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GENOTYPING IDENTIFICATION OF LOCALLY ISOLATED *EIMERIA* SPECIES USED IN EVALUATION OF IMPORTED CHICKEN COCCIDIOSIS VACCINES

By

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ABSTRACT

A multiplex polymerase chain reaction (PCR) assay was used for the detection of the circulating *Eimeria* species in the domestic chicken flocks in 2 Egyptian governorates. Three types of intestinal samples were collected from freshly dead birds diagnosed to have coccidial infestation. These samples were pooled from duodenal, cecal and the remained intestinal contents separately. The oocysts of each sample were washed, purified and concentrated. DNA was extracted from each sample and utilized in a multiplex PCR assay using 7 pairs of species-specific primers. *Eimeria* acervulina (E. acervulina) and E. praecox were detected in the pooled duodenal samples, only E. tennella was detected in the cecal samples, and the known seven *Eimeria* species were detected in the mixed intestinal samples. The results of this work assure the infestation of the Egyptian chicken flocks with all the known seven *Eimeria* species.

INTRODUCTION

Avian coccidiosis is an intestinal disease caused by protozoan parasites of the genus Eimeria, occurs worldwide. It is considered to be one of the most economically important diseases of domestic poultry. Seven distinct *Eimeria* species can infect chickens, causing intestinal lesions of variable extent and severity, reducing the absorptive function of the mucosa, and thus leading to weight loss, diarrhoea, poorer feed conversion and higher mortality of the affected flocks (**McDougald and Reid, 1997**).

For many years, prophylactic use of anticoccidial feed additives has been the primary means of controlling coccidiosis in poultry industry and has played a major role in the growth of this industry. However, development of anticoccidial resistance has threatened the economic stability of poultry industry. So, live *Eimeria* species vaccines are being increasingly used in the field (**Allen and Fetterer, 2002; Chapman *et al.*, 2002**).

In the present study we tried to detect the already circulating *Eimeria* species in the field; as an initial step toward the isolation, identification and purification of all the Egyptian species of Eimeria. Each species of which will be used in the challenge of the vaccinated experimental chickens to determine the protection percentage produced by the examined vaccines against each *Eimeria* species.

As a consequence, the aim of the present study is the detection of the circulating *Eimeria* species in the Egyptian domestic fowl using multiplex PCR technique.

MATERIAL AND METHODS

Parasite:

a. Field samples:

Samples were collected from balady flocks that had no history of previous vaccination against coccidiosis from poultry farms in Monofia and Kalubia governorates. Samples were taken from three locations of the intestine of the freshly dead birds (that died due to coccidiosis), the first sample was collected from the duodenal content, the second was from the cecal content and the third was from the content of jejunum, ilium and rectum (the rest portions of the intestine). Duodenal samples were pooled together also the same was done for cecal samples and intestinal samples.

Samples were preserved in 2% potassium dichromate and transported to the laboratory for further investigations.

Eimeria oocysts of each sample were washed, concentrated, purified and sporulated following the standard procedures described by **Long *et al.*, (1976)**.

b. Vaccinal samples:

Commercial coccidiosis vaccine (Coccivac-D); Shering-Plough; Animal Health Corporation, USA, Batch No. 159/03 was used as positive control.

DNA extraction:

DNA extraction and precipitation was done followed the procedures described by **Zhao *et al.*, (2001)**, briefly: 5 x 10^7 oocysts of each sample were cleaned with sodium hypochlorite solution (5-6% active chlorine) for 10 minutes

at 4°C, washed 3 times with deionized water and resuspended in extraction buffer (10mM Tris-HCl, pH 8.0; 50mM EDTA, pH 8.0). The oocysts and sporocysts were fully disrupted by vortexing with half the volume of 425-600um acid washed glass beads. The lysate was centrifuged at 14000 g for 10 minutes to eliminate debris and digested with DNAse free RNAse A (Stratagene Co.) (20ug/ml) at 37°C. A further digestion with proteinase K (Stratagene Co.) (100ug/ml) and SDS (0.5%) was carried out at 50°C for 2 hours.

The DNA was then extracted once with 1 volume of phenol, phenol/chloroform and chloroform then precipitated with ethanol and ammonium acetate. The pellet was washed with 70% ethanol and resuspended in TE buffer (10mM Tris-HCl, pH 8.0; 0.1mM EDTA; pH 8.0). DNA was quantified by absorbance at 260 nm (using spectrophotometer, Beckmann, USA).

Primers:

Species-specific primers designed by **Fernandez *et al.*, (2003)** were used. Table (1) lists the primers used for the multiplex PCR assay and their respective sequences. All the primers presented a length varying from 21 to 29 bases, with a melting temperature around 62°C.

Polymerase Chain Reaction (PCR):

Single standard PCR amplifications were used for individual reactions of each primer pair in order to obtain a common reaction condition for the seven *Eimeria* species. This condition was then adapted for multiplex PCR reactions following the recommendations of **Henegariu *et al.*, (1997) and Fernandez *et al.*, (2003)**.

Multiplex amplifications were typically performed with 200 uM dNTps, 2.4 mM $MgCl_2$, 5 u of Taq polymerase and 10 X amplification buffer in a final volume of 50 ul. Different primer concentrations were used as described in Table (1).

DNA template mixtures were composed of 50 ng of genomic DNA of each pooled sample. Cycling conditions consisted of an initial denaturation at 96°C for 5 minutes and 30 cycles for 1 minute at 94°C, 1 minute at 65°C and 1 minute at 72°C with a final extension step of 72°C for 10 minutes. All amplification reactions were performed with a T-gradient thermal cycler (Biometra Co.).

All the amplification products were analyzed by separation on 1.5% agarose gels stained with ethidium bromide, and visualized using UV transilluminator.

Table (1): Primer sequences of *Eimeria* species of domestic fowl used for the multiplex PCR assay

Species	Primer designation	Primer sequence	Amplicon size (bp)	Primer conc.
E. acervulina	Ac-01-F	AGTCAGCCACACAATAATGGCAAACATG	811	0.7μM
	Ac-01-R	AGTCAGCCACAGCGAAAGACGTATGTG		
E. brunette	Br-01-F	TGGTGGCAGAACCTACAGGGCTGT	626	0.85μM
	Br-01-R	TGGTCGCAGACGTATATTAGGGGTCTG		
E. tenella	Tn-01-F	CCGCCCAAACCAGGTGTCACG	539	0.55μM
	Tn-01-R	CCGCCCAAACATGCAAGATGGC		
E. mitis	Mt-01-F	AGTCAGCCACCAGTAGAGCCAATATTT	460	0.55μM
	Mt-01-R	AGTCAGCCACAAACAAATTCAAACTCTAC		
E. praecox	Pr-01-F	AGTCAGCCACCACCAAATAGAACCTTGG	354	0.70μM
	Pr-01-R	GCCTGCTTACTACAAACTTGCAAGCCCT		
E. maxima	Mx-01-F	GGGTAACGCCAACTGCCGGGTATG	272	0.55μM
	Mx-01-R	AGGAAACCGTAAAGGCCGAAGTCCTAGA		
E. necatrix	Nc-01-F	TTCATTTCGCTTAACAATATTTGGCCTCA	200	0.70μM
	Nc-01-R	ACAACGCCTCATAACCCCAAGAAATTTTG		

* Primer conc.: primer concentration is the concentration of each primer pair that utilized in a multiplex PCR reaction.

RESULTS

Amplification of DNA from vaccinal sample in individual reactions produced a single fragment (PCR product) in each reaction, when the primer pair specific for *Eimeria acervulina* (*E. acervulina*) was used the produced fragment was of 811 bp length, lane 2, (Fig. 1).

This result was repeated with each *Eimeria* species when its specific primer pair was used and the length of the produced fragments were 626 bp for *E. brunetti*, 272 bp for *E. maxima*, 460 bp for *E. mitis*, 200 bp for *E. necatrix*, 354 bp for *E. praecox* and 539 bp for *E. tenella*; lanes 3, 4, 5, 6, 7 and 8 respectively as shown in Fig. (1).

Amplification of DNA from the same vaccinal sample in a multiplex PCR assay (using the seven primer pairs) produced 7 fragments representing the seven *Eimeria* species and indicating their presence in the examined sample as shown in Fig. (1), lane 9.

Amplification of DNA from the duodenal samples in a multiplex PCR assay using the seven primer pairs produced 2 fragments of 811 bp and 354 bp

length that are specific for *E. acervulina* and *E. praecox* respectively as shown in Fig. (2), lane 2.

Amplification of DNA from the cecal samples in a multiplex PCR assay using the seven primer pairs produced only one fragment of 539 bp length indicating the presence of only *E. tenella* (Fig. 2 lane 3).

Finally, amplification of the DNA of the samples of the remained intestine under same conditions produced seven fragments of 811 bp, 626 bp, 272 bp, 460 bp, 200 bp, 354 bp and 539 bp indicating the presence of *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* and *E. tenella* respectively as shown in Fig. (2) lane 4.

DISCUSSION

The PCR based diagnostic assay were used for the simultaneous detection of the 7 *Eimeria* species that infect domestic fowl. The test can be performed using either individual or single-tube multiplex reactions with similar results, thus this test is highly economic, saving time and money, highly specific and sensitive (**Fernandez *et al.*, 2003**).

The multiplex PCR assay utilized here could also be converted in the future to a quantitative assay by the use of fluorescent probes in a real-time PCR equipment (**Fernandez *et al.*, 2003**). Using this approach, one could detect the number of *Eimeria* species and concentration of each in a given coccidial vaccine vial. In our study, we could detect the different species of *Eimeria* circulating in Egypt which could be for the first time in Egypt; but we still need more time, investigations and efforts to purify and obtain well identified and pure oocysts of each *Eimeria* species separately.

Our study detect and discriminate the seven *Eimeria* species that were known to infect the domestic fowl not only in Egypt but worldwide. This result agreed with many authors (**Braunius, 1986; Litjeus, 1986; McDougald *et al.*, 1986 and McDougald *et al.*, 1987**).

Although our samples were collected from poultry farms had no history of previous vaccination against coccidiosis, we could not assure the origin of the detected *Eimeria* species is field strains or vaccinal strains. So, further investigations are required to differentiate between field isolates of *Eimeria* and the vaccinal strains of each *Eimeria* species.

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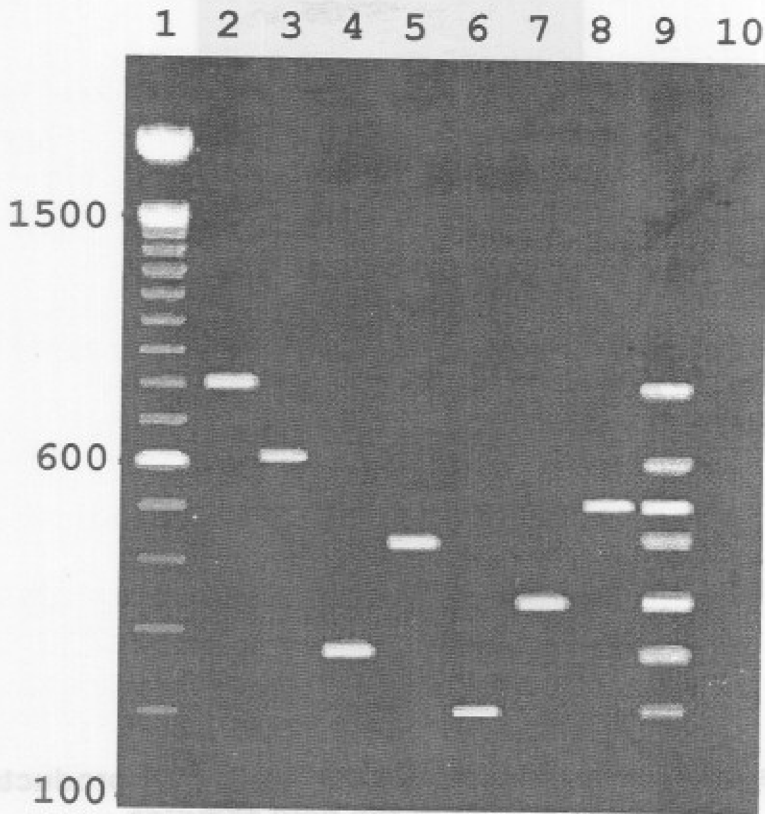

Fig (1): Agarose gel electrophoresis of individual and multiplex PCR products using the mixed DNA templates of the "Coccivac-D" that contains all the *Eimeria* species

Lane (1): DNA ladder.
Lane (2): The individual PCR product of *E. acervulina.*
Lane (3): *E. Brunette.*
Lane (4): *E. maxima.*
Lane (5): *E. mitis.*
Lane (6): *E. necatrix.*
Lane (7): *E. praecox.*
Lane (8): *E. tenella.*
Lane (9): The multiplex PCR products of the 7 *Eimeria* species.
Lane (10): Control with no starting DNA.

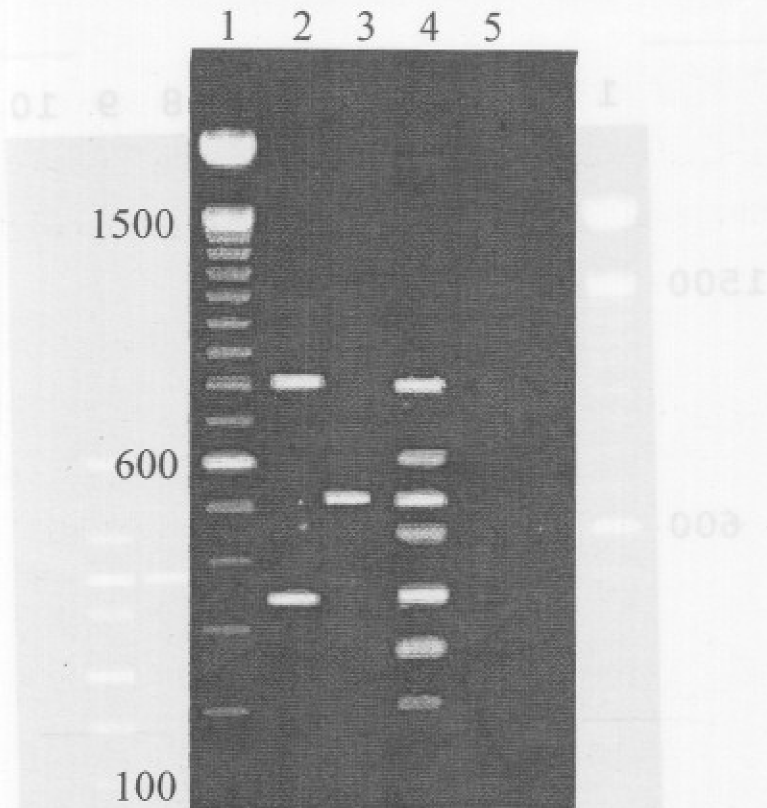

Fig (2): Agarose gel electrophoresis of multiplex PCR products using the DNA templates of the field samples

Lane (1): DNA ladder.

Lane (2): The multiplex PCR product of the pooled duodenal samples showing presence of *E. acervulina* and *E. praecox*

Lane (3): cecal samples showing presence of *E. tenella*

Lane (4): Mixed intestinal contents showing presence of the 7 species.

Lane (5): Control with no starting DNA.

الملخص العربي

توصيف عترات كوكسيديا دواجن معزولة محلياً باستخدام تقنيات الهندسة الوراثية والتى تستخدم فى معايرة لقاحات الكوكسيديا المستوردة

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أستخدم تفاعل البلمرة التسلسلى المركب لتحديد أنواع الإيميريا المتسببة فى مرض الكوكسيديا الذى يصيب قطعان الدجاج فى مصر .حيث تم تجميع عينات من أمعاء دجاج حديث النفوق فى مزارع مصابة بالكوكسيديا فى محافظتين من محافظات مصر .وقد تم تقسيم كل الامعاء إلى ثلاثة أجزاء، الأثنى عشر، الأعورين وباقى الأمعاء .ثم جمعت عينات الأثنى عشر معا وكذا عينات الأعورين معاً وأبقت باقى الأمعاء ثم تم غسل وتنقية وتركيز حويصلات الايميريا الموجود فى كل جزء من الأمعاء على حدة .وقد تم استخلاص الحامض النووى الديوكسى من هذه الحويصلات حيث استخدم فى تفاعل البلمرة التسلسلى المركب والمحتوى على سبعة ازواج من البريمرات الخاصة بالسبعة أنواع المعروفة عالمياً من الايميريا وقد دلت نتائج التفاعل على وجود نوعين من الايميريا فى عينات الأثنى عشر وهى أيميريا اسيرفيولينا وايميريا براكوكس .كما دلت على وجود نوع واحد فقط من الايميريا فى عينات الأعورين وهى ايميريا تينيلا .كما دلت النتائج على وجود كل السبعة أنواع المعروفة من الايميريا فى عينات باقى الأمعاء .واخيرا هذه الدراسة أكدت يقيناً وجود السبعة أنواع المعروفة عالمياً من الايميريا فى قطعان الدجاج المصرى.