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Original Article

Molecular Characterization of *Eimeria* Species Naturally Infecting Egyptian Baldi Chickens

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Abstract

Background: Coccidiosis is a serious protozoal disease of poultry. The identification of *Eimeria* species has important implications for diagnosis and control as well as for epidemiology. The molecular characterization of *Eimeria* species infecting Egyptian baladi chickens was investigated.

Methods: *Eimeria* species oocysts were harvested from intestines of naturally infected Egyptian baldi chickens. The morphometry characterization of oocysts along with COCCIMORPH software was done. The DNA was extracted initially by freezing and thawing then the prepared samples was subjected to commercial DNA kits. The DNA products were analyzed through conventional polymerase chain reaction by using amplified region (SCAR) marker.

Results: The PCR results confirmed the presence of 7 *Eimeria* species in the examined fecal samples of Egyptian baldi breed with their specific ampilicon sizes being *E. acervulina* (811bp), *E. brunette* (626bp), *E. tenella* (539bp), *E. maxima* (272bp), *E. necatrix* (200bp), *E. mitis* (327bp) and *E. praecopx* (354bp). A sequencing of the two most predominant species of *Eimeria* was done, on *E. tenella* and *E. máxima*. Analysis of the obtained sequences revealed high identities 99% between Egyptian isolates and the reference one. Similarly, *E. maxima* isolated from Egyptian baldi chickens showed 98% nucleotide identities with the reference strain. Only single nucleotide substitution was observed among the Egyptian *E. tenella* isolates (A181G) when compared to the reference one. The Egyptian isolates acquired 4 unique mutations (A68T, C164T, G190A and C227G) in compared with the reference sequence.

Conclusion: This is the first time to identify the 7 species of *Eimeria* from Egyptian baladi chickens.

Introduction

Coccidiosis is a serious protozoal disease of poultry; characterized by damage to the intestinal epithelium, leading to inefficient feed conversion, and reduction in weight gain resulting in economic loss (1, 2). It is caused by protozoa of the genus *Eimeria*. Infection with one or several of the 7 *Eimeria* species infecting chickens including *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox*, and *E. tenella*, leads to variable signs from subclinical enteric infection to sub-acute mortality (3).

The baladi breeds became hybrid breeds, most of which are raised by growers for two or six weeks before being sold to village women. Village households raise the chickens as a source of eggs and meat or small income (4).

The identification of *Eimeria* species has important implications for diagnosis and control as well as for studying their epidemiology and population biology. Traditionally, species of *Eimeria* have been identified by morphometry and morphological features of the sporulated oocysts (using a microscope) and the specific host from which they originate (5). But, these criteria can be unreliable. For example, individual oocysts of *E. brunetti and E. maxima* can be the same or very similar in size and shape, thus preventing unequivocal identification. Biochemical, immunological and molecular techniques can overcome the limitations of traditional approaches for parasite identification (6, 7). In particular, DNA approaches, such as arbitrarily-primed PCR (AP-PCR), sequencing and specific PCR approaches, have proven useful for the identification, detection or characterization of *Eimeria* species. The PCR has been utilized to `fingerprint' avian *Eimeria* species (8, 9).

Therefore, the aim of this study was molecular identification of *Eimeria* species infecting Egyptian baladi chicken in Beni-Suef province.

Materials and Methods

Samples collection

A total of 700 gut samples of Egyptian baladi chickens suspected for coccidiosis from different farms of baladi chickens and small holders rearing system located in Beni-Suef province were microscopically examined for *Eimeria* species oocysts. A total of 140 positive fecal samples were collected from gut samples of the examined intestine to undergo the study.

Morphological identification

Oocyst morphology and size were determined by measuring length and width of 50 oocysts having similar morphological features using ocular micrometer (10, 11). Furthermore, Coccimorph identification of *Eimeria* species was identified with COCCIMORPH soft-ware (http://www.coccidia.icb.usp.br/coccimorph /). The software was downloaded from the Internet and the oocyst images (400× magnification) were uploaded for species identification as described online (12).

Molecular identification of Eimeria species by polymerase chain reaction (PCR)

The molecular identification of *Eimeria* species was carried out on pooled fecal samples. Fourteen pools of *Eimeria* species, each pool represented 10 fecal samples (a total of 140 samples). Coccivac D a living non attenuated vaccine containing 7 *Eimeria* species was used as control positive.

DNA extraction from fecal samples

DNA extraction was done according to Guven et al. (22) with slight modification. Fifty cycles of freezing, using of liquid nitrogen and thawing, in a shaking water path at 50C°, were carried out for complete rupturing of oocysts walls without adding sodium hypochlorite or use of glass beads. During this process, 10ul was taken and were examined under

the microscope (40 X) to ensure complete oocyst wall destruction. DNA extraction kit was used for genomic DNA extraction from tissue. DNA extracted according to the kits instructions (Biobasic, Inc. Canada, Cat. No. BS427**)**.

Polymerase chain reaction (PCR)

Samples were analyzed by PCR with the SCAR primers for *E. tenella, E. acervulina, E. necatrix, E. maxima, E. brunette* and *E. praecox* (9). In addition, ITS1 gene was performed for *E. mitis* (13, 14); this was showed in Table 1. PCR amplifications were individually made for each primer pair using 40 pmol of each primer, 1 U of platinum Taq DNA polymerase, dNTPs, $MgCl_2$ (Invitrogen®) and 5µL of target DNA in a 25 µL reaction volume (9). The PCR reaction conditions were as follow: initial denaturation cycle at 95°C for five minutes, 30 cycles of 94°C for 45 sec., annealing at 57°C: 63°C for 30 sec for each species primer (as mentioned at Table 1) and 72°C for one and half minutes. The final extension step was at 72°C for seven minutes. Amplifications were carried out in 0.2 mL polypropylene tubes using a Labnet International, Inc software v3.3.4c, Multigene model: Tc9600-G.

Table 1: The primers used in PCR running

Species	Primer Name	Primer sequences (5'→ 3')	Amplicon size (bp)	Annealing temperature
E. acervulina	Ac-01F	AGTCAGCCACACAATAATGGCAAACATG	811	60◦C
	Ac-01R	AGTCAGCCACAGCGAAAGACGTATGTG		
E. brunetti	Br-01F	TGGTCGCAGAACCTACAGGGCTGT	626	63◦C
	Br01R	TGGTCGCAGACGTATATTAGGGGTCTG		
E. tenella	Tn-01F	CCGCCCAAACCAGGTGTCACG	539	60◦C
	Tn-01R	CCGCCCAAACATGCAAGATGGC		
E. praecox	Pr-01F	AGTCAGCCACCACCAAATAGAACCTTGG	354	58◦C
	Pr-01R	GCCTGCTTACTACAAACTTGCAAGCCCT		
E. mitis	Mt-01F	TATTTCCTGTCGTCGTCTCGC	327	57◦C
	Mt-01R	GTATGCAAGAGAGAATCGGGA		
E. maxima	Mx-01F	GGGTAACGCCAACTGCCGGGTATG	272	58◦C
	Mx-01R	AGCAAACCGTAAAGGCCGAAGTCCTAGA		
E. necatrix	Nc-01F	TTCATTTCGCTTAACAATATTTGGCCTCA	200	57◦C
	Nc-01R	ACAACGCCTCATAACCCCAAGAAATTTTG		

F = foward primer; R = reverse primer.

Sequencing of 2 isolated Eimeria species from the Egyptian baladi chickens for confirmation of PCR results

The gel extraction and purification of PCR products was done. Confirmation of the results of PCR was applied by direct sequencing of 539 bp and 272 bp of SCAR marker gel purified PCR products of the selected most prevalent *Eimeria* species (*E. tenella* and *E. maxima*, respectively) isolated from Egyptian baladi. Briefly, PCR-products were excised from gel and were purified with Wizard® SV gel and PCR clean up system according to the Manufacturer's instructions. The DNA was dried and shipped for direct sequencing; which was performed by Macrogen Inc**.** (908 World Meridian Venture Center #60-24, Gasan-dong Geumchun-gu, Seoul 153-781, Korea) in both forward and reverse directions using the same primer sets that have been used for amplification of each PCR product. Sequencing was done on an Applied Biosystems 310 automated DNA sequencer using cycle sequencing ABI prism Big Dye terminator chemistry (a terminator cycle sequencing ready reaction kit) (Perkin-Elmer/Applied Biosystems, Foster City, CA USA). A BLAST analysis was initially performed to establish

sequence identity to Gen Bank accessions (15). Comparative sequences analyses were performed using CLUSTAL W Multiple Sequence Alignment Program, version 1.83 (http://www.genome.jp/tools/clustalw/).

Results

Identification of Eimeria species infecting Egyptian baldi chickens

Morphological identification

The morphological features of the isolated *Eimeria* species revealed that 7 species were suspected from the examined Egyptain baldi chickens (*E. acervulina, E. maxima, E. mitis, E. necatrix, E. brunetti ,E. tenella* and *E. praecox)* Table 2.

COCCIMORPH *identification*

Identification of *Eimeria* spp. using COCCIMORPH software revealed suspected 7 species of *Eimeria*.

Molecular identification

The results of molecular identification proved the presence of 7 *Eimeria* species in the examined Egyptian baldi chicken fecal samples. The primers were sufficiently sensitive and specific enabling the discrimination of seven *Eimeria* species. The amplified fragments presented different sizes: *E. acervulina* (811 bp), *E. brunette* (626 bp), *E. tenella* (539 bp), *E. mitis* (310 bp), *E. praecox* (354 bp), *E. maxima* (272 bp) and *E. necatrix* (200 bp) (Fig. 1 & Fig. 2).

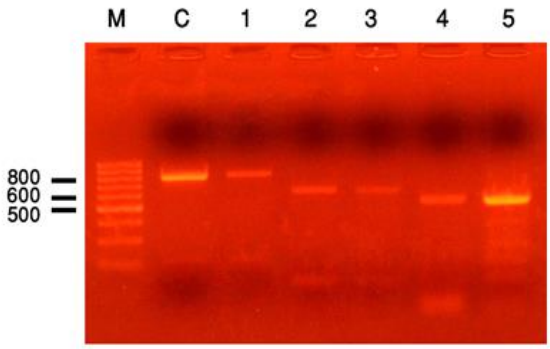

Fig. 1: Amplicone of *E. acervulina*(811bp) Amplicone of *E. burnetti* (626bp)/ Amplicone of E. tenella (539bp)/ Lane M molecular weight marker 100bp**,** lane C control positive for *E. acervulina* 811bp, lane 1 pool No 3 positive for (*E. acervulina* 811bp), lane 2 control positive for *E. brunetti* 626bp, lane 3 pool No 3 positive for (*E. brunetti* 626bp), lane 4 control positive for *E. tenella* 539bp and lane 5 pool No positive for (*E. tenella* 539bp)

Table 2: Morphological and molecular identification of *Eimeria* species isolated from Egyptian baladi chickens

Pool No	Identified *Eimeria* species by morphology							Identified *Eimeria* species by PCR						
	Ac	Br	Te	Pr	Mi	Ma	Ne	Ac	Br	Te	Pr	Mi	Ma	Ne
1	+		+		+	+	+			+		+	+	+
2	+		+			+	+			+			+	+
3	+	+	+			+	+	+	+	+			+	+
4			+	+		+	+			+	+		+	+
5			+				+			+				+
6	+					+	+		+				+	
7			+			+				+				
8	+		+			+	+			+			+	+
9			+		+	+	+			+			+	
10						+	+						+	
11						+							+	
12			+				+			+				+
13					+	+						+	+	
14			+			+	+			+			+	

Pools from 1-14 are fecal samples of Egyptian baldi chickens./ Ac. *E. acervulina*, *Br. E. brunette*, *Te. E. tenella*, Ma. *E. maxima*, Ne. *E. necatrix*, *Mi. E. mitis*, Pr. *E. praecopx* (354bp)

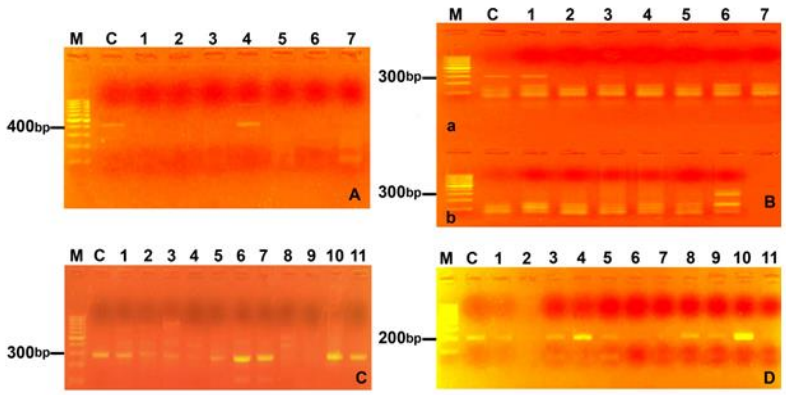

Fig. 2: A. Amplicone of *E. praecox* (354bp). Lane M molecular weight marker 100bp**,** lane C control positive for *E. praecox*. Lane 4 represents the pool No 4, the only positive pool for *E. praecox* from 14 pool. B. Amplicone of *E. mitis* (327bp). Lane M molecular weight marker 100bp**,** lane C control positive for *E. mitis*. Lane 1a represent pool No 1, lane 6 b represent pool No 13 C. Amplicone of *E. maxima* (272bp). Lane M molecular weight marker 100bp**,** lane C control positive for *E. maxima*. Pools No 1, 2, 3, 5, 6, 7, 10 and 11 were positive for *E. maxima*. D. Amplicone of *E. necatrix* (200 bp). Lane M molecular weight marker 100bp**,** lane C control positive for *E. necatrix*. Pools No 1, 3, 4, 5, 8, 9 and 10 were positive for *E. necatrix*

AY571634.1 E. tenella Tn-K04-539 CCGCCCAAACCAGGTGTCACGAATACACACAAAAGAGGAATGACCCTCATGCAATATCGCCACTAGTACTATTAAAACTGCCCACCTATTTAAGATGCAATTTTTCACTATAAGGAAGAA [120]
Egyptian native E._tenella ..... [120]

AY571634.1 E. tenella Tn-K04-539 AAAACGAAGAGACGAATCGCTCATGCGGCAGTACGTTGCGGGGTGCAGCTACGGTGTTCTAAAAGACGAAGAACTACAAATGATATTTGCAGAAATCGACAAAAATTGCGACGGGTGAGC [240]
Egyptian native E._tenella .....G..... [240]

AY571634.1 E. tenella Tn-K04-539 CTCATTGCTCTCTTCTTTCCTGTTGGAAAAAATTGCCGAACTTTAGCTAGAACTAAGCCCACTGTGAGTGTAGGCTGCGTAGTGCTCTACAGTCTTTGGGACTTTTAACTCTTCTAAATC [360]
Egyptian native E._tenella ..... [360]

AY571634.1 E. tenella Tn-K04-539 ACACTGAGCTGCCACACTGAGCTGCCGATGCGGAATGAGTATACGTAGAGCTGTGACTGATTCTCACAATGTTGATGGTGCTGTGCAGGTATGTCGACTTTTATGAATTCTGCGACTTGA [480]
Egyptian native E._tenella ..... [480]

AY571634.1 E. tenella Tn-K04-539 TGACGGCGGCGGACTGAAGCAGTCTGTGGACCAGAGAGCCATCTTGCATGTTTGGGCGG [539]
Egyptian native E._tenella ..... [539]

AY571588.1 E. maxima Mx-A09-1008 GGGTAACGCCAACTGCCGGGTATGTTGTTGCTAAAATACTTCAGTAGTCGCGAGGGTGCACTGCGATAAGCTTTGGACACCATTGGATATGTGGAAAAGTTAACTCGTATGGGTCTATAC [120]
Egyptian native E._maxima .....T..... [120]

AY571588.1 E. maxima Mx-A09-1008 ATACGTAGCGAGGGATTATAAGGCTACATGATCCAAGAATGAACGAACTTAGTGCCCTTTCAATGTTCAGCACATGGCAGCAACCCCCCTATGGCTAAGCTAGACTCGGGGAGGAAAACA [240]
Egyptian native E._maxima .....T.....A.....T.....G..... [240]

AY571588.1 E. maxima Mx-A09-1008 AATATCTAGGACTTCGGCCTTTACGGTTTGCT [272]
Egyptian native E._maxima ..... [272]

Fig. 3: Deduced nucleotide sequences of the 539bp of *E. tenella* and 272bp of *E. maxima* RAPD-SCAR marker gene
*Egyptian native means Egyptian baladi **Dots indicates identical nucleotides

Sequence analysis of RAPD-SCAR marker gene

Four PCR products were sequenced including isolates obtained from native Egyptian baladi chickens infected with the most prevalent *Eimeria* species (*E. tenella* and *E. maxima*). Sequencing was done using *Eimeria* species-specific primers. Sequences *E. tennela* revealed 99% overall identities between Egyptian isolates and the reference one (Gen Bank accession No. AY5716 34.1). Similarly, *E. maxima* isolated from baldi breed showed 99% nucleotide nucleotide identities with the reference strain (Gen Bank accession No. AY571588.1). Only single nucleotide substitution was observed among the Egyptian *E. tenella* isolates (A181G) when compared to the reference one. On the other hand Egyptian *E. maxima* isolates acquired 4 unique mutations (A68T, C164T, G190A and C227G) when analyzed with the reference sequence (Fig. 3).

Discussion

Initially, 7 *Eimeria* species were recorded morphologically and molecularly in baldi chickens in Beni Suef province. *Eimeria* species identified were *E. tenella, E. acervulina, E. necatrix, E. maxima, E. mitis, E. brunette* and *E. praecox*. In Egypt, no study reported the 7 species of Eimeria species but numerous studies revealed the morphological features of *Eimeria* species**;** 5 *Eimeria* species *(E. necatrix, E. tenella, E. acervulina, E. mitis and E. maxima*) were recorded among the examined Egyptian native chicks (16, 17). Moreover, 6 *Eimeria* species were reported from 4 Egyptian governorates (Qalubeia, Sharkeia, Fayoum and Giza) which were *E. necatrix, E. acervulina, E. pracox, E. maxima, E. mitis* and *E. tenella* (18). This variation with the present work may be attributed to the area of the study. Worldwide, many studies estimated the 7 Eimeria species in different breeds and localities (19- 22).

Traditionally, identification of *Eimeria* spp. has been based on the morphological characteristics of oocysts, parasite biology, clinical signs of the affected animals, and the typical macroscopic lesions assessed during necropsy. However, in a natural setting mixed infections of different *Eimeria* spp. are commonly encountered and morphological characteristics and pathological changes may overlap, hindering accurate diagnosis and undermining detection of subclinical disease (23). COCCIMORPH tool is, an innovative approach developed for identification of eimerian oocysts of poultry and rabbits through which digital images of unidentified sporulated *Eimeria* ocysts are uploaded for species identification based on sporulated oocyst morphology (12). COCCIMORPH tool recorded the same findings of microscopical examination in both of baldi chickens.

Due to the thick and resistant oocyst wall of *Eimeria* spp. (24), several means of breaking down the oocyst wall have been described, including hot phenol incubation (25), repeated freezing and thawing (26), enzyme digestion after sodium hypochlorite incubation (27), passage through a high pressure cell (28), grinding in liquid nitrogen (29) and grinding by glass beads (9) and they mentioned that the most commonly used protocol for DNA extraction from *Eimeria* species is based on glass bead grinding of oocysts combined with classic phenol-chloroform DNA extraction. In the present study, we overcame this problem by freezing in liquid nitrogen and thawing in a water bath at 50 °C. This caused breaking of the oocysts wall and liberation of oocysts content facilitate DNA extraction by using of tissue DNA extraction kits under our laboratory conditions. This method for DNA extraction was like that of Guven et al. (22) except freezing and thawing for 50 times and without using sodium hypochlorite, glass beads or stool DNA extraction kits.

The measurements of the oocysts can vary due to changes in metabolism of parasites or birds, and even in the value of the shape morphometric indices that may overlap and lead to misleading conclusions regarding the spe-

cies (30). Consequently, application of molecular tools for identification and characterization of these parasites has been carried out to ensure isolation of 7 *Eimeria* spp. in Beni-Suef province by PCR technique.

The results of molecular diagnosis by conventional PCR technique using amplified region (SCAR) marker proved the presence of 7 *Eimeria* species in the examined fecal samples of baldi chickens with their specific ampilicon sizes (*E. acervulina* (811bp), *E. brunette* (626bp), *E. tenella* (539bp), *E. maxima* (272bp), *E. necatrix* (200bp), *E. mitis* (327bp), *E. praecopx* (354bp). PCR findings coincide with the morphological findings of baldi chicken *Eimeria* species.

PCR confirmed the presence of 7 *Eimeria* species in Beni Suef province for the first time. In Egypt, this result more or less as of other study (9), even they used the same primers but they reported *Eimeria* species in broilers chickens by use of multiplex PCR *(E. necatrix, E. acervulina, E. pracox, E. maxima, E. mitis and E. tenella)*. Moreover, using of the same primers (SCAR) detected 7 *Eimeria* species by multiplex PCR (31, 11). Besides, 7 *Eimeria* species was recorded by PCR amplification with species-specific primers for the internal transcribed spacer (ITS) sequence or the small RNA subunit sequence of each of the seven species of *Eimeria* (30, 32, 33). On other hand, the PCR results of *Eimeria* species shouldn't detect 7 species but may give 2 species only; *Eimeria praecox* and *Eimeria mitis* (34). This variation of PCR results may be because of locality, used primers, methods of DNA extraction and DNA quantity in the used samples. All these studies were investigated *Eimeria* species other breeds of chickens. Therefore, the present study was unique in investigation of *Eimeria* species in Egyptian baladi chickens.

Sequencing of the excised DNA bands obtained after amplification of DNA of the two most prevalent *Eimeria* species, *E. tenella* and *E. maxima*, using *Eimeria* species-specific primers was done. Blast analysis of the obtained sequences confirmed the species specificity of the used primers as previously reported (9); *E. tenella* multisequence alignment indicated high nucleotide identities (99%) between Egyptian isolates and the reference one. Similarly, *E. maxima* isolated from Egyptian baladi breed showed 98% nucleotide identities with the reference strain. Only single nucleotide substitution was observed among the Egyptian *E. tenella* isolates (A181G) when compared to the reference one. On the other hand Egyptian *E. maxima* isolates acquired 4 unique mutations (A68T, C164T, G190A and C227G) when analyzed with the reference sequence. The significance of these mutations cannot be assessed specifically with the non-translatable nature of the target SCAR marker into functional amino acids. Further studies would be done using different primer sets for amplification of other genes.

Conclusion

Baldi chickens were found infected by the 7 species of *Eimeria* first time in Egypt. The identification of *Eimeria* species was confirmed by PCR. Mutations and differences of *Eimeria* spp genomes between *Eimeria* species isolates compared with the reference strains have to be studied further.

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