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Molecular Diagnosis of *Eimeria* and *Clostridia* in Simultaneously Infected Chickens

[1]*M.A. Kutkat,* [2]*H.A. Shalaby,* [2]*R.M. El. Khateeb,* [2]*N.M. Abu Elezz,*
[2]*A.A. Zayed,* [3]*A.B. Abd El-Razik,* [4]*S.A. Nassif and* [5]*M.M. Amer*

[1]Department of Poultry Diseases, National Research Center, Dokki, Giza, Egypt
[2]Department of Parasitology and Animal Diseases, National Research Center, Giza, Egypt
[3]Department of Genetic and Ain Shams Center of Genetic Engineering and Agriculture Biotechnology, Agriculture Ain Shams University, Cairo, Egypt
[4]Control Laboratory for Evaluation of Veterinary Biologicals, Abbasia, Cairo, Egypt
[5]Department of Poultry Diseases, Fact. Vet. Med., Cairo Univ., Egypt

Abstract: A total number of 95 intestinal and caecal samples were collected form chickens of 19 flocks suspected to have simultaneous infection with coccidia and clostridia. All the flocks were of local breads, reared on deep litter system and 30 to 90 days old. The study was conducted on 4 Egyptian governorates (Qalubeia, Sharkeia, Fayoum and Giza) over a period of one year. The collected samples were transported on ice bags to the laboratory, each sample was divided into two parts, the first was kept on 2% potassium dichromate for preservation of the *Eimeria* oocysts that were detected by microscopic examination, while the second was used for clostridial isolation. Sixty six out of ninety five samples, proved to have *Eimeria* oocysts, were subjected to DNA extraction that utilized in multiplex PCR. *E. necatrix, E. maxima, E. praecox, E. acervulina, E. mitis and E. tenella* were detected with variable degrees. On the other hand, 65 out of 95 samples were positive for clostridia and were subjected to DNA extraction and subsequent multiplex PCR. Only *Clostridium perfringens* type A was detected, out of them 57 samples were proved to have infection with *Eimeria* spp. It was concluded that Multiplex PCR is diagnostic assay used for the simultaneous detection of the six *Eimeria* species and *C. perfringens* type A that infect domestic fowl in Egypt.

Key words: Chickens · Coccidia · Clostridia · Simultaneous infection · Multiplex PCR

INTRODUCTION

Coccidiosis of domestic fowl is a widespread enteric disease caused by obligatory intracellular protozoa of the genus *Eimeria*. Seven distinct *Eimeria* species, *E. tenella, E. acervulina, E. brunetti, E. necatrix, E. praecox, E. maxima and E. mitis*, 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, poor feed conversion and higher mortality of the affected flocks [1]. It is recognized as the parasitic disease that has the greatest economic impact on the commercial poultry industry. Current expense for preventive medication exceeds $90 million in the USA and over $300 million world wide. While the control of coccidiosis has relied mainly on the preventative use of anticoccidial drugs (coccidiostats), together with the induction of species-specific natural immunity in chickens flocks [2, 3] this widely used approach is costly and has led to serious problems with drug resistance in *Eimeria* population [4]. Due to these drug resistance problems, live vaccines are now finding wide application [2, 3]. Because different species and/or strains can vary in pathogenicity, drug resistance and other biological parameters, the specific diagnosis is important for the purity control of the strains used to prepare live vaccines, thus avoiding potential cross-contamination [5]. Increasingly, molecular tools have been developed and relied upon for the specific diagnosis of coccidiosis [6].

The damage in the gut epithelium that caused by coccidiosis has been shown to predispose to necrotic enteritis in chicken [7]. The disease is an enterotoxaemia caused by type A and C of the enteric bacterium

Corresponding Author: Dr. Hatem A. Shalaby, Department of Parasitology and Animal Diseases, National Research Center, P.O. Box: 12622, Giza, Egypt

Clostridium perfringens [8] and manifests itself when the presence of high numbers of bacteria coincides with significant damage to the gut epithelium [9]. Since the causative organisms of both necrotic enteritis and coccidiosis often share sites in the bird's intestine, it might be expected that there would be interactions between the two diseases. Many investigators showed that the simultaneous infection with *E. necatrix* and *C. perfringens* increased clostridial population in the intestine of the chickens and had synergic effect on mortality and oedema in the upper intestine [10, 11].

The purpose of this study was to identify the prominent circulating species of *Eimeria* and *C. perfringens* which present in simultaneous infection in some poultry farms in four Egyptian governorates using polymerase chain reaction (PCR) assay as a first step toward prevention and purity control of this syndrome.

MATERIALS AND METHODS

Samples: A total number of 95 intestinal and caecal samples were collected form chickens of 19 flocks suspected to have simultaneous infection with coccidia and clostridia (Fig. 1). All flocks were of local breads, reared on deep litter system and of 30 to 90 days old. The study was conducted on 4 governorates (Qalubeia, Sharkeia, Fayoum and Giza) over a period of one year. The collected samples were transported on ice bags to the laboratory, each sample was divided into two parts; the first was kept on 2% potassium dichromate for preservation of the *Eimeria* oocysts that were detected by microscopic examination, while the second was used for clostridial isolation. The *Eimeria* oocysts were left for sporulation.

Gas Pack Anaerobic Jar: Barid and Tatlock anaerobic jar was used under room temperature for the production of anaerobiosis by using disposable hydrogen carbon dioxide bags with socket; when the water was introduced into the socket, hydrogen and carbon dioxide were produced inside the closed jar [12].

Media: Cooked meat medium [13] was used for enrichment, purification and preservation of pure isolates of clostridial organisms. Sheep blood agar medium [14] was used for the cultivation and detection of haemolytic activity of the isolates as well as for anaerobic isolation.

Fig. 1: Coccidian and clostridian simultaneous infection in chicken with necrotic foci in intestine

For biochemical identification of *C. perfringens*, five medium were used; A-Sugar fermentation medium [14], Gelatin agar medium [15], Nitrate agar medium (Oxiod), Litmus milk medium [14] and Indol production medium [16].

Vaccinal Strains of Coccidia: Commercial coccidiosis vaccine (coccivac-D) Shering plough, Animal Health Corporation, USA. Batch No.66/03 was used as positive control. This vaccine contained 7 *Eimeria* spp. (*E. tenella, E. acervulina, E. brunetti, E. necatrix, E. praecox, E. maxima and E. mitis*).

Isolation of DNA: For *Eimeria* oocysts, DNA extraction and precipitation was done according the procedure described by [17]. For *C. perfringens*, DNA was extracted and purified as described by [18].

Polymerase Chain Reaction (PCR): For *Eimeria* species, specific primers were designed by [5]. Table (1) listed 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. Single standard PCR amplification was used for individual reactions of each primer pair in order to obtain a common reaction for the seven *Eimeria* species. This condition was then adapted for multiplex PCR reactions after the procedure of [5, 19, 20,].

For detecting *C. perfringens*; specific primers corresponding to each toxin were designed by using the sequence data obtained from Genbank (National Institutes of Health) and were synthesized with a DNA synthesizer (Expedite 8905; Perseptive Co.) (Table 2). A multiplex PCR reaction was carried out according to procedure of [21, 22]. All the amplification products were analyzed by separation on 1.5% agarose gel stained with ethidium bromide and visualized using UV transilluminator.

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

Specis	Primer Designation	Primer sequence	Amplicon Size(bp)	Primer conc
E. acervuline	Ac-01-F	AGTCAGCCACACAATGGCAAACATG	811	0.7&M
	Ac-01-R	AGTCAGCCACAGCGAAAGACGTATGTG		
E.burnetti	Br-01-F	TGGTGGCAGAACCTACAGGGCTGT	626	0.85&M
	Br-01-R	TGGTCGCAGACGTATATTAGGGGTCTG		
E. tenella	Tn-01-F	CCGGCCCAAACCAGGTGTCACG	539	0.55&M
	Tn-01-R	CCGCCCAAACATCGAAGATGGC		
E. mitis	Mt-01-F	AGTCAGCCACCAGTAGAGCCAATATTT	460	0.55&M
	Mt-01-R	AGTCAGCCACAAACAAATTCAAACTCTAC		
E. praecox	Pr-01-F	AGTCAGCCACCACCAAATAGAACCTTGG	354	0.70&M
	Pr-01-R	GCCTGCTTACATCAAACTTGCAAGCCCT		
E. maxima	Mx-01-F	GGGTAACGCCAACTGCCGGGTATG	272	0.55&M
	Mx-01-R	AGGAAACCGTAAAGGCCAAGTCCTAAGA		
E. necatrix	Nc-01-F	TTCATTTCGCTTAACAATATTTGGCCTCA	200	0.70&M
	Nc-01-R	ACAACGCCTCATAACCCCAAGAAATTTTG		

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

Table 2: Primer sequences of *C. perfrungens* used for multiplex PCR assay

Primer(direcation)	Primer sequence	Location	Amplicon Size(bp)
CAP(Alpha toxin)			
Forward	5-GTTGATAGCGCAGGACATCTTAAG-3	511-535	402
Reverse	5-CATGTAGTCATCTGTTCCAGGATC-3	913-889	
CPB(Beta toxin)			
Forward	5-ACTATACAGACATTCAACC-3	589-613	236
Reverse	5-TTAGGAGCAGTTAGAACTACAGAC-3	824-801	
CPE(epsilon toxin)			
Forward	5-ACTGCAACTACTACTCATAGTGTG-3	436-459	541
Reverse	5-CCTGGTGCCTTAATAGAAAGACTCC-3	976-953	
CPE(iota toxin)			
Forward	5-GCGATGAAAAGCCTACACCACTAC-3	563-586	317
Reverse	5-GGTATATCCTCCACGCATATAGTC-3	879-856	

RESULTS

A total number of 66 out of 95 field samples were found to be positive for *Eimeria* spp. infection, representing an overall infection rate of 69.47 % (Table 3).

Amplification of DNA from the vaccinal sample in a multiplex PCR assay (using seven primer pairs) produced 7 fragments representing 7 *Eimeria* spp. as shown in Fig.2 Lane 2. Amplification of DNA from the *Eimeria* isolates in multiplex PCR assay using seven primer pairs produced 4 and 6 fragments representing 4 and 6 *Eimeria* species. (Fig.2 Lane3and4), respectively. Fifty percentage of the positive samples was proved to contain 4 *Eimeria* spp. which were *E. necatrix; E. maxima, E. praecox and E. acervulina.* The other fifty percentage of the positive samples was proved to contain 6 *Eimeria* spp. which were *E. maxima, E. praecox, E. acervulina; E. mitis; E. tenella and E. necatrix,* as shown in Table 4.

A total of 65 isolates of *C. perfringens* organisms were isolated and identified out of 95 examined samples of internal organs with an overall infection of 68.42% as shown in Table 3.

Amplification of DNA from the *C. perfringens* isolates in multiplex PCR produced one fragment, representing species of *C. perfringens* type A. (Fig.3 Lanes 2, 3, 4, 5, 6, 7, 8, 9 and 10). Out of them 57 samples were proved to have infection with *Eimeria* spp. with an overall infection of 60.0% of the suspected cases (Table 3).

Table 3: *Eimeria* spp. and *C. perfringens* isolation from collected samples

No. of outbreaks	Governorate	*Eimeria* spp.	%	*C. perfringens*	%	Simultaneously Infected cases	%
1	Qalubeia	5/5	100	3/5	60	3/5	60
2	Qalubeia	5/5	100	3/5	60	3/5	60
3	Qalubeia	4/5	80	4/5	80	4/5	80
4	Qalubeia	2/5	40	3/5	60	2/5	40
5	Qalubeia	3/5	60	3/5	60	3/5	60
6	Sharkeia	3/5	60	3/5	60	3/5	60
7	Sharkeia	5/5	100	4/5	80	4/5	80
8	Sharkeia	4/5	80	4/5	80	4/5	80
9	Sharkeia	2/5	40	3/5	60	2/5	40
10	Fayoum	3/5	60	2/5	40	2/5	40
11	Fayoum	3/5	60	4/5	80	3/5	60
12	Fayoum	4/5	80	5/5	100	4/5	80
13	Fayoum	2/5	40	3/5	60	2/5	40
14	Giza	3/5	60	4/5	80	3/5	60
15	Giza	4/5	80	3/5	60	3/5	60
16	Giza	5/5	100	4/5	80	4/5	80
17	Giza	2/5	40	4/5	80	2/5	40
18	Giza	4/5	80	3/5	60	3/5	60
19	Giza	3/5	60	3/5	60	3/5	60
Total & % of +ve samples		66/95	69.47	65/95	68.42	57/95	60.0

Table 4: Multiplex PCR for detection of *Eimeria* species in the collected intestinal samples

Amplicon size (bp)	*Eimeria* species	Samples	%
811	*E. acervulina*	66/66	100
626	*E. brunetti*	0/66	0
539	*E. tenella*	33/66	50.0
460	*E. mitis*	33/66	50.0
354	*E.praecox*	66/66	100
272	*E. maxima*	66/66	100
200	*E. necatrix*	66/66	100

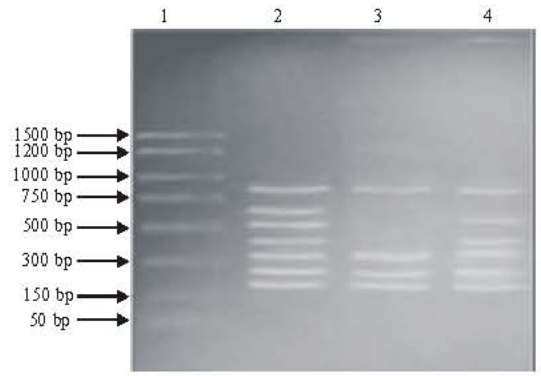

Fig. 2: Multiplex PCR of the coccidiosis vaccine and some isolates of *Eimeria* collected from simultaneous infection by Coccidia and Clostridia in local bread chickens. Lane I: 50 bp DNA ladder, lane 2: Multiplex PCR products of the coccidian vaccine (coccivac D), lane 3: Multiplex PCR products of some isolates of *Eimeria* contaning *E. necatrix; E. maxima; E. praecox* and *E. acervulina* and lane 4: multiplex PCR products of some isolates of *Eimeria* contaning *E. necatrix; E. maxima; E. praecox; E. acervulina; E. mitis* and *E. tenella*

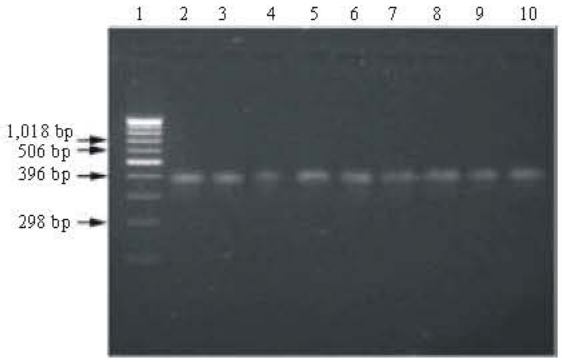

Fig. 3: DNA amplification for some isolated strains of *Clostridium perfringens* by the multiplex PCR. It produced one fragment in lanes 2, 3, 4, 5, 6, 7, 8, 9 and 10 representing *C. perfringens* type A (396bp), while Lane 1 is DNA size marker (Fermenter, I-kb ladder).

DISCUSSION

Species identification of *Eimeria* had been classically performed using morphological and pathological criteria, including oocyst shape and size, prepatent period, sporulation time, intestinal site and shape of the lesions and characteristics of the endogenous stages in the intestinal mucosa [23]. However, these approaches could be unreliable, particularly given that multiple species of *Eimeria* could simultaneously infect the host and because there could be an "overlap" in the sizes of oocysts and the sites of infection in the intestines for some species [24]. Hence, some of the limitations of the traditional methods have been overcome by the development of

molecular methods. Indeed, in the current work, PCR based diagnostic assay were used for the simultaneous detection of the seven *Eimeria* species that infect domestic fowl. The test could be performed using either individual or multiplex PCR reactions with similar results, thus it was highly economic, saving time and money, highly specific and sensitive [5].

The present work revealed that the detection percent of *Eimeria* spp. and *C. perfringens* from cases suspected to have simultaneous infection reached to 69.47 and 68.42% in local breads of chicken flocks, respectively. The amplification of DNA from the *Eimeria* isolates detected *E. necatrix, E. maxima, E. praecox and E. acervulina* in 33 out of 66 positive samples and *E. necatrix, E. maxima E. praecox, E. acervulina, E. mitis and E. tenella* in the other 33 samples. In that sense, [5] had reported an approach for the development of reliable and species-specific markers for *Eimeria* spp. of domestic fowl. They described the generation of a novel set of Sequence-Characterised Amplified Region (SCAR) markers and the development of an integrated, cost-effective and simple multiplex PCR assay that permitted the simultaneous discrimination of the seven *Eimeria* species that infected the domestic fowl. The DNA amplification of isolated *C. perfringens* proved *C. perfringens* type A in 57 out of 66 *Eimeria* spp. positive samples. This appeared to be in line with. [10] who suggested that the simultaneous infection with *E. necatrix* and *C. perfringens* increased the clostridial population in the intestine of the chickens and had synergic effects on mortality and oedema in the upper intestine. [25] reported that *C. perfringens* type A, *E. acervulina* and *E. necatrix* produced necrotic entritis (NE) in chickens and high mortality. Damage of the intestine caused by *Eimeria* spp. was thought to be involved in increasing the susceptibility of chickens to outbreaks of NE caused by *C. perfringens* Type A [11] and cases of NE associated with *C. perfringens* had increased in countries that had stopped using antibiotic growth promoters [26, 27]. The acute form of NE led to increased mortality in broiler flocks which could account for 1% mortality losses per day for several consecutive days during the last weeks of the rearing period [26]. In the model developed by [11], a virulent coccidial challenge with *E. maxima* sufficient to cause clinical coccidiosis exacerbated a virulent clostridial challenge. Also,[11] observed reduced NE lesion development following a virulent clostridial challenge in Paracox-5 vaccinated broilers due to preventing severe coccidial lesions that might predispose birds to NE. Many investigators detected seven species of *Eimeria*: *E. necatrix, E. maxima, E. praecox, E. acervulina, E.mitis, E. tenella and E. brunetti* in Egypt [28] but no literature have identified the prominent circulating species of *Eimeria* and *C. perfringens* which present in simultaneous infection.

It could be concluded that under field conditions, 6 species of *Eimeria*: *E. necatrix, E. maxima, E. praecox, E. acervulina, E. mitis* and *E. tenella* could play a significant role in the occurrence of necrotic enteritis by different percentages when a sufficient number of toxigenic strain of *C. perfringens* type A present. In addition the multiplex PCR is diagnostic assay used for the simultaneous detection of the six *Eimeria* species and *C. perfringens* type A that infect domestic fowl in Egypt.

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