadisiyah Governorate (Jarad, 2020) A study in Gilan in Iran on broiler or broiler chickens showed that the infection rate with *C.parvum* parasite was 2%, and this type, unlike the rest of the species, can infect chickens and turkeys without showing clinical signs. , The study showed that the percentage of molecular examination is higher than that of microscopic examination (Shahbazi *et al*., 2020) The study differed with a study in Bangladesh, where the rate of infection of chickens with the parasite was 1.0% (Kabir *et al*., 2020).

In domestic pigeons, the parasite infection was high, amounting to 50% by molecular examination method, and thus it agreed with a study in Egypt, where the infection rate was high in domestic pigeons (Abou Elez *et al*., 2023), The study also differed with a study in Babylon, where the infection amounted to 11/2), i.e. 18.18% (Altamimi & Al-Aubaidi, 2020) as well as(Oliveira *et al*., 2017), in a study in Brazil on carrier pigeons and domestic pigeons. The molecular level is higher than the microscopic examination. As for turkeys, it was the highest percentage in birds, it amounted to 62.5, and it agreed with a study in Germany, where the percentage of turkeys was the highest among poultry, and *C.parvum* was the most prevalent, and the strains of the type were close to 100-99 (%) of the nucleotides of human strains of patients in addition to to animal strains, and thus poultry may be a source of infection with the parasite (Helmy *et al*., 2017) As for ostriches, the infection rate was 57.14. A parasite infection rate was recorded in

farmed birds in a study in the Czech Republic on ostriches, chickens, and geese. Types of it were diagnosed and genetic patterns determined, as well as a new type of parasite in ostriches *C.ornithophilus* (Holubova *et al*., 2020). A study in Brazil stated that the rate of parasite prevalence was 33.3%, in chickens it was 15.4% (20/130) (and in quail 40% (2/5)) and in turkeys 100% (1/1). The parasite species *C.baileyi*, *C. meleagridis* ,*C. Galli* (da Cunha *et al*., 2018) In China, a study was conducted on domestic chickens, the general prevalence rate of *C.meleagridis* was 7.8% and C.baileyi by 4.8%, and found subtypes that were genetically identical to those identified in humans, and this indicates transmission between chickens and humans (Lin *et al*., 2022). In a study in China on wild birds, a number of species that infect birds were diagnosed, including *C.parvum.* The study stated that birds are among the most important hosts that can transmit the parasite and cause human and animal infection in areas adjacent to the habitats of wild birds. Because of the lack of control over wild birds, the possibility of transmission and spread of the disease increases.(Wang *et al*., 2021) And in China, on wild birds, the type *C.baileyi* was found by 3%, and the type *C.parvum* by 58% (Jian *et al*., 2021), And a study that included chickens, geese and ducks, the result of the study was the emergence of *C.meleagridis* species *C. xiaoi*, *C.gali C.baileyi*, and the latter was more prevalent (Gong *et al*., 2021).

The proportions of genotypes in each type of bird

The study showed the genotypes of *C.parvum* parasite in various types of birds (domestic chickens, domestic pigeons, turkeys and ostriches) and they were IIa, IId, IIe,. Type IIa was recorded in turkeys, 100%, and in ostriches, the proportion of the type was 33.3% of the samples sent to Genbank. The genotype IId was recorded in domestic pigeons, by 100% of the samples of sent pigeons. As for type IIe, it was found in domestic chickens, by 100%, and in ostriches. 66.6% of the samples sent, In a study in Bangladesh, where type IIa was reported in Sonali chicken and broiler chicken, subspecies IIaA13G2R1, IIaA11G2R1 were diagnosed (Kabir *et al*., 2020), Patterns IIe, IIc are genetically distinct subtypes and are related to the development of these parasites. Host adaptation may play a role in the emergence of new species. These patterns seem to be found only in human *C. parvum*. The glycoprotein gp60, along with other proteins, determines the appearance of patterns. measurable parasite genomics, particularly in the context of parasite-host interaction (Widmer & Lee, 2010) A study in New Zealand reported that type IIc, IIe appear as sporadic individual

cases (Knox *et al*., 2021), in contrast to types IIa,IId which are common in ruminants and transmitted through them to humans(Nader *et al*., 2019). Type IIe is considered the most common in the samples of the study sent, although previous studies reported that it is very rare and the numbers mentioned are very small. It was found in 11 studies, 6 of which were in countries where sanitation is poor, and one in a mixture of countries, most of which were in the lowest quartile. of sanitation and has not been reported in countries with good sanitation (King *et al*., 2019).

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