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FINGERPRINTING OF *EIMERIA STIEDAE* (LIVER COCCIDIOSIS) OF RABBIT IN EGYPT BY USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

(With 3 Tables and 3 Figures)

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البصمة الوراثية للإيميريا ستيدى (الكوكسيديا الكبدية) للأرانب فى مصر باستخدام التكبير العشوائى المتعدد الأوجه

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تم عزل ثلاث معزولات للإيميريا ستيدى، من ثلاث أماكن جغرافية مختلفة فى مصر (البحيرة – أسيوط – القليوبية) وبواسطة استخدام اختبار التكبير العشوائى المتعدد الأوجه (RAPD technique) لتحديد البصمة الوراثية للثلاث عترات المعزولة باستخدام أربعة بوادئ (4 primers) لتحديد أوجه الشبه والخلاف بين الثلاث معزولات. ووجد أن هناك عدد من الحزم المختلفة وأيضاً المتشابه للحامض النووى DNA بين العترات الثلاثة المعزولة وتم تحديد درجة التشابه بين المعزولات الثلاثة المعزولة وكان التشابه كبير بين المعزولات لإيميريا ستيدى المعزولة من محافظة البحيرة وأسيوط والتشابه أقل بين الإيميريا ستيدى من محافظة البحيرة ومحافظة القليوبية. وهذا يعنى وجود تقارب كبير فى التركيب الوراثى بين معزولات الإيميريا ستيدى المعزولة من محافظة البحيرة وأسيوط، وأبعد نسبياً عن الإيميريا ستيدى المعزولة من القليوبية والبحيرة. ومن هذا يتضح ان اختبار RAPD technique له قدرة عالية لتحديد الإختلافات الوراثية فى الحامض النووى لمعزولات الإيميريا ستيدى.

SUMMARY

Random amplified polymorphic DNA (RAPD technique) gives characteristic fingerprints or genetic polymorphism for *Eimeria stiedae* isolates. The isolates were collected from three different localities in Egypt (Al-Bahyra, Assiut and El-Kalybia provices). Four oligonucleotides primers reflected different phylogenetic relationship among the 3 *Eimeria stiedae* isolates. The degree of similarity between

the 3 isolates reflected the presence of both species specific and even strain-specific band, the statistical analysis suggested a closer phylogenetic relationship between Al-Bahyra and Assiut *Eimeria stiedae* isolates which are distantly related to El-Kalybia isolates RAPD technique could easily differentiate with a great potential the minor changes in the genomes of the related genotypes of *Eimeria stiedae* isolates in Egypt.

Key words: *Parasitology, eimeria stiedae, liver coccidiosis, rabbit*

INTRODUCTION

Hepatic coccidiosis is considered as one of the major parasitic diseases affecting rabbits. The disease causes high mortality in young rabbits especially those between 4-8 weeks old (Wang and Tsai, 1991).

Hepatic coccidiosis caused by *Eimeria stiedae* occurred in the liver, the vital organ for protein synthesis and storage of the most of nutritive body materials. The disease causes severe interruption of feeding and digestive processes, dehydration, and high mortality (Calnek *et al.,* 1997). The objective of this study was isolation and identification of *Eimeria stiedae* from 3 different Egyptian localities including Al-Bahyra (isolate 1), Assiut (isolate 2) and El-Kalybia (isolate 3), amplification of the isolated *Eimeria stiedae* DNA using RAPD technique and detection of the homogenicity and heterogenisty among the amplified DNA of *Eimeria stiedae* from these different localities.

MATERIALS and METHODS

1. Parasitic strains:

Three isolates of *Eimeria stiedae* oocysts (field strain isolates) were obtained from the gall bladder content of naturally infected rabbits in different localities Al-Bahyra (isolate No. 1), Assiut (isolate No. 2) and El-Kalybia (isolate No. 3). In-vitro sporulation of each isolate was developed in 2.5% potassium dichromate solution according to (Soulsby, 1982). These strains were stored at 4°C until used.

2. Isolates purification and propagation:

The purification and propagation of the field strain, were done according to Zhang *et al.* (1996). 30,000 freshly sporulated oocysts of *Eimeria stiedae* of each isolate were inoculated orally in a group of five coccidia free rabbits. 17 days post infection, all rabbits were slaughtered and the gall bladder contents were examined for oocysts and allow to

sporulate. Three serial passages of each isolate in coccidia free rabbit were done to obtain pure strains. Each isolate was propagated by inoculation of other 5 coccidia free rabbits and the resulted oocysts were sporulated as mentioned before.

3. Sporocyst and sporozoite preparation and purification:

5 x 10^7 oocysts were used and treated according to a method derived from that of Doran and Augustin (1973), Hosek *et al.* (1988). The resulted sporocysts were washed in PBS and pelleted by centrifugation at 2500 g for 10 minutes. Excystation was performed by incubation for 3 minutes at 39°C in 1% biliary salt and 4% trypsin. The reaction was stopped by the addition of PBS at 4°C. The sporozoites were washed twice in PBS and pelleted by centrifugation at 9000 g, for 6 minutes and used for genomic DNA extraction without further purification.

4. DNA isolation:

The sporozoites were resuspended in 300 µl of lysis buffer (0.1 M Tris-HCl, 0:1 M NaCl, 50 mM ethylene diamine tetraacetic acid (EDTA), 1% sodium dodecyl sulphate (SDS), and 5 gm of protinerase k/ml (Appligere, pH 8). After incubation overnight at 40°C and centrifugation at 1100 g, DNA was extracted by phenol/chloroform method that described by Sambrook *et al.* (1989), then precipitated by ethanol and dried.

5. RAPD amplification:

The RAPD reactions were performed in a final volume of 25 ul containing 20 ng of parasite DNA, 15 ng of the decamer primer (Eight primers each of 8-mer oligonucleotides) and synthesized by Biotechnology Center for Services and Research in Faculty of Veterinary Medicine, Cairo University. (E13, E11, O11, O19, A2, O4, C19, B8) 250 mM MdNTP, 1.5 mM $MgCl_2$, 1x reaction buffer (supplied by enzyme manufacture) and 1 M of taq DNA.

The PCR reaction consisted of an initial denaturation of 5 minutes at 94°C and 40 cycle of 1 minute at 94°C, 1 minute at 40°C and 1.5 minute at 72°C, with a final extension step at 72°C for 8 minutes. All reactions were performed in a PTC-100/96 V thermocycler.

Each amplification included a tube without DNA template as negative control. The amplified PCR products were mixed with a loading buffer (0.25% bromophenol blue in 30% glycerol) and subjected to electrophoresis through 1.5% agarose gels in 1x TBE buffer according to Sambrook and Russel (2001). Gels were stained with ethidium

bromide (0.5 ug/ml), visualized under UV light and photographed with a Polaroid camera using type 667 film.

The 8 used primers are demonstrated as follow:

E13	CCCGATTCGG
E11	GAGTCTCAGG
O11	GACAGGAGGT
O19	GGTGCAGGTT
A2	TGCCGAGCTG
O4	AAGTCCGCTC
C19	GTTGCCAGCC
B8	GTCCGCTC

RESULTS

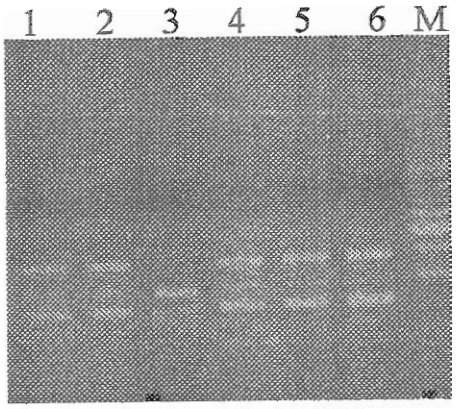


Fig. 1: RAPD profile of *Eimeria stiedae* genomic DNA isolates amplified by 2 random oligonucleotide primer

Lane 1, 4: Isolate No. (1) Al Bahyra
Lane 2, 5: Isolate No. (2) Assiut
Lane 3, 6: Isolate No. (3) Al Kalybia
Lane 1, 2, 3: Amplified PCR product using primer E11
Lane 4, 5, 6: Amplified PCR product using primer E13
Lane 7 (M): Hae III digest DNA marker supplied by Fin Zym-Finland

Table 1: RAPD analysis using two different primers for three *Eimeria stiedae* isolates from Al-Bahyra, Assiut and Al-Kalybia.

E11 Primer	GAGTCTCAGG		
MW	Strain 1	Strain 2	Strain 3
640	1	1	1
603	0	1	1
520	1	1	0
427	0	0	1
350	0	1	1
330	1	0	0
310	1	1	1
280	1	1	1
E13 Primer	CCCGATTCGG		
MW	Strain 1	Strain 2	Strain 3
630	1	0	1
570	0	1	0
560	1	0	0
530	0	0	1
480	1	1	0
380	1	0	0
320	1	1	1
300	1	1	1
290	0	1	0
270	1	1	1

Strain (1): Al-Bahyra
Strain (2): Assiut
Strain (3): Al-Kalybia

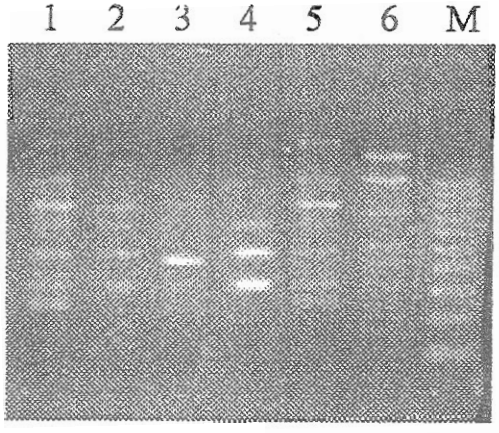


Fig. 2: RAPD profile of *Eimeria stiedae* genomic DNA isolates amplified by 2 random oligonucleotide primer

Lane 1, 4: Isolate No. (1) Al Bahyra
Lane 2, 5: Isolate No. (2) Assiut
Lane 3, 6: Isolate No. (3) Al Kalybia
Lane 1, 2, 3: Amplified PCR product using primer O11
Lane 4, 5, 6: Amplified PCR product using primer O19
Lane 7 (M): 100 base pair ladder (Biotool Spain)

Table 2: RAPD analysis using two different primers for three *Eimeria stiedae* isolates from Al-Bahyra, Assiut and Al-Kalybia

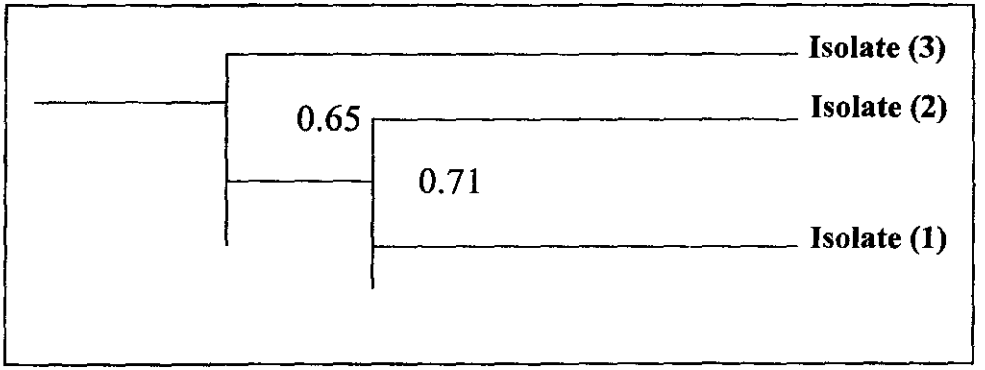
O11 Primer	GACAGGAGGT		
MW	Strain 1	Strain 2	Strain 3
310	1	1	1
295	1	1	1
290	0	0	1
280	1	1	1
200	1	1	1
140	1	0	0
O19 Primer	GGTGCACGTT		
MW	Strain 1	Strain 2	Strain 3
330	0	1	0
310	1	0	1
300	1	1	1
290	1	0	0
280	1	1	0
210	1	1	0
160	0	0	1

Strain (1): Al-Bahyra
Strain (2): Assiut
Strain (3): Al-Kalybia

Table 3: The degree of similarity among *E. stiedae* isolates using Dice Coefficient of PCR-RAPD amplified bands

E. stiedae isolates	Relation Coefficient		
	Al-Bahyra	Assiut	Al-Kalybia
Al-Bahyra	100.0	71.4	63.4
Assiut	71.4	100.0	66.7
Al-Kalybia	63.4	66.7	100.0

Fig. 3: Adendrogram showing the phylogenetic relationship among the 3 *E. stiedae* isolates of Al-Kalybia, Assiut and Al-Bahyra



Primer E11 as shown in the electrophonetogram (Fig.-1) and (table-1) revealed a total number of 8 different amplified DNA bands for the three isolates under investigation. The electrophonetogram of the isolates, displays five different polymorphic bands of molecular sizes bps 603, 520, 427, 350 and 330 bps. Close inspection of the data shows 2 different diagnostic bands each characterizing specific isolate. One diagnostic band of MW 427 bps appeared in isolate 3 while the second band (330 bps) characterized the banding pattern of isolate 1. The 3 remaining bands are monomorphic exhibiting the molecular sizes of 640, 310 and 280 bps.

As far as primer E13 is concerned, electrophonetogram (Fig. 1, Table 1) displays the highest number of bands (i.e. 10 bands). The 3 monomorphic bands exhibit the molecular weights of 320, 300 and 275 bps. However, the seven polymorphic bands show molecular weights of 630, 570, 560, 530, 480, 380, and 290 bps. Isolate (1) shows 2 positive diagnostic bands of MWs 560 and 380 bps. Isolate (2) shows a positive diagnostic bands of molecular sizes 570 and 290 bps and isolate (3) shows only one positive diagnostic band of 530 bps.

The electrophonetogram of primer O11 displays 4 monomorphic and only 2 polymorphic bands (Fig.-2 and Table-2). The monomorphic bands exhibit molecular weights of 310, 295, 280 and 200 bps. The 2 polymorphic bands shows molecular weight of 290 and 140 bps. The band of 290 bps is considered to be diagnostic for the isolate 3, while the other band is positively diagnostic for isolate (1). Data presented in Fig. (2) and Table (2) (primer O19), shows 6 polymorphic and only one monomorphic bands for the 3 isolates. The monomorphic band has a molecular size of 300 bps while the polymorphic ones show molecular weights of 330, 310, 290, 280, 210 and 160 bps.

The bands of 290, 330 and 160 bps are positively diagnostic for isolates 1, 2 and 3 respectively.

The degree of similarity among the 3 studied isolates was numerically estimated using the Dice Coefficient method (Table-3). The similarity matrix was employed to generate the dendogram as illustrated in Fig. (3). The highest coefficient value (71.4) was scored between isolates 1 and 2. The lowest value (63.4) was estimated between isolates 1 and 3. The intermediate coefficient value (66.7) was recorded between isolates 2 and 3.

DISCUSSION

Liver coccidiosis of rabbits is caused by *Eimeria stiedae*. The RAPD method providing specific fingerprints to differentiate between isolates or strains of different protozoa isolates or strains of different protozoa including Trypanosomes (Waitumbi and Murphi, 1993), *Babesia bovis* (Ali *et al.*, 2002), *Theileria annulata* (Gamal El-Din *et al.*, 1998), *Eimeria* (MacPherson and Gajad Har, 1993, Shirley and Bumstead, 1994) and *Eimeria tenella* (Gamal El-Din *et al.*, 2003).

The 4 different decamer primers used in PCR-RAPD study revealed species-specific DNA bands. The degree of similarity among the 3 studied *Eimeria stiedae* isolates using 4 oligonucleotide primers was tested and the results were scored as shown in Table 1, 2, 3 and 4 and Figures 1, 2, 3 and 4.

The obtained results showed different levels of similarity as reflected by the nature of polymorphism. All primers showed different levels of polymorphism (dissimilarity). Primer E13 and O19 showed the highest level of polymorphism as reflected by the presence of seven and six polymorphic bands respectively. E11 primer showed an intermediate number of polymorphic bands (5 bands). On the contrary, primer O11 showed the lowest level of polymorphism as reflected by the presence of only 2 polymorphism bands. Therefore, primers E11 and O19 are considered as the informative ones, considering the polymorphic bands when present, it is known as a positive diagnostic mark. On contrast, it is considered as a negative diagnostic mark when absent. The obtained data is in a good agreement with the observation documented by Procunier *et al.* (1993) and Shirley and Bumstead (1994) who used RAPD method to compare between different strains of *E. tenella* and *E. acervulina*. They mentioned that the degree of relationship may vary according to the strains within species and probably according to the used primer. This finding was also confirmed by Williams *et al.* (1990) who mentioned that RAPD technique can differentiate and clarify even the minute difference between the isolates of the same species.

Eimeria stiedae isolates of Al-Bahyra and Assiut (1 and 2 isolates) are clustered in one phylogenetic group (71.4) and separated from Al-Kalybia isolate (isolate 3). This suggests a closer phylogenetic relationship between isolate Al-Bahyra and Assiut (1 and 2 isolates) which are distantly related and comparatively far from the isolated *E. stiedae* of Al-Kalybia (isolate 3).

Our results revealed that the isolates of both Al-Bahyra and Assiut (1 and 2 isolates) are closely similar to each other, but Al-Kalybia strain was not-similar to other 2 strains and this finding directs the attention that may refere to trials for vaccine preparation should takes in considcration both similarity and non-similarity between different *Eimeria* strains in order to avoid vaccination failure.

Finally, PCR-RAPD technique represents a highly sensitive powerful method of mapping the genomic DNA polymorphism of *E. stiedae.*

REFERENCES

Ali, M.M.; Farah, A.W.; Gamal El-Din, H.Y. and Daoud, A.M. (2002): PCR technique for differentiation of different isolates of *Babesia bovis* from various geographical regions in Egypt. J. Egypt. Vet. Med. Assoc., 62 (3): 119-129.

Calnek, B.W.; Barne, H.J.; Beard, C.W.; McDougald, L.R. and Saif, Y.M. (1997): Diseases of Poultry. Iowa State, University Press Ames, Iowa, USA, pp. 865-883.

Doran, D.J. and Augustin, P.C. (1973): Comparative development of *Eimeria tenella* from sporozoites to oocysts in primary kidney cells cultures from Gallinaceous birds. J. Protozoal, 20 (5): 658-661.

Gamal El-Din, H.Y.; Abbas, A.M. and El-Shater, S.A.A. (1998): Characterization of *Theileria annulata* isolates in Egypt by random amplified polymorphic DNA marker. J. Egypt. Vet. Med. Assoc., 58 (4): 629-644.

Gamal El-Din, H.Y.; Hegazy, N.A.M.; Amani, W. F.; Romany, M.M. and Daoud, A.M. (2003): Avian coccidiosis: Intra-specific variation of *Eimeria tenella* in chicken using RAPD technique in Egypt. Egypt. Vet. Med. Assoc., 63 (5): 9-21.

Hosek, J.E.; Todd, K.S. and Kuhlenschmide, M.S. (1988): Improved method for high-yield excystation and purification of infective sporozoites of *Eimeria* spp. J. Protozool., 35: 583-589.

MacPherson, J.M. and Gajad Har, A.A. (1993): Differentiation of seven *Eimeria* species by random amplified polymorphic DNA. Vet. Parasitol., 45: 257-266.

Procunier, J.D.; Fernado, M.A. and Barta, J.R. (1993): Species and strain differentiation of *Eimeria* spp. of the domestic fowl using DNA polymorphism amplified by arbitrary primers. Parasitol. Res., 79: 98-102.

Sambrook, J. and Russel, D.W. (2001): Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor laboratory Press, Cold Spring, Harbor.

Sambrook, J.; Fritsch, E.F. and Maniatis, T. (1989): Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor. New York.

Shirley, M.W. and Bumstead, L. (1994): Intra-specific variation within *Eimeria tenella* detected by random amplification of polymorphic DNA Parasitol. Res., 80: 346-351.

Soulsby, E.J.L. (1982): Helminths, arthropods and protozoa of domesticated animals. Bailiere and Tindall and cassel Ltd, 7th Ed., London.

Waitumbi, J.N. and Murphy, N.B. (1993): Inter and intra-species differentiation of Trypanosomes by genomic finger-printing with arbitrary primers. Mol. Biochem. Parasitol., 58: 181-186.

Wang, J.S. and Tsai, S.F. (1991): Prevalence and pathological study on rabbit hepatic coccidiosis in Taiwan. Proceed. National Sci. Life Sci., 15 (4): 240-243.

Williams, J.G.K.; Kabelik, A.R.; Livak, K.J.; Rafalski, J.A. and Tingey, S.V. (1990): DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18: 6531-6535.

Zhang, J.; Wilson, E.; Yang, S. and Healey, M.C. (1996): Increasing the yield of *Eimeria tenella* oocysts in primary chicken kidney cells. Avian Dis., 40: 63-67.