Intra-Specific Variation Of *Eimeria Necatrix* Of Chicken Using Random Amplified Polymorphic DNA (RAPD) Technique In Egypt

El-Khatib, R.M.; Gamal El-Din, H.Y.; Hegazy, N.A.M. and Daoud, A.M.
*Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo.

ABSTRACT

RAPD technique and electrophoresis in polyacrylamide gel provided characteristic fingerprints for *Eimeria necatrix* isolates.

The isolate of *Eimeria necatrix* was collected from 3 different localities in Egypt (El-Giza, Kena and Damanhour) 4 oligonecleotides reflected different phylogenetic relation among the 3 E. necatrix, isolate.

The degree of similarity between the three isolates reflected the presence of both species-specific and even strain-specific bands, the statistical analysis suggested a doser phylogentic relationship between El-Giza and Damanhour. *Eimeria necatrix* isolates which are distantly relation to Kena.

RAPD technique could easily differentiate with a great potential the minor change in the genomes of the related genotypes of *Eimeria necatrix* isolates in Egypt.

INTRODUCTION

Coccidiosis of domestic fowl is a world disease caused by obligatory intracellular protozoa of the genus Eimeria. It is responsible for important economic losses in poultry production. The disease is characterized by enteric lesions of variable extent and severity, reducing the absorptive function of the intestinal mucosa, thus leading to weight loss, diarrhea, poorer feed conversion and mortality in the affected flock *(1,2)* developed the random amplified polymorphic DNA (RAPD) technique which is based on the amplification of anonymous targets by the use of arbitary primers.

This generate fingerprint of multiple bands. Because no previous knowledge of nucleotide sequence is required, RAPD has been used for discriminating parasite population, such as Eimeria *(3-8)*.

The objectives of this study were isolation and identification of *Eimeria necatrix* from 3 different Egypt localities El-Giza (isolate 1), Kena (isolate 2) and Damanhouer (isolate 3) and amplification of the isolated *Eimeria necatrix* DNA using RAPD technique. Detection of the homogenicity and heterogenicity among the amplified DNA of *Eimeria necatrix* from the different localities.

MATERIAL AND METHODS

1. *Eimeria necatrix* isolates

Three *Eimeria necatrix* oocysts (field isolates) were taken from the intestinal mucosal lesions of naturally infected chicken *(9)* from different localities El-Giza (isolate No. 1), Kena (isolate No. 2) and Damanhouera (isolate No. 3). In-vitro sporulation of each isolate was developed in 2.5% potassium dichromate solution *(10)*. The isolate were stored at 4°C until used.

2. Isolates purification and propagation

Three *Eimeria necatrix* isolates were purified by infecting 7 days old chicks with single oocysts and there after maintained by passage in another groups of chicks *(10,11)*.

3. DNA extraction

DNA extraction was carried out, according to *(12)*, 5 x 10^7 oocysts of each sample were cleaned with sodium hypochlorite solution for 10 minutes at 4°C, washed three times with deionized water and resuspended in extraction buffer (10 mM Tris-Cl pH 8.0, 50 mM EDTA pH 8.0). The ooysts and sporocysts were completely broken down by vortexing. The lysate was centrifuged at 14,000 g for 10 minutes to eliminate debris and then digested with DNase se-free RNase A (20 ug/ml) at 37°C for 1 hour. A further digestion with proteinase-K (100 ug/ml) and

SDS (0.5%) was carried out at 50°C for 2 hours the DNA was then extracted once with one volume of phenol, phenol/chloroform and chloroform and then precipitated with ethanol and ammonium acetate. The pellet was washed with 70% ethanol and resuspended in TE (10 mM Tris-Cl pH 8.0, 0.1 mM EDTA pH 8.0).

4. RAPD amplification

Eight 8-mer oligonucleotides (E13, O11, A2, C19, B7, O1, O4 and B8) were synthesized by Biotechnology Center for Services and Research in Faculty of Veterinary Medicine, Cairo University. Each primer was used with each of the isolate for random amplification of genomic DNA *(2,13)*.

Amplification was carried out in a total reaction volume of 50 ul containing 10 ng *Eimeria necatrix* DNA, 1x PCR buffer, 2.5 ul of 50 mM $MgCl_2$, 5 ul of 2 mM dNTps, 1.0 ul taq polymerase and 2 ul of primer. The reaction mixture was overlaid with 100 ul of mineral oil. The DNA was initially denaturated for 4 minutes at 94°C. The PCR was carried out one minute at 94°C, one minute at 35°C and 2 minutes at 72°C for a total of 35 cycles.

5. Gel electrophoresis

The PCR were electrophorized *(7)* in polyacrylamide gel using Tri-borate EDTA buffer. The gel containing separated DNA was stained in the ethidium bromide stain *(14)* standard molecular Hae III digest DNA marker supplied by Biotool, Spain.

The 8 primers used are as follows:

A2	TGCCGAGCTG
A8	GTCCGCTC
B7	GGTGACGCAG
O1	GGCACGTAAG
E13	CCCGATTCGG
O11	GACAGGAGGT
C19	GTTGCCAGCC
O4	AAGTCCGTC

RESULTS

Primer A2 as shown in Fig. 1. and Table 1. revealed a total number of 9 different amplified DNA bands for the three isolates under study.

The electro-phonetogram of the isolates displays 8 different polymorphic bands of molecular size 1600, 1500, 900, 800, 700, 500, 400 and 300 bps.

Close inspection of the data shows 7 different diagnostic bands characterizing specific isolate. Isolate 1 displays a number of 3 bands of molecular sizes 900, 800 and 400 bps. However, isolate 2 shows no diagnostics bands while isolate 3 exhibits 4 different diagnostic bands at molecular weights 1600, 1500, 700 and 300 bps. The electrophonetogram of the 3 isolates revealed only one monomorphic band of molecular size 1100 bps.

As far as primer B8 is concerned the electrophonetogram of the 3 isolates displays the highest number of bands (i.e. 10 bands). Isolate 1 shows 2 different positive diagnostic bands (i.e. 1100 and 700 bps) no diagnostic bands appeared for isolate 2 while isolate 3 revealed 3 different diagnostic bands of molecular size 1300, 1000 and 400 bps.

Primer B7 as shown in Fig. 2. and Table 2. revealed a total number of 7 different amplified DNA bands for the 3 isolates under investigation. The electrophonetogram of the isolates displays 4 polymorphic bands of molecular size 250, 175, 150 and 125 bps. The 3 remaining bands are monomorphic exhibiting the molecular sizes of 200, 100 and 75 bps.

Isolate 1 shows nither positive nor negative diagnostic bands as far as primer B7 is concerned. However, isolate 2 displays only one negative marker band (125 bps), while isolate 3 exhibits 2 negative bands of 175 and 150 bps.

The elecrophonetogram of primer O1 shows a number of 9 different amplified bands Fig. 2 and Table 2.

The 3 bands of molecular sizes 200, 125 and 100 bps are monomorphic, the remaining size bands of molecular sizes 300, 275, 225, 175, 150 and 75 bps are polymorphic. Moreover, isolate 1 and 3 each show only one positive diagnostic bands of molecular size 175 and 75 bps respectively.

Table 1. RAPD analysis using two different primers for three *Eimeria necatrix* isolates from Giza, Kena and Damanhour

Primers 5-sequence-3	A2 TGCCGAGCTG			Primers 5-sequence-3	B8 . GTCCGCTC		
	Eimeria necatrix isolates of				*Eimeria necatrix* isolates of		
Polymorphic and monomorphic band	Giza	Kena	Damanhour	Polymorphic and monomorphic band	Giza	Kena	Damanhour
1600	0	0	1	1600	0	0	0
1500	0	0	1	1500	0	0	0
1400	0	0	0	1400	0	0	0
1300	0	0	0	1300	0	0	1
1200	0	0	0	1200	1	1	0
1100	1	1	1	1100	1	0	0
1000	0	0	0	1000	0	0	1
900	1	0	0	900	1	0	1
800	1	0	0	800	1	0	1
700	0	0	1	700	1	0	0
600	0	0	0	600	1	0	1
500	1	0	1	500	1	0	1
400	1	0	0	400	0	0	1
300	0	0	1	300	0	0	0

Table 2. RAPD analysis using two different primers for three *Eimeria necatrix* isolates from Giza, Kena and Damanhour

Primers 5-sequence-3	B7 GGTGACGCAG			Primers 5-sequence-3	O1 GGCACGTAAG		
	Eimeria necatrix isolates of				*Eimeria necatrix* isolates of		
Polymorphic and monomorphic band	Giza	Kena	Damanhour	Polymorphic and monomorphic band	Giza	Kena	Damanhour
300	0	0	0	300	0	1	1
275	0	0	0	275	1	1	0
250	1	1	0	250	0	0	0
225	0	0	0	225	0	1	1
200	1	1	1	200	1	1	1
175	1	1	0	175	1	0	0
150	1	1	0	150	1	1	0
125	1	0	1	125	1	1	1
100	1	1	1	100	1	1	1
75	1	1	1	75	0	0	1

Table 3. Degree of similarity among E. necatrix isolates using Dice Coefficient of PCR-RAPD amplified bands

	Giza	Kena	Damanhour
Giza	100	60	96
Kena	60	100	62.5
Damanhour	96	62.5	100

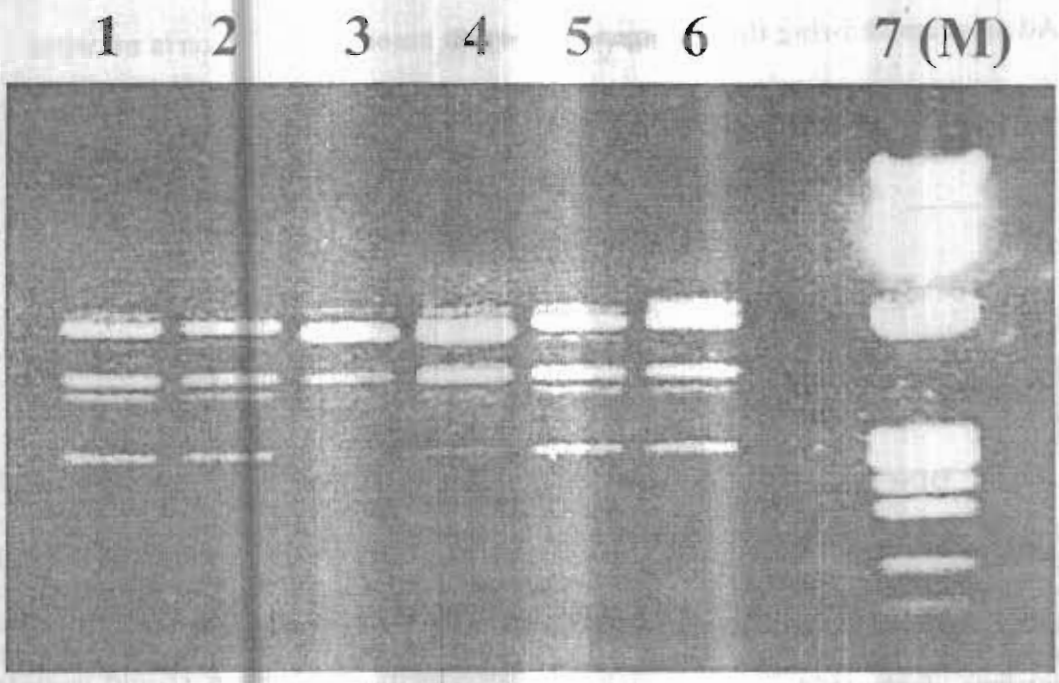


Fig. 1 . RAPD profile of *Eimeria necatrix* genomic DNA isolates amplified by 2 random oligonucleotide primers

Lane 1, 4: Isolate No. (1) Lane 2, 5: Isolate No. (2)
Lane 3, 6: Isolate No. (3) Lane 1, 2, 3: amplified PCR product using primer A2
Lane 4, 5, 6: amplified PCR product using primer B8
Lane 7 (M): Hae III DNA digest, Biotool, Spain

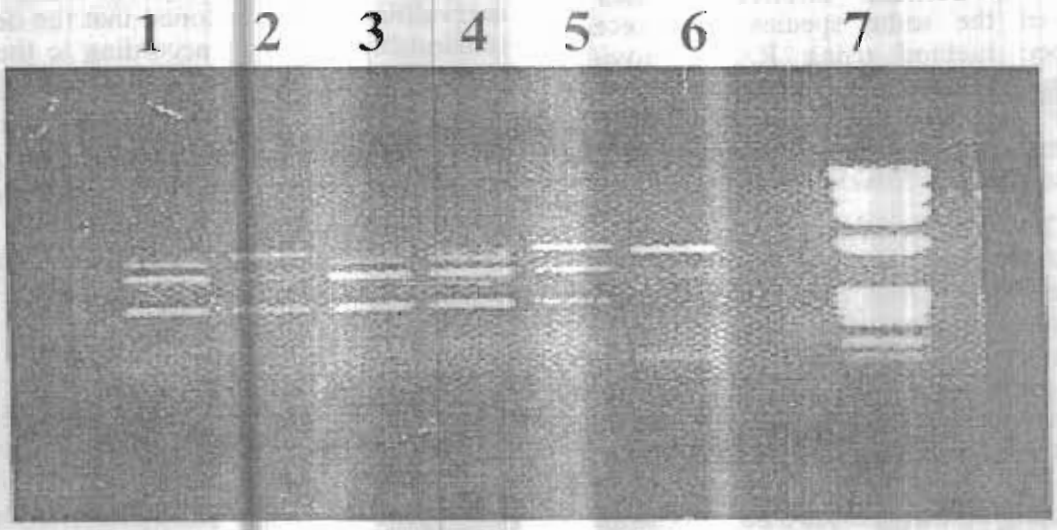
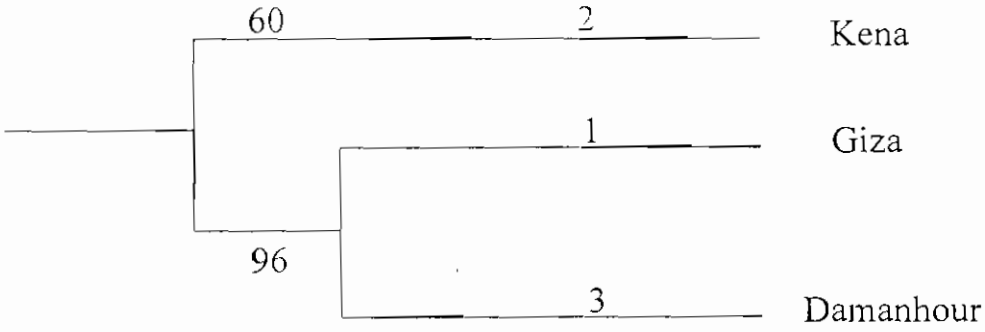


Fig. 2 . RAPD profile of *Eimeria necatrix* genomic DNA isolates amplified by 2 random oligonucleotide primers

Lane 1, 4: Isolate No. (1) Lane 2, 5: Isolate No. (2)
Lane 3, 6: Isolate No. (3) Lane 1, 2, 3: amplified PCR product using primer B7
Lane 4, 5, 6: amplified PCR product using primer O1
Lane 7 (M): Hae III DNA digest, Biotool, Spain

Fig. 3. Adenogram showing the phylegenoic relation among the 3 Eimeria necatrix



DISCUSSION

Avian coccidiosis is one of the major parasitic diseases affecting poultry. The disease is universally found wherever chicken are raised. Economic losses from coccidiosis are mortalitics, weight loss, low feed conversion rate, decrease in egg production as well as delayed sexual maturity in pullets *(15)*. The majority of outbreaks of coccidiosis were caused by E. necatrix (62.75%) followed by E. acervulina and E. tenella (12.86% and 9.91% respectively) *(16)*.

Classic taxonomic method can not differentiate between different isolates or strains of the same species. The recently developed method using RAPD providing specific fingerprints to differentiate between isolates or strains of different protozoa including Babesia bovis *(17)*. *Theileria annulata (18)* and *Eimeria (3,5)*.

The 4 different decamr primers used in PCR-RAPD study revealed genotype-specific fragment. These DNA fragments or bands can be used as diagnostic or marker bands to characterize a given isolate (genotype) for a given primer. In this respect, the most indicative used primer is A2 (i.e. 7 bands) followed by B8 (i.e. 5 bands) and then O1 (i.e. 2 bands). On the contrary, the primer B7 is the least indicative one (i.e. zero bands) compared to the others. The degree of similarity among the 3 standard E. necatrix isolates using 4 oligonucleotide primer was tested and the results were scored as shown in Tables 1, 2 and 3 and Fig. 1, 2 and 3. The similarity between the 2 species revealed a great similarity between El-Giza and Damanhour to a proportion of 96% while Giza and Kena revealed 60% while damanhour and Kena revealed similarity to 62.5%.

Our results revealed that the isolates of both the Giza and the Damanhour one closely similar to each other, but Kena strain was non-similar to other 2 strain that may refer to trials for vaccine preparation should consider both similarity and non-similarity results showuld be considered in order to avoid vaccination failure for trials of vaccine preparition.

The obtained data is in a good agreement with the observation to documented by *(4)* and *(5)* who used RAPD method to compare different strains of E. tenella and E. acervulina. They mentioned that the degree of relationship may vary according to the strains within aspects and probably according to the used primer.

This finding was confirmed by *(2)* who mentioned that RAPD technique can differentiate and clarify even the minute difference between the isolates of the same species.

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الملخص العربى

التصنيف الداخلى لعترة الايميريا نيكاتركس فى الدواجن باستخدام التكبير العشوائى المتعدد الأوجه فى مصر

رجب محمد الخطيب حامد يوسف جمال الدين
نصر أحمد محمد حجازى أحمد محمود داود
* معهد بحوث الأمصال واللقاحات البيطرية – العباسية

بواسطة استخدام اختبار التكبير العشوائى المتعدد الأوجه للحامض النووى DNA (PCR-RAPD Technique) لتحديد البصمة الوراثية لعترة الإيميريا نيكاتركس المعزولة من ثلاث اماكن جغرافية مختلفة (الجيزة – قنا – دمنهور) وتم التصنيف الوراثى باستخدام ٤ بوادئ (4 primers) لتحديد أوجه التشابه والاختلاف بين العترات الثلاثة المعزولة.

وقد وجد العديد من الحزم المختلفة للحامض النووى DNA المشتركة بين المعزولات الثلاثة وعلى الجانب الأخر تم تحديد الحزم النووية DNA الخاصة لكل واحد.

كان التشابه كبير بين عترة الإيميريا المعزولة من محافظات الجيزة ودمنهور والتشابه أقل بين الإيميريا نيكاتركس المعزولة من محافظات الجيزة وقنا.

وهذا يعنى وجود تقارب كبير فى التركيب الوراثى بين عترات الإيميريا نيكاتركس المعزولة من محافظة الجيزة ودمنهور وأبعد نسبيا من الجيزة وقنا.

وعلى ذلك يعتبر اختبار RAPD Technique له قدرة فعالة وعالية لتحديد الاختلافات الوراثية فى الحامض النووى لعترات الإيميريا نيكاتركس.