j.Egypt.net.med.Assoc 78, no 4. 565 – 574 (2018)

MOLECULAR CHARACTERIZATION OF DIFFERENT EIMERIA SPECIES FROM FIELD COCCIDIAL INFECTION IN BROILER CHICKENS IN SOME GOVERNORATES IN EGYPT

BY

Enas, S. A*, Youssef. I.Y* and Nassif. S.A**

*Department of Poultry and Rabbit Diseases - Faculty of Veterinary Medicine-Cairo University.

**Central Laboratory for Evaluation of Vet. Biologics.

ABSTRACT

Avian coccidiosis is one of the most important diseases affecting the intensive poultry industry worldwide. Coccidia are almost universally found wherever chickens are raised, and it is exceedingly rare to find a commercial chicken flock not affected. In our study, molecular based identification (conventional PCR) on positive fecal and intestinal samples for chicken coccidiosis from broiler farms. PCR results revealed the prevalence of *Eimeria tenella* 100% then *Eimeria necatrix* 14.28 % from positive cecal and fecal samples, *Eimeria acervulina* 10.71% from positive intestinal and fecal samples. Our sequence revealed 98% identity with coccivac -B *E. tenella* ITS-1 sequence.

Key words:

Eimeria, chicken, PCR, ITS_1 region, sequencing, Egypt.

INTRODUCTION

Coccidiosis remains one of the major menaces for poultry industry throughout the world **(Hafez, 2011)**. Diagnosis of Eimeria infection and differentiation of species is according to the consideration of clinical signs in the host and the morphological features of the parasite **(Yao-Chi Su, 2003)**. Recently, diagnosis of Eimeria infection and identification of different Eimeria species based on polymerase chain reaction (PCR) amplification of DNA was reported by **(Yao-Chi Su, 2003)**. The accurate identification of Eimeria species has important implications for diagnosis and disease control, but also to the epidemiology and biology studies, creation of new vaccines and selection of anticoccidial drugs **(Tsuji *et al.,* 1997, Woods *et al.,* 2000, Morris and Gasser, 2006, Sun *et al.,* 2009; Lee *et al.,* 2010)**. The internal transcribed spacer 1 (ITS1) from within ribosomal DNA (rDNA) gene was investigated by **(Molloy *et al,*1998, Schnitzler *et al*,1998, Yao-Chi Su 2003 and Lee *et al,***

2011) to identify and differentiate coccidial infection in chicken to genus and species level. ITS-1 sequence was also used to study the intrastrain variation of Eimeria in chickens **(Lew *et al.*, 2003, Su *et al.*, 2003).**

The aim of our work is planned to confirm traditional method of diagnosis for performing accurate diagnosis and identification of different *Eimeria* species in broiler farms.Whom were suffering clinical coccidiosis as, the specific diagnosis of *Eimeria* species in chickens using a PCR based approach is basic to a better understanding of the epidemiology and dynamics of the disease that underlies the effective prevention and control of coccidiosis. In addition, the other aim of work is to differentiate the field strains from the vaccinal strains by sequencing ITS-1 region of the most prevalent coccidial strains for more effective preventive strategies as sequence similarity could accurately predict vaccine efficacy later.

Material and methods:

Samples:

One-hundred and forty intestinal and fecal content from positive samples of previously examined chicken broiler farms of suspected outbreaks of chicken coccidiosis from seven governorates according to parameters of (recording clinical signs, dropping score (0-4), lesion score (0 - 4) and mortality % at time of examination as described in our previous work **(Enas *et al.*, 2018).**

Chemicals:

QIAamp DNA stool Mini Kit used for DNA extraction from coccidial positive samples. QIAquick PCR Product extraction kit, Bigdye Terminator V3.1 cycle sequencing kit, Ethanol 96%, Emerald Amp GT PCR mastermix (Takara), Gel Pilot 100 bp ladder supplied from QIAGEN (USA), Agarose 1.5%, Ethidium bromide solution 10 mg / ml and Tris borate EDTA (TBE) electrophoresis buffer (1x).

Glasses, instruments and apparatus:

PCR tubes 0.2 ml capacity, balance, microwave, monochannel micropipette (2-20 µl), sterile filter tips, calibrated cylinders, glass flasks, T3 Thermal cycler, type II A biosafety cabinet, gel documentation system, deionizer, applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA) and double distillator.

Parasite and DNA extraction:

Oocysts were collected from positive samples taken from each intestinal portion of each farm

separately and subjected for preservation and sporulation in potassium dichromate 2.5%. Preparation of samples using QIAGEN tissue laser LT in samples, followed by DNA extraction according to QIAamp DNA stool mini kit instructions, and analyzed by agarose gel electrophoresis following standard procedures of (**Sambrook *et al.*, 1989**).

Amplification of the ITS1 and sequence analyses:

The ITS1 regions of suspected species were amplified from genomic DNA using the species-specific primers. The sizes of the PCR products were calculated using a 100-bp ladder.

(Table 1): *Eimeria* species and its specific primers.

Gene	Sequence		Amplified product	Reference
E. tenella **ITS1**	**F**	**AATTTAGTCCATCGCAACCCTTG**	**278 bp**	**Lee *et al.*, (2011)**
	R	**CGAGCGCTCTGCATACGACA**		
E. necatrix **ITS1**	**F**	**TACATCCCAATCTTTGAATCG**	**778 bp**	**Schnitzler *et al.*, (1998)**
	R	**GGCATACTAGCTTCGAGCAAC**		
E. acevulina **ITS1**	**F**	**GGCTTGGATGATGTTTGCTG**	**586 bp**	
	R	**CGAACGCAATAACACACGCT**		
E. maxima **ITS1**	**F**	**GTG GGA CTG TGG TGA TGG GG**	**505 bp**	**Schnitzler *et al.*, (1999)**
	R	**ACC AGC ATG CGC TCA CAA CCC**		

Nucleotide sequencing of the ITS-1rDNA:

PCR product was purified using QIAquick PCR Product extraction kit. (Qiagen Inc. Valencia CA) following the manufacturer's recommendations. Purified PCR product was sequenced using Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA). The result was analyzed using Bio edit software. Sequence comparisons were performed using the BLASTN program from the National Center for Biotechnology information website http://www.ncbi.nlm.nih.gov/. BLAST and the sequences were aligned using CLUSTAL-W version 1.8 **(Thompson *et al.* 1994)**. A neighbor-joining tree was constructed using MEGA version 6 **(Tamura *et al.* 2013)**. Reference ITS-1 sequences from GenBank (accession number MF034720.1, KY117141.1, GQ153636.1, GQ856291.1, HQ263221.1, FJ447468.1, GQ856308.1, EU586057.1, KY117135.1, KY117147.1, JX477100.1, MF537631.1, KY117139.1, JQ061004.1, JQ061001.1, KY117150.1, KY117137.1, GQ153631.1,

FJ449692.1, KY117132.1, EU931579.1, JQ061002.1, KY117151.1, KY117148.1, KY117143.1, KY117134.1 , GQ856289.1, JQ061000.1, KY117152.1, KY117138.1, GQ153633.1, GQ856298.1 and JQ061003.1) and ITS-1 sequence of *E. tenella* in Coccivac-B (accession number JQ060999.1) were used in the alignment and in construction of the phylogenetic tree.

RESULTS

Amplification of different *Eimeria* ITS-1:

All cecal and fecal samples were positive for *E. tenella* infection (56 samples). Nine samples were positive for *E. acervulina*. Eight samples were positive for *E. necatrix*. Amplification of the ITS1 genomic rDNA of *E. tenella*, *E. necatrix* and *E. acervulina* which were produced an amplicon size of 278, 778 and 586 bp respectively from different positive samples as illustrated in (Table 2) , Fig.(1,2).

Nucleotide sequencing of the ITS-1rDNA:

We sequenced one of the positive samples from cecal portion (sample no. 23 from Gharbia governorate) which was highly positive for *E. tenella* only after results of PCR Fig. (3). We compared our sequence (accession number MG969842.1) with thirty-three published ITS-1 sequences in GenBank and ITS-1 sequence of coccivac-B (vaccinal strain). The vaccinal *E. tenella* sequence showed 98% similarity with our sequence. Our sequence revealed that 99% identity with Egyptian Giza strain (MF537631.1), 98% identity with Behera isolate (JQ061000.1), 99% identity with Matrouh isolate (JQ061001.1), 97% identity with Gharbia isolate (JQ061002.1), 96% identity with Alexandria isolate (JQ061003.1) and 98% identity with Kafer El-sheik isolate (JQ061004.1).

Table (2): Identification of different *Eimeria* species using PCR protocol.

Sample	Conventional PCR											
	Dropping				Duodenal portion		Jejunal Portion		illiual Portion		Cecal Portion	
	T	N	A	M	A	M	A	M	A	M	T	N
1	+	-	-	-	-	-	-	-	-	-	+	.
2	+	-	-	-	-	-	-	-	-	-	+	-
3	+	-	-	-	-	-	-	-	-	-	+	-
4	+	-	-	-	-	-	-	-	-	-	+	-
5	+	-	-	-	-	-	-	-	-	-	+	-
6	+	-	-	-	-	-	-	-	-	-	+	-
7	+	-	-	-	-	-	-	-	-	-	+	-
8	+	-	-	-	-	-	-	-	-	-	+	-
9	+	-	-	-	-	-	-	-	-	-	+	-
10	+	-	-	-	-	-	-	-	-	-	+	-
11	+	-	-	-	-	-	-	-	-	-	+	-
12	+	-	-	-	-	-	-	-	-	-	+	-
13	+	-	-	-	-	-	-	-	-	-	+	-
14	+	-	-	-	-	-	-	-	-	-	+	-
15	+	-	-	-	-	-	-	-	-	-	+	-
16	+	-	-	-	-	-	-	-	-	-	+	-
17	+	-	-	-	-	-	-	-	-	-	+	-
18	+	-	-	-	-	-	-	-	-	-	+	-
19	+	-	-	-	-	-	-	-	-	-	+	-
20	+	-	-	-	-	-	-	-	-	-	+	-
21	+	-	+	-	+	-	-	-	-	-	+	-
22	+	+	-	-	-	-	-	-	-	-	+	+
23	+	-	-	-	-	-	-	-	-	-	+	-
24	+	-	-	-	-	-	-	-	-	-	+	-
25	+	+	-	-	-	-	+	-	+	-	+	+
26	+	+	+	-	+	-	+	-	+	-	+	+
27	+	-	+	-	-	-	+	-	-	-	+	-
28	+	+	+	-	-	-	+	-	-	-	+	+

(+): positive sample **(-) : negative sample**

T: E.tenella **A: E.acervulina**

N: E.necatrix **M: E.maxima**

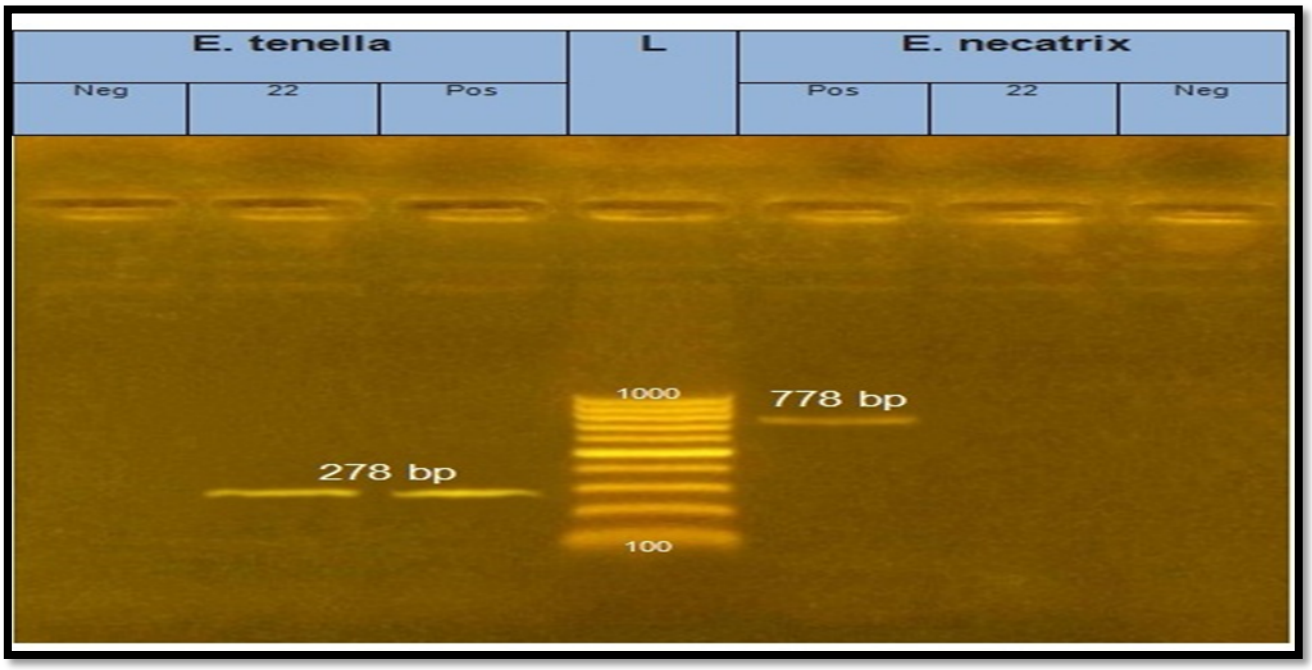

Fig. (1): PCR results targeting ITS-1 of the genomic rDNA of *E. tenella* and *E. necatrix* with an expected size of 278 and 778 bp. respectively M 100-bp ladder DNA with positive control and negative control.

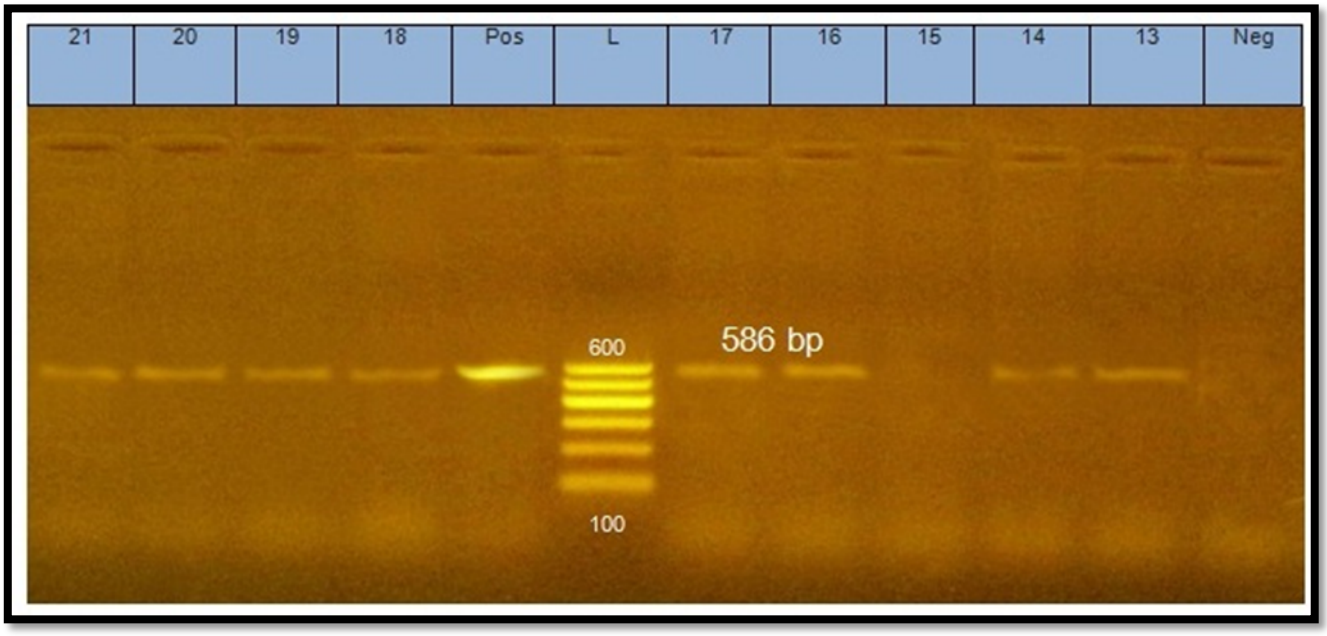

Fig. (2): PCR results targeting ITS-1 of the genomic rDNA of *E. acervulina* with an expected size of 586 bp. M 100-bp ladder DNA with positive control and negative control.

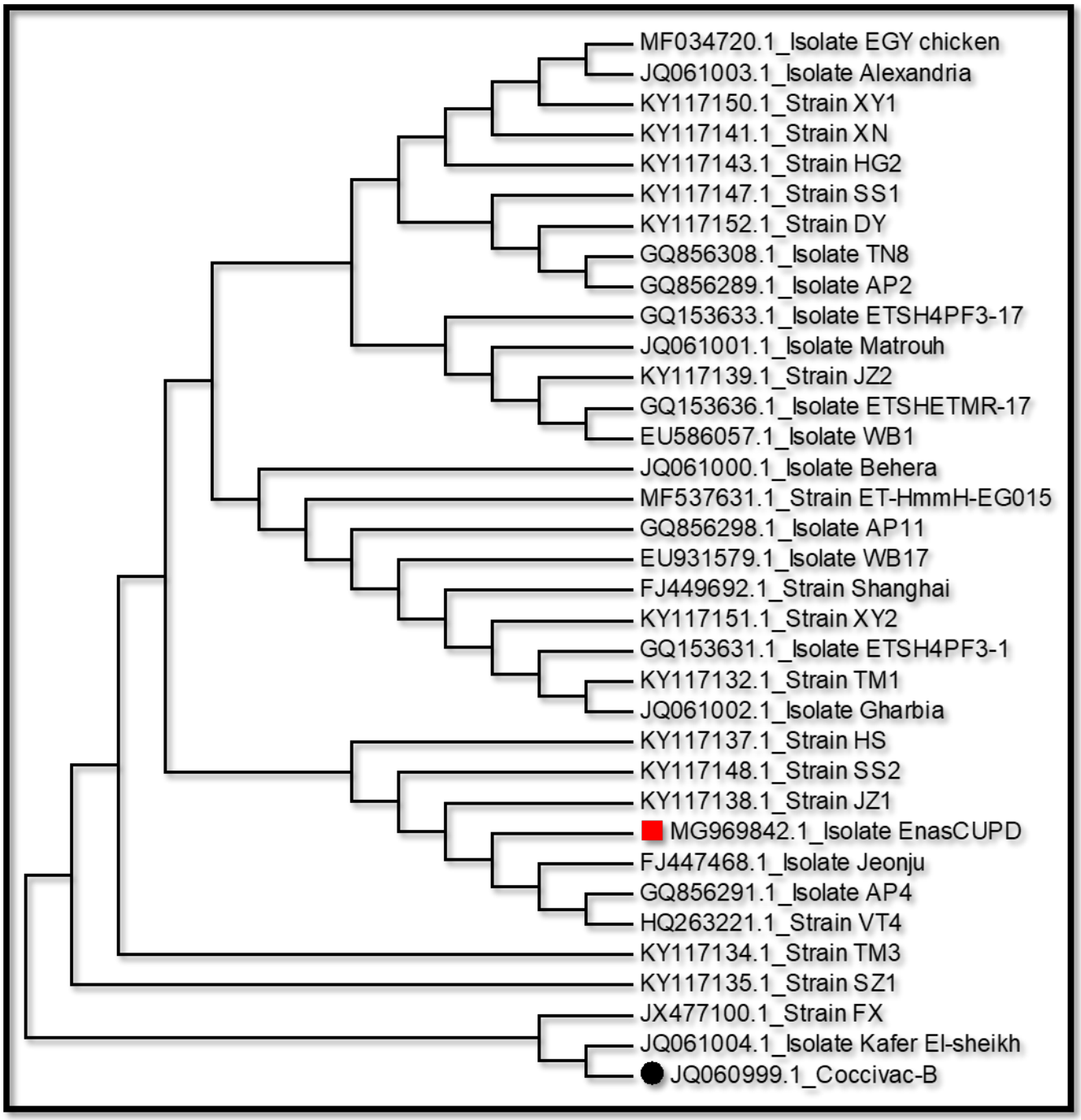

Fig. (3): Dendogram of the genetic relationship based on the sequence data of ITS-1 of rDNA isolated from Coccivac B® and our field strains of *E. tenella* and other reference sequences from GenBank, which used in the phylogenetic tree.

DISCUSSION

The results of identification of *Eimeria* species using conventional PCR revealed that, *Eimeria tenella* 100% then *Eimeria* necatrix 14.28 % from positive cecal and fecal samples, *Eimeria acervulina* 10.71% from positive intestinal and fecal samples. The present results agreed with those reported by (**Kumara*, et al.*, 2014*).* They found that 100% of samples was *E. tenella* using nested PCR and similar to **(Güven *et al.*, 2013)** who found that species-specific PCR assays confirmed the presence of *E. maxima*, *E. tenella, E. acervulina* and *E. praecox*, and nested PCR results showed the presence of *E. mitis* and *E. brunetti* from fecal and litter samples. In addition, agreed with **(Abd El-Hamid, 2007)** who reported that in Egypt, the most common *Eimeria* spp. is *E. tenella* where the infection rate is 20–100% and mortality is 20–60% with severe reduction in body gain and feed efficiency. The present results disagreed with those of **Shakshouk, 1984.** He reported that, the prevalence of *E. tenella* was 67.8% and *E. necatrix* 32.2%. The results of **Ali, 2006** disagreed with those results. He reported that the prevalence of *E. tenella*, and *E. necatrix* in 12 broiler flock were 75%, 17% respectively. In the meantime it disagreed with **(Carvalho *et al.*, 2011)** who reported that, *E. praecox* and *E. maxima* were identified in 100% of broiler chicken flocks using PCR. **(Gyorke *et al.*, 2013)** who identified four *Eimeria* species as *E. acervulina* (91%), *E. tenella* (61%), *E. maxima* (22%) and *E. praecox* (13%) in broiler farms from Romania. Our sequence revealed 98% identity with coccivac -B *E .tenella* ITS-1 sequence that is nearly agreed with the results by **(Awad *et al.*, 2013)** who reported that the vaccine *E. tenella* ITS-1 sequences shared 96.6 % similarity with the Kafer El-Sheikh strain. 94 % similarity with the Gharbia strain, 90 % similarity with the Alexandria strain and 78.4 % similarity with the Matrouh and Behera strains.

CONCLUSION

PCR results revealed that highly incidence of infection was for *E. tenella* in 100% of positive samples in both younger and growing ages of birds from examined broiler farms. While, incidence of *E. necatrix* was 14.28 % of positive samples in growing age (50-70 days) and incidence of *E. acervuline* was 10.71% of positive samples in growing age (50-70 days) than younger age (16 - 43 days).

REFERANCE

Abd El-Hamid, H.S., (2007): A Project on Studies of the Research Work Done on Chicken Coccidiosis in Egypt Presented to the Academy of Scientific Research and Technology, Ministry of High Education, Egypt, pp. 1–73.

Ali, A. M. N., Sahar, S. Mohamed, A.M., Badawy, A.A., Taha, M.M. (2006): "Identification of different *Eimeria* species isolated from broiler and replacement layers by using multiplex polymerase chain reaction in Egypt." Proc. 7th. Sci. Conference of the Egyptian Veterinary Poultry Association (EVPA). PP: .259-265.

Awad, A.M., El-Nahas, A.F., Abu-Akkada, S. S. (2013): Evaluation of the protective efficacy of the anticoccidial vaccine Coccivac-B in broilers, when challenged with Egyptian field isolates of *E. tenella*. Parasitol Res .112:113 - 121.

Carvalho, F. S., A. A., Wenceslau, M., Teixeira, J. A. M., Carneiro, A. D. B., Melo, G. R., Albuquerque (2011): Diagnosis of *Eimeria* species using traditional and molecular methods in field studies. Veterinary Parasitology 176, 95 - 100.

Enas, S. A., Youssef, I. Y., Nassif, S.A. (2018): Epidemiological studies on prevalence of different *Eimeria* species affecting broilers in Egypt. Journal of the Egyptian Veterinary Medical Association. 78 (4), 551-563.

Hafez, H.M., (2011): Enteric diseases of poultry with special attention to Clostridium perfringens. Pak. Vet. J., 31:175-184.

Kumar, S., R., Garg, A., Moftah, E. L., Clark, S. E., Macdonald, A. S., Chaudhry, O., Sparagano, P. S., Banerjee, K., Kundu, F. M., Tomley, D. P., Blake (2014): An optimised protocol for molecular identification of *Eimeria* from chickens. Veterinary Parasitology 199, 24 - 31.

Lee, L., Hong, S., Chung, Y. and Kim, O. (2011): Sensitive and specific identification by polymerase chain reaction of *Eimeria tenella* and *Eimeria maxima*, important protozoan pathogens in laboratory avian facilities. Lab Anim Res, 27(3), 255-258.

Lee, B.H., Kim, W.H., Jeong, J., Yoo, J., know, Y., Jung, B.Y., Know, J.H., Lillehoj, H.S., Min, W. (2010): Prevalence and cross-immunity of Eimeria species on Korean chicken farms. J. Vet. Med. Sci., doi:10.1292/jvms.09-0517.

Lew, A.E., Anderson, G.R., Minchin, C.M., Jetson, P.J., Jorgensen, W.K. (2003): Inter- and intra-strain variation and PCR detection of the internal transcribed spacer 1 (ITS-1) sequences of Australian isolates of *Eimeria* species from chickens. Vet Parasitol 112:33–50.

Molloy, J.B., Eaves, F.W., Jeston, P.J., Minchin, C.M., Stewart, N.P., Lew, A.E., Jorgensen,W.K. (1998): Detection of *Eimeria acervulina* using the polymerase chain reaction. Avian Dis. 42, 119-123.

Morris, G.M., Gasser, R.B. (2006): Biotechnological advances in the diagnosis of avian coccidiosis and the analysis of genetic variation in *Eimeria*. Biotechnol. Adv. 24, 590 - 603.

Güven, E., R. B., Beckstead, S., Kar, Z., Vantasever, Z., Karaer (2013): Molecular identification of *Eimeria* species of broiler chickens in Turkey. Ankara Üniv Vet Fak Derg, 60, 245-250.

Gyo¨rke, A., Pop, L., Cozma, V. (2013): Prevalence and distribution of *Eimeria* species in broiler chicken farms of different capacities. Parasite 2013, 20, 50.

Sambrook, J.; Fritscgh, E.F. Mentiates (1989): Molecular cloning. A laboratory manual. Cold spring Harbor Laboratotry press, New York.

Schnitzler, B.E., Thebo, P.L., Mattsson, J.G., Tomley, F.M., Shirley, M.W. (1998): Development of a diagnostic PCR assay for the detection and discrimination of four pathogenic Eimeria species of the chicken. Avian Pathol. 27, 490 - 497.

Schnitzler, B.E., Thebo, P.L., Tomley, F.M., Uggla, A., and Shirley, M.W. (1999): PCR identification of chicken Eimeria: a simplified read-out. Avian Pathol. 28, 89 -93.

Shakshouk, A.G. (1984): Studies on chicken coccidiosis with special reference to drug scattering. Thesis. M.Sc., Avian and Aquatic Animal Med. Fac. of Vet. Med. Alex. Univ. Egypt.

Su, Y., Fei, A.C., Tsai, F. (2003): Differential diagnosis of five avian Eimeria species by polymerase chain reaction using primers derived from the internal transcribed spacer 1 (ITS-1) sequence. Vet Parasitol 117:221–227.

Sun, X.M., Pang, W., Jia, T., Yan, W.C., He, G., Hao, L.L., Bentué, M., Suo, X. (2009): Prevalence of Eimeria species in broilers with subclinical signs from fifty farms. Avian Dis. 53, 301-305.

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

Thompson, J.D., Higgins, D.G., Gibson, T.J., and Clustal, W. (1994): Improving the sensitivity and progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucl Acids Res 22:4673-4680.

Tsuji, N., Kawazu, S., Ohta, M., Kamio, T., Isobe, T., Shimura, K., Fujisaki, K. (1997): Discrimination of eight chicken Eimeria species using the two-step polymerase chain reaction. J. Parasitol. 83, 966 - 970.

Woods, W.G., Richards, G., Whithear, K.G., Anderson, G.R., Jorgenses, W.K., Gasser, R.B. (2000): High-resolution electrophoretic procedures for the identification of five Eimeria species from chickens, and detection of population variation. Electrophoresis 21, 3558-3563.

Yao-Chi Sua, Andrew Chang-Young Fei, Fang-Mei Tsai (2003): Differential diagnosis of five avian Eimeria species by polymerase chain reaction using primers derived from the internal transcribed spacer 1 (ITS-1) sequence. Veterinary Parasitology .117, 221-227.