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Genetic Variation Among Five Egyptian Field Isolates of *Eimeria tenella* Detected by Random Amplified Polymorphic DNA Assay

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Abstract: Five identified field isolates of *Eimeria tenella* from 5 Egyptian provinces (Behera, Khafr El-Sheikh, Alexandria, Gharbia and Matrouh) were examined for genetic polymorphism by the random amplified polymorphic DNA (RAPD) assay. Seven different oligonucleotide RAPD primers were used to amplify DNA from each isolate. Following single gel isolation of each isolate, the five isolates were confirmed to be *E. tenella* depending on single PCR assay targeting ITS-1 of the genomic rDNA *of E. tenella*. RAPD-PCR for the five *E. tenella* isolates has amplified from 1 to 35 DNA fragments ranging in size from 50 to 2000 bp. Genetic polymorphism were detected among the five isolates indicated by polymorphism in each primer alone and in the average polymorphism among all primers (29.2%) in each isolate. The inferred phylogenetic tree on the fingerprinting of all five isolates showed that, Matrouh isolate is genetically different from other isolates however; it is more related to Behera and Khafr El-sheikh isolates followed by Alexandria and Gharbia isolates. These results were compared to the results of evaluating the pathogeneticity of these five isolates of *E. tenella* and it could be concluded that *E. tenella* from different geographical regions in Egypt are genetically different which could affect on the degree of their pathogenecity.

Key words: *Eimeria tenella* · RAPD-PCR · Genetic Polymorphism · Pathogenecity

INTRODUCTION

Coccidiosis is one of the most important diseases in commercial poultry production worldwide. It is caused by infection with one or more of the seven *Eimeria* species infecting chickens [1]. It is responsible for 6-10% of all broiler mortalities and the annual global economic losses are estimated of about 1.5 billion US$1 [2, 3]. Seven distinct *Eimeria* species are actually considered pathogenic to chickens: *E. tenella*, *E. acervulina*, *E. maxima*, *E. necatrix*, *E. brunetti*, *E. praecox* and *E. mitis*. Identification of these species has important implications for the diagnosis and control of the disease, as well as for studying their epidemiology and population biology. Molecular approaches for improving the accuracy of *Eimeria* species identification have been developed to overcome the limitations of traditional methods [4]. Molecular approach involves the use of some genetic markers as diagnostic assays and, in particular primers specific for internal transcribed spacers-1of *Eimeria* (ITS-1) of nuclear ribosomal DNA (rDNA) which separate the ribosomal genes, have been developed for the detection of the seven *Eimeria* species in fecal and intestinal samples [5, 6]. Another approach is random amplification of polymorphic DNA (RAPD) [7, 8] which relies on the amplification of genomic DNA fragments using single primer provides the ability to screen the entire genome. RAPD has been used to study variation within species of *Eimeria* from chickens [9].

Eimeria tenella is considered among the most pathogenic *Eimeria* organisms parasitizing growing chickens, causing considerable financial loss to the poultry industry. In Egypt, studies indicted that it is the most common *Eimeria spp.* where the infection rate is 20- 100% and mortality is 20- 60% with severe reduction in body gain and feed efficiency [10]. However, there is no available data about intraspecific genetic variation of *E.tenella* in Egypt. Because different species and/or strains can vary in pathogenicity, drug resistance and other biological parameters, their precise discrimination is

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important for epizootiological studies as well as for checking the purity of reference and/or vaccine strains [11, 12]. The presence of antigenic variation within *E. tenella* isolates has been described in samples collected from different geographical areas [13-15]. The local *Eimeria* isolates which cause coccidiosis could possibly differ from the vaccine strains, as a sequence they probably do not confer enough immunity against the *Eimeria* found on those poultry farms.

The aim of the present study was to identify *E.tenella* isolates from five different geographical regions in Egypt (Behera; Khafr El-Sheikh; Alexandria; Gharbia and Matrouh) by using ITS-1 specific primer for *E. tenella* and using RAPD markers to determine genetic variation among those isolates. Furthermore, compare our data with pathogenicity data of these five isolates done by Abuakkada and Awad [10].

MATERIALS AND METHODS

Parasite Isolation and Propagation: Five field isolates identified as *E.tenella*, representing five different provinces (Behera, Khafr El-Sheikh, Alexandria, Gharbia and Matrouh) were used in this study. The first 4 provinces located north Egypt with sharing boundaries and Matrouh located west-north side. These isolates were collected from floor- raised, naturally *Eimeria* - infected broiler farms. Oocysts identified as *E. tenella* were harvested from the cecal contents of these birds by the method of Davies *et al*. [16]. They were allowed to sporulate in 2.5% potassium dichromate with aeration. After sporulation, the oocysts were preserved in potassium dichromate and stored at 4°C until used. Pure isolates of *E. tenella* were obtained through isolation of a single oocyst from each field isolate by means of agar plate method according to Tsutsumi [17]. Two-week-old broiler chicks, reared from day- old, were used to obtain *E. tenella* single oocyst line. Each chick was raised on a home- made metal cage (30x22x22 cm) from the first day of life until the end of the experiment under strict hygienic measures. Each of the 5 isolates was inoculated into the crop of each chick according to Tsutsumi [17]. Fecal material was allowed to collect on waterproof paper placed in the dropping pans. The droppings were collected, washed and then sporulated. Birds were killed on the 10^{th} day PI. The ceca, including their contents were placed in 2.5% potassium dichromate solution to induce sporulation. Sporulated oocysts obtained from this experiment were used to infect 14-days old coccidium-free chicks, raised on wire-floored cages, for propagation of the five pure isolates of *Eimeria tenella* to provide a sufficient number of oocysts necessary for DNA extraction.

DNA Extraction by Glass-Bead Grinding: The potassium dichromate solution was removed from the sporulated oocysts by several centrifugations in sterile distilled water, followed by sterilization of oocysts by sodium hypochlorite treatment (4% available chlorine, 30 min, 4°C) according to Wagenbach *et al*. [18]. Oocyst suspension was then pelleted by centrifugation at 3000 rpm for 15 min, followed by another 3X washing in sterile distilled water. The oocysts pellet was resuspended in sterile distilled water. DNA was extracted using a glass bead grinding method described by Hnida and Duszynski [19]. In 2-ml round bottom microcentifuge tube, glass-beads of Ø 0.5 mm was added and the tube was vortexed vigorously. Breakage was monitored using a compound microscope 40x at 2min intervals until all the oocysts and their sporocysts appeared to be ruptured after approximately 10 min. The suspension containing freed sporozoites was added to 300 µl of lysis buffer containing 10µl proteinase K (20 mg/ml) and incubated at 37°C overnight. DNA was then extracted by phenol/chloroform extraction method followed by ethanol precipitation [20]. The air-dried DNA pellet was resuspended in TE buffer.

Specific PCR Amplification and Identification of the PCR Products: Optimized single PCR assays targeting ITS-1 sequences of the genomic rDNA of E. *tenella* were developed to ensure that all the five isolates are *E. tenella*. The reverse and forward primers targeting the ITS-1 gene were selected according to Haug *et al*. [21].

ETF2.a 5'-AATTTAGTCCATCGCAACCCTTG-3
ETR.b 5-CGAGCGCTCTGCATACGACA-3

The expected amplicon size was 278 bp. The amplification was done for the DNA of the five *E. tenella* isolates. One negative control tube without template DNA was run with this primer. It was carried out in 25 µl reaction volumes containing 2 µl test DNA sample (5 ng/µl), 50 pmol *E. tenella* specific primers (forward and reverse), 3.5 mM $MgCl_2$, 2.5 µl 10X PCR Gold Buffer, 200 mM of each deoxynucleotide triphosphate (dNTP) and 0.4 U Taq Polymerase (Promega). The cycling profile was: 95°C for 5 min in precycle, followed by 40 cycles of 95°C denaturation for 1 min, primer annealing 48°C for 1 min and extension at 72°C for 1 min. Final primer extension continued for an additional 10 min to allow the complete

Table 1: Oligonucleotides used in RAPD-PCR, G-C%, melting temperature and annealing temperature

Primer name	Primer sequence	G-C%	Calculated	Annealing temp.
111	AGTAGA CGGG	60	32	35
114	TGACCG AGAC	60	32	35
115	TTC CGC GGG C	80	36	42
127	ATCTGG CAGC	60	32	34
132	ACG GAT CTCC	60	32	37
134	AAC ACACGAG	50	40	35
137	GGT CTC TCCC	70	34	40

Tm: Melting temperature

elongation of all amplifications. Each sample (8 µl) was mixed with 2 µl loading buffer and analyzed by electrophoresis in 2% agarose gels stained with 0.5 mg/ml ethidium bromide. The PCR products were identified by size using a 100 bp ladder.

Random Amplified Polymorphic DNA - Polymerase Chain Reaction: RAPD PCR was performed using the primers designated 111, 114, 115, 127,132, 134 and 137 which h ave been published earlier by Procunier [22]. The nucleotide sequences of these primers and G-C content are listed in Table 1. The calculated melting temperatures (Tm) of each primer were estimated as follows: Tm = 4(G + C) + 2 (A + T). For PCR purposes Tm-5 is a good annealing temperature [23]. Based on the calculated Tm of each primer, the optimum annealing temperature were determined experimentally. We examined 2°C below and 5°C above TM for each primer until reach the exact annealing temperature (Table 1). RAPD-PCR was carried out in 25 µl reaction volumes containing the same components as that described with *E.tenella* specific primer except the volume of the RAPD primers were 100 pmol in each reaction mixture. The cycling profiles the same as the specific primer, with 40 cycles. 12.5 µl of PCR product were mixed with 2µl loading buffer and loaded in 2.5% agarose gels.

Statistical Analysis, Data Analysis of RAPD and Dendrogram Construction (Phylogenic Tree): The DNA bands were scored for their presence as (1) or absence as (0) in the RAPD profile of the five isolates. Comparative data generated from each isolate/primer combination were totaled. The amplified fragments DNA lengths within isolates were used to determine the specific and common fragments which are reproducible in all amplification. The polymorphism in each primer based on the number of unique bands divided by the total number of bands.

The similarity coefficients were calculated which depend on the number of bands amplified from each isolate and the number of shared bands [22, 24]. Distance matrix data were used to construct a dendrogram using the unweighted pair-group with arithmetic mean (UPGMA). The cluster analysis and dendrogram construction was performed with Statistica 5 [25].

RESULTS

Specific PCR for E. Tenella: Targeting ITS-1 genomic rDNA of *E.tenella* confirmed that the five *Eimeria* isolates were *E. tenella* with an amplicon size of 278 bp (Fig. 1).

RAPD-PCR: Total numbers of reproducible fragments amplified by the six primers among the five *E. tenella* isolates were 180 of which 61 were polymorphic fragments as shown in Fig. 2. A, B, C, D and recorded in Table 2. The amplification was done with 1-35 DNA bands ranged from 50-2000 bp. All used primers showed high polymorphism which ranged from 23.3 to 48.5 % except primer 127 which showed low polymorphism (Fig. 2 C).

Primer 111 produced 20 bands, ranged in size from ~150-1500 bp. Among these bands 15 were shared, five were unique, one in Behera isolate, two in Alexandria isolate and two in Gharbia isolate (Tables 2, 3).

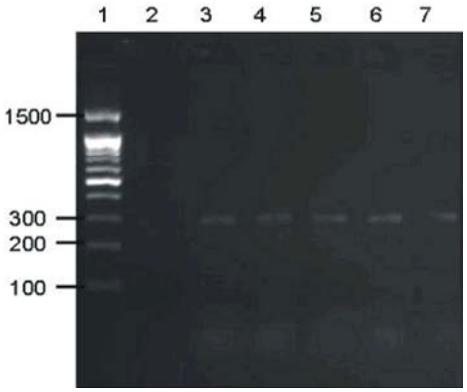

Fig. 1: PCR results targeting ITS-1 of the genomic rDNA of *E. tenella* with expected size 278 bp. Lane 1, 100 bp ladder DNA (1500 bp length), lane 2 negative control without DNA, lane 3-7 with DNA isolated from *E.tenella* isolates from 5 Egyptian province

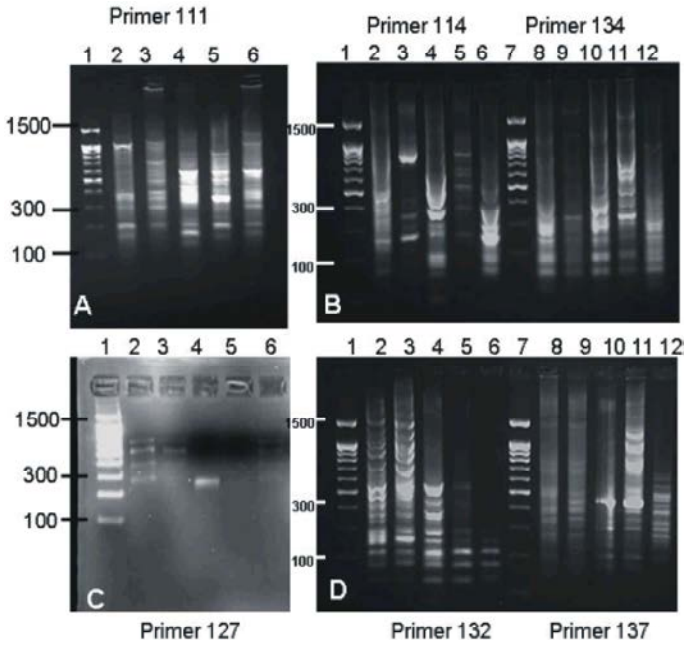

Fig. 2: RAPD-PCR fingerprints generated for the 5 *E.tenella* isolates, Panel A primer 111 (lanes 2–6), Panel B primer 114 (lanes 2–6) and primer 134 (lanes 8-12), Panel C primer 127 (lanes 2–6), Panal D with primer 132 (lanes 2–6) and primer 137 (lanes 8-12). Lane 1 in panel A–C and lane1, 7 in panel B and D, are ladders

Table 2: Summary of analysis for DNAs amplicon (bands) among the five *E.tenella* isolates using 6 RAPD primers

Primer name	Range of amplicon (bp)	Range of amplicon/ individual	Total Amplicon	Polymorphic amplicon	Polymorphism %	Similarity %
111	150-1500	9-14	20.0	8.0	40.0	60.0
114	50-2000	11-16	35.0	17.0	48.5	51.4
115	50-1500	5-15	27.0	10.0	37.0	62.9
127	250-800	1-4	4.0	0.0	0.0	100.0
132	100-1700	5-26	35.0	11.0	31.4	68.0
134	100-1600	12-21	30.0	7.0	23.3	76.0
137	150-2000	10-12	29.0	7.0	24.1	75.8
Average			25.5	8.5	29.2	70.6

Table 3: Unique positive and negative specific markers among the tested *E.tenella* isolates using RAPD primers.

Primer	No of Unique						No of Unique					
name	+ve bands	Behera	K-El-sheik	Alex-andria	Gharbia	Matrouh	-ve bands	Behera	K- El-sheik	Alex-andria	Gharbia	Matrouh
111	5	1	0	2	2	0	3	1	1	0	0	1
114	16	3	3	1	3	2	2	0	0	0	1	1
115	10	0	0	4	6	0	0	0	0	0	0	0
132	9	4	2	2	1	0	2	0	1	0	0	1
134	7	1	1	1	4	0	8	2	3	0	2	1
137	4	0	0	0	3	1	3	1	1	1	0	0

Table 4: Similarity values among the five *E.tenella* isolates based on overall RAPD primers

E. tenella isolates	Behera	K- El Sheikh	Alexandria	Gharbia	Matrouh
Behera					
K- El Sheikh	0.67				
Alexandria	0.49	0.50			
Gharbia	0.39	0.45	0.56		
Matrouh	0.61	0.54	0.53	0.55	

Primer 114 was a highly polymorphic primer (48.5%); it has produced 35 bands in the five isolates of *E. tenella* ranged in size from about 50 -2000 pb, from which, 16 unique positive and two unique negative bands were detected in the 5 isolates (Table 3). The distributions of these unique bands among the isolates are shown in Table 4.

Primer 115 produced 27 bands, ranged in size from 50-1500 bp with 62.9 % polymorphism. Among these bands 10 unique positive bands, four in Alexandria isolates and six in Gharbia isolate. This primer can be used as a specific marker to characterize Alexandria and Gharbia isolates (Tables 2, 3).

Primer 127 produced only four bands in the five isolates ranged from 250-800 bp. All these bands were shared. No unique bands were observed for any isolate (Fig. 2 C).

Primer 132 produced 35 bands in the 5 isolates, ranged in size from 100-1700 bp with 31.4% polymorphism. Among these bands nine are unique positive and three unique negative. Primer 132 could be used as specific genetic markers to characterize Behera isolate as it showed four unique bands.

Primer 134 produced 30 bands in the five isolates, ranged in size from 100- 1500 bp with 23.3% polymorphism. Among these bands, seven unique positive and eight negative bands were detected. The RAPD primer 134 could be used as a specific marker to characterize *E. tenella* isolates from Gharbia as it showed four unique bands (Fig. 2, Tables 2, 3).

Primer 137 produced 29 bands in the five isolates, ranged in size from ~150-2000 bp with 24.1% polymorphism. Among these bands, nine were unique positive and three were negative. Primer 137 could be used as specific genetic markers to characterize Gharbia isolate as it showed 3 unique bands (Fig. 2, Tables 2, 3).

Genetic similarity, dendogram analysis and relationship among the five Eimeria isolates: Construction of dendrogram based on genetic distance clustered the five *E. tenella* isolates as shown in Fig. 3. The dendrogram based on RAPD markers among the five isolates produced one main cluster which then subdivided into two sub-clusters. Within the first sub-cluster, Behera with K. El-Sheikh together separated from Matrouh isolate and in the second sub-cluster Gharbia with Alexandria isolates (Fig. 3). However, similarity values among the 5 isolates for each primer are listed in Table 2 and for overall primers are shown in Table 4. The mean similarity among the 5 isolates was 70.6 (Table 3). The highest similarity value for overall primer was between Behera and K. El-Skeikh (0.67), followed by Behera and Matrouh (0.61), while the lowest one was between Behera and Gharbia (39.0) (Table 4).

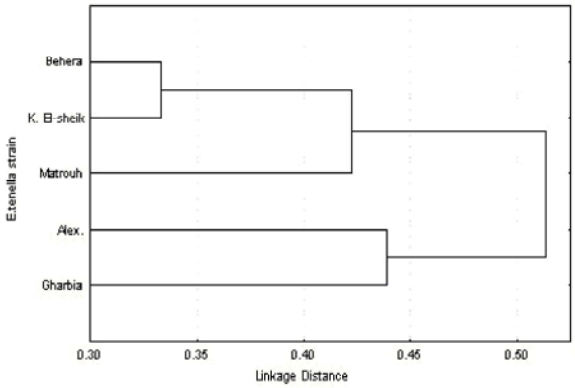

Fig. 3: Dendrogram of genetic relationship among the five *E. tenella* strains based on overall RAPD primers

Comparison between pathogenicity data and genetic polymorphism among the five isolate indicate that, Matrouh isolate (G5) showed the most pathogenicity through increased mortality and oocysts count, decreased Hb% and RBCs count compared with the other group. The phylogenetic tree indicates that G5 genetic structure is present in a separate line from the other groups (Fig. 3). Furthermore, G1 and G2 (Behera and Kafer El-Sheik) showed similarity in their genetic structure indicted in the phylogenetic tree (Fig. 3). The similarity index between Matrouh and Behera isolate (0.61) is high value. Groups 3, 4 (Alexandria, Gharbia isolate), showed similarity in their genetic structure as indicated in the phylogenetic tree (Fig. 3).

DISCUSSION

Different evolutionary mechanisms such as migration, selection and genetic drift play a fundamental role in the genetic distribution in natural populations. These mechanisms have important consequences from an epidemiological and medical point of view (strain typing, pathogenicity, vector specificity and susceptibility to drugs and vaccines) [26-28].

There are differences among *Eimeria tenella* isolates around the world. The antigenic variation observed in *E. tenella*, *E. maxima* and *E. acervulina* isolates has been described in samples collected from different geographical regions [14, 15, 29]. Thus, regional *Eimeria* isolates evaluation is very important to verify their pathogenicity and eventually the possible utilization in the production or evaluation of vaccines.

Multiple species of *Eimeria* frequently infect a single host, leading to an overlap of the affected zones in the intestines, variation in the nature and extent of lesions and considerable overlap in oocyst size and shape [30, 31]. In the present study, it was confirmed that the five *Eimera* isolates, obtained from field cases from five different geographical regions and undergone single oocyst isolation, were *Eimeria tenella* by using ITS-1 primer specific for *E.tenella* with amplicon size 278 bp. Many studies have used primers derived from internal transcribed spacer 1 (ITS-1) for differential diagnosis of *Eimeria* species [5, 6, 21, 32]. While the ribosomal genes themselves tend to be conserved, these spacers are relatively heterogenous in length and sequence among species, such that specific primers may be designed to the flanking gene sequences. Like other regions of nuclear rDNA, these spacers evolve in a concerted fashion and are repetitive in nature [33], making any PCR assay (based on their use) sensitive.

Depending on seven RAPD primers, amplification were performed with bands ranged from 1-35 DNA segments ranged from 50 and over 2000 bp. Nowzari *et al.* [34] in a similar study, obtained 1-14 bands but with higher molecular weight ranged from 240 to 3000 bp. All primers used in this study showed a large number of bands except primer 127 which showed 1-4 bands, this primer produced smearing pattern which has been reported by Procunier *et al*. [22] and Nowzari *et al*. [34].

RAPD has been used to study variation within species of *Eimeria* from chickens. Shirley and Bumstead [9] examined seven strains of *E. tenella* of varying biological phenotypes. The RAPD primer used in this study showed great variation among the *Eimeria tenella* isolates obtained from the five different provinces. This variation is indicated by the polymorphism percentage in each primer alone or the average polymorphism among all primers (36.8%) for each isolate. Some researchers have used the calculation of similarity coefficients to gain a measurement of degree of relatedness [22, 24]. In this study, the similarity in the seven RAPD primers among the 5 isolates was 70.6%. Nowzari *et al*. [34] demonstrated that intra-specific similarity coefficient within five isolates of *E. tenella* in Iran was 82%. This indicated that the polymorphism among the 5 Egyptian isolates of *Eimeria tenella* in our study was high although they all are restricted to north Egypt.

The impact of coccidiosis outbreaks on the productivity of broiler chicken farms can be substantial, depending on the severity of disease caused by particular species and strains of *Eimeria*. In this study, the genetic diversity of five *Eimeria tenella* isolates was analyzed parallel with a separate study concerning the pathogenecity of the same five isolates of *Eimeria tenella* [10]. They have assessed pathogenicity of the five isolates by calculating body weight gain, feed conversion ratio, lesion scores, dropping nature scores, cecal scrapings, mortality percentage, oocysts count and hematological parameters. They have found that, there were different degrees of pathogenicity among the isolates. Matrouh strain was the most pathogenic one followed by Behera, while Alexandria and Gharbia isolates almost had the same pathogencity. The most pathogenic strain was Matrouh one, which isolated from North West region of Egypt far from the other four provinces. The results of Abou-Akkadda and Awad [10] coincidences with our genetic study on the five *Eimeria* isolates. Kawazoe *et al*. [29] and Nowzari *et al*. [34] suggested that the possibility of strain antigenic differences and pathogencity could be more frequent depending on geographic regions of the farms.

Schwarz *et al*. [35] examined the genome of *Eimeria* species population in relation to the performance in four broiler chicken farms; they demonstrated the diversity in *Eimeria* species variants associated with different pathogenicity and performance in the four farms.

Vaccination of chickens with live oocysts has become a more widely used method for controlling avian coccidiosis as resistance to anticoccidial medication increases. However, some coccidia strains are not useful in multi-species vaccines because antigenic variation has made them generally less protective [36]. In addition, the composition of a vaccine must be appropriate for each, geographical region, production system and age of the chickens to maximize its efficacy [37, 38]. In the present study, detection of genetically different isolates of *E.tenella* in five Egyptian provinces with different pathogencity raises the question about the efficacy of commercial anticcocidial vaccines already used in Egyptian markets. A further study will follow to compare the five isolates *E.tenella* isolates with the commercial vaccinal strains to explore its efficiency.

CONCLUSIONS

It could be concluded that there were genetic polymorphisms detected between *E. tenella* isolates from the five Egyptian provinces by using RAPD analysis. Comparing these results with that of the pathogenicity of the five isolates of *E. tenella* revealed that *E.tenella* from different geographical regions are genetically different and this could affect on degree of pathogenicity.

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