

RESEARCH

Open Access



Genetic diversity and drug sensitivity studies on *Eimeria tenella* field isolates from Hubei Province of China

Li Tan, Yalin Li, Xin Yang, Qiyun Ke, Weiqiang Lei, Mudassar Niaz Mughal, Rui Fang^{*}, Yanqin Zhou, Bang Shen Junlong Zhao^{*}

Abstract

Background: Avian coccidiosis is an intracellular intestinal parasitic disease, caused by intracellular intestinal parasites from the genus *Eimeria*, among which *Eimeria tenella* is one of the most pathogenic species and causes great economic losses. Frequent applications of anticoccidial drugs have resulted in the development of drug-resistance in *E. tenella*. In the present study, we sought to determine the genetic diversity of *E. tenella* isolates prevalent in chicken farms in Hubei Province of China and examine their sensitivity to three anticoccidial drugs. The results provide useful information for the prevention and control of coccidiosis in this region.

Methods: *Eimeria tenella* oocysts were isolated from faecal samples collected from different commercial broiler production farms in Hubei Province, China. After oocyst sporulation and animal inoculation for expansion of the field isolates, DNA and RNA were extracted from excysted sporozoites for molecular characterization. Species identification of field isolates were performed by polymerase chain reaction (PCR) amplification of the internal transcribed spacer 1 (ITS1) region of ribosomal DNA. Random amplified polymorphic DNA (RAPD) was used for population genetic analysis. Subsequently, sequences of the major sporozoite surface antigen (SAG), micronemal protein 2 (MIC-2) and cytochrome *b* (*cytb*) genes from genomic DNA, and the *Eimeria tenella* cation-transport ATPase (*EtCat* ATPase) gene from cDNA were obtained for genotyping using multi-sequence alignments. Finally, sensitivity of the field isolates to three commonly used anticoccidial drugs (diclazuril, decoquinate and maduramycin) were tested to assess the prevalence of drug resistance in *E. tenella* in Hubei Province of China.

Results: Analysis of the ITS1 sequences indicated that all the isolates were *E. tenella*. RAPD analysis and multi-sequence alignments of the SAG, MIC-2, *EtCat* ATPase and *cytb* showed genetic diversity among these isolates. Finally, drug sensitivity tests demonstrated that all field isolates were sensitive to diclazuril but resistant to decoquinate (except for the isolates from eastern Hubei) and maduramicin.

Conclusions: Population genetic analysis indicated that genetic polymorphisms among field isolates were closely related with their regional distributions. Drug sensitivity testing demonstrated that *E. tenella* isolates in Hubei Province were sensitive to diclazuril, but resistant to maduramycin and decoquinate. The results presented here provide important information for the control and preventions of coccidiosis in the Hubei Province of China.

Keywords: *Eimeria tenella*, Genetic diversity, Maduramycin, Decoquinate, Diclazuril

* Correspondence: fangrui19810705@163.com;
zhaojunlong@mail.hzau.edu.cn
State Key Laboratory of Agricultural Microbiology, College of Veterinary
Medicine, Huazhong Agricultural University, Wuhan 430070, Hubei, People's
Republic of China

Background

Avian coccidiosis, an intestinal parasitic disease caused by *Eimeria* spp., has caused great economic losses to the poultry industry worldwide [1, 2]. When chickens are infected with *Eimeria tenella*, clinical signs include lethargy, feather dishevelment and bloody feces. The main pathological changes include thickening of the intestinal wall and petechial hemorrhages.

Eimeria tenella is one of the most pathogenic species of *Eimeria*. Accurate identification is essential for the prevention and control of *E. tenella*. Many studies have focused on genetic diversity of *E. tenella* [3, 4]. As a useful molecular marker, the ITS1 fragment has been widely used for species identification of *Eimeria* [5]. Clark et al. used phylogenetic analysis of ITS sequence data to define species diversity between and within populations for all seven *Eimeria* species of chickens [6]. Schwarz et al. examined the genetic diversity of *Eimeria* species in Arkansas (AR) and North Carolina (NC) by analyzing ITS [7]. Williams et al. developed a RAPD technique based on the amplification of undefined targets by arbitrary primers to detect genetic polymorphisms [8], the technique has been widely used for the analysis of *Eimeria* genetic diversity [9, 10]. Such analysis can help us to estimate the phylogenetic relationship among different *Eimeria* isolates [11].

Currently, the main method to control *Eimeria* infection is anticoccidial drugs [12, 13]. Maduramycin, decoquinate and diclazuril are three chemotherapeutic agents. Maduramycin is thought to kill coccidium by interrupting their normal intracellular ion balance and influence of Na^+ - K^+ -ATPase activity [14]. Decoquinate interferes with the electron transport in the mitochondrial cytochrome system and CytB is an important part of the system [15]. The working mechanism of diclazuril is still unknown. Because of the prolonged use of anticoccidial drugs, resistance to such drugs has been frequently reported [16]. In addition, MIC2 and SAG genes are thought to be involved in host cell adhesion and invasion [17, 18]. Therefore, we aimed to compare the genetic diversity among field isolates using the four selected genes: *EtCat* ATPase and *cytb* genes which are drug targets; MIC2 and SAG genes which are important for interactions between parasites and host.

In the present study, the genetic diversity and drug sensitivity of *E. tenella* field isolates from Hubei Province of China were analyzed. The results should provide useful information for prevention and control of coccidiosis in this region.

Methods

Animals

Coccidia-free, 0-day-old chickens were purchased from Charoen Pokphand Group (Wuhan, China). Chickens were housed in a clean, coccidia-free environment in an

isolated brooder room, and fed with commercial broiler feed and water [19].

Parasite material

Faecal samples were collected from eight different local commercial broiler production farms in Hubei Province between January 2012 and November 2013 (Additional file 1: Table S1). *Eimeria* oocysts obtained from the faecal samples were purified by saturated sodium nitrate flotation and sporulated using standard procedures [20].

Specific pathogens free (SPF) chickens (14-day-old) obtained from Charoen Pokphand Group were orally inoculated with 5×10^4 sporulated oocysts. Seven days post-inoculation, the chickens were sacrificed and necropsies were conducted. The caecal contents were collected for the isolation of *Eimeria* oocysts using the protease digestion method. The protease digestion and sporulation method were as follow: (i) the caeca from *Eimeria* oocysts infected groups were homogenized using a tissue grinder and 2 mg/ml of trypsin was added; (ii) this suspension was incubated in a water bath kettle for 1 h at 39 °C, sieved (180 diameter mesh) and transferred into a 500 ml centrifuge tube; (iii) the suspension was centrifuged at $1000 \times g$ for 5 min; (iv) the sediment was suspended in 2% w/v aqueous potassium dichromate and transferred to a 50 ml conical tube for oocysts sporulation in an aerobic incubator for 4 days at 28.6 °C. Sporulated oocysts were stored in 2.5% potassium dichromate at 4 °C [21] and were enumerated using the McMaster's method under the microscope [22].

DNA and RNA extraction

For DNA extraction, 10^4 sporulated oocysts from each sample were washed with 1 mM sodium hypochlorite solution for 10 min at 4 °C, then washed three times with deionized water. The oocyst walls were ruptured using a grinding tissue homogenizer for released sporozoites (the volume 200 μl and homogenized), then 100 μl (1 mg/ml) of chicken bile, 100 μl of buffer suspension solution GA, and 20 μl (20 mg/ml) of proteinase K (Genomic DNA Kit, TIANGEN, Beijing, China) were added and the mixture was incubated at 56 °C for 2.5 h. DNA was extracted using a Genomic DNA Kit (TIANGEN), according to the manufacturer's instructions. DNA concentration was measured using a NanoDrop 2000 nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). In parallel, total RNA from sporozoites (5×10^4) was extracted using TRIzol reagent, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). First strand cDNA was synthesized by a reverse transcription (RT) reaction using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA).

ITS1 amplification and RAPD analysis

Extracted DNA was used as a template to amplify the ITS1 region of *Eimeria* spp., as described by Schnitzler et al. [23, 24]. RAPD random primers were synthesized by Sangon Biotech (Shanghai, China), and the conditions used for RAPD typing were the same as described by Fernandez et al. [10]. The sequences of the random primers used for RAPD amplification are listed in Additional file 1: Table S2. To examine the amplification results, all amplicons were assessed by electrophoresis through 1% agarose gels and then visualized by ethidium bromide staining.

Polymorphism analysis

Polymorphisms in *cytb*, MIC-2, SAG and *EtCat* ATPase genes were identified by sequencing the corresponding genes. The *cytb* and SAG genes were PCR amplified from the genomic DNA of isolated isolates using PrimeSTAR® Max DNA Polymerase (TaKaRa, Tokyo, Japan). The primers used are listed in Additional file 1: Table S3 and the following PCR program was used: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at primer-dependent temperatures, and 60 s extension at 72 °C, followed by a final 7 min extension at 72 °C. The annealing temperatures were 60 °C for the *cytb* gene, 58 °C for MIC-2 and 55 °C for SAG. *EtCat* ATPase gene sequence is unavailable, the cation-transporting ATPase gene is highly conserved across *Eimeria* species, therefore, the primers used for amplifying *E. tenella* cation-transporting ATPase gene were designed based on the *E. acervulina* clone Dui-10 cation-transporting ATPase gene complete coding region (EU590120.1). The *EtCat* ATPase gene were amplified from the cDNA of field isolates for sequence alignments using similar PCR reaction conditions. All PCR products were subject to agarose gel electrophoresis and visualized by ethidium bromide staining. PCR products for ITS-1 amplification and polymorphism analysis were sent to Sangon Biotech (Shanghai, China) for sequencing using primers in both directions.

Drug sensitivity tests

Selected field isolates were tested for their sensitivity to three coccidiostat drugs maduramycin, decoquinate and diclazuril. The design of drug-sensitivity tests in Additional file 1: Table S4. In a coccidia-free environment, 170 14-day-old SPF chickens were weighed and randomly divided into 17 groups, each containing ten chickens. All 17 groups were inoculated with *E. tenella* except one, which was not infected and used as a control. Infected chickens were given 5 mg/kg maduramycin, or 1 mg/kg diclazuril, or 30 mg/kg decoquinate, or no treatment as control, in fodder from the time of infection. Chicken faeces were collected from each post-infection groups

between 5–7 days single droppings per day to evaluate the relative number of oocysts per gram of feces (OPG). At 21-day of age (7 days post-infection), all the chickens were individually weighed, sacrificed, and necropsied. The weight gain of chickens was recorded. The coccidial lesions present in the chickens were scored as category 0–4 following the methods described by Johnson & Reid [25]. Subsequently the anti-coccidial index (ACI) was calculated to assess drug effectiveness.

Sequence analysis

Alignment of the ITS1 sequences from *Eimeria* isolates was performed using MAFFT version 7 (<http://mafft.cbrc.jp/alignment/server/index.html>). Pairwise percentage identity was determined using BioEdit v.7.0 (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>). RAPD results are analyzed by using SAHN program of the NTSYS-pc software (version 2.02 K,

Applied Biostatistics, Inc, NY, USA). The amino acid sequences of the *EtCat* ATPase gene from selected parasites were compared by the DNAMAN software (<http://www.lynnon.com/dnaman.html>). Phylogenetic analyses were conducted using MEGA, version 5.0.

Results

Isolation and species identification of *Eimeria tenella* field isolates from local farms in China

Twenty-one *Eimeria* field samples were isolated from different local commercial broiler production farms in Hubei Province (Fig. 1). To confirm the species identity of these *Eimeria* samples, seven pairs of species-specific primers (for *E. acervulina*, *E. brunetti*, *E. mitis*, *E. necatrix*, *E. maxima*, *E. praecox* and *E. tenella*, respectively) amplifying ITS1 region were used. The identification results of *Eimeria* species using species specific primers amplifying the internal transcribed spacer 1 (ITS1) region in Additional file 1: Table S5. The results indicate that all the field isolates were *E. tenella* since they produced a specific 278 bp band using the *E. tenella*-specific primer (one example is given in Fig. 2). This fragment obtained from each strain was subject to DNA sequencing and the results showed that they were 97–100% identical to the ITS1 sequence of *E. tenella* (GenBank No. AF026388), further confirming that these isolates are *E. tenella* (GenBank No. KY117132–KY117152). Pairwise comparison of ITS1 sequences from these isolates revealed high sequence identities ranging from 92.5–100% (Table 1), suggesting homology among these isolates. Phylogenetic relationship based on the ITS1 of *E. tenella* north (Suizhou isolate), *E. tenella* east (Huanggang isolate), *E. tenella* south (Jingzhou isolate), *E. tenella* middle (Tianmen isolate), *E. tenella* UK (GenBank: LN609779), *E. tenella* US (GenBank: LN609784), *E. maxima* (GenBank: AF065095), *Neospora caninum* (GenBank: AF029702) and

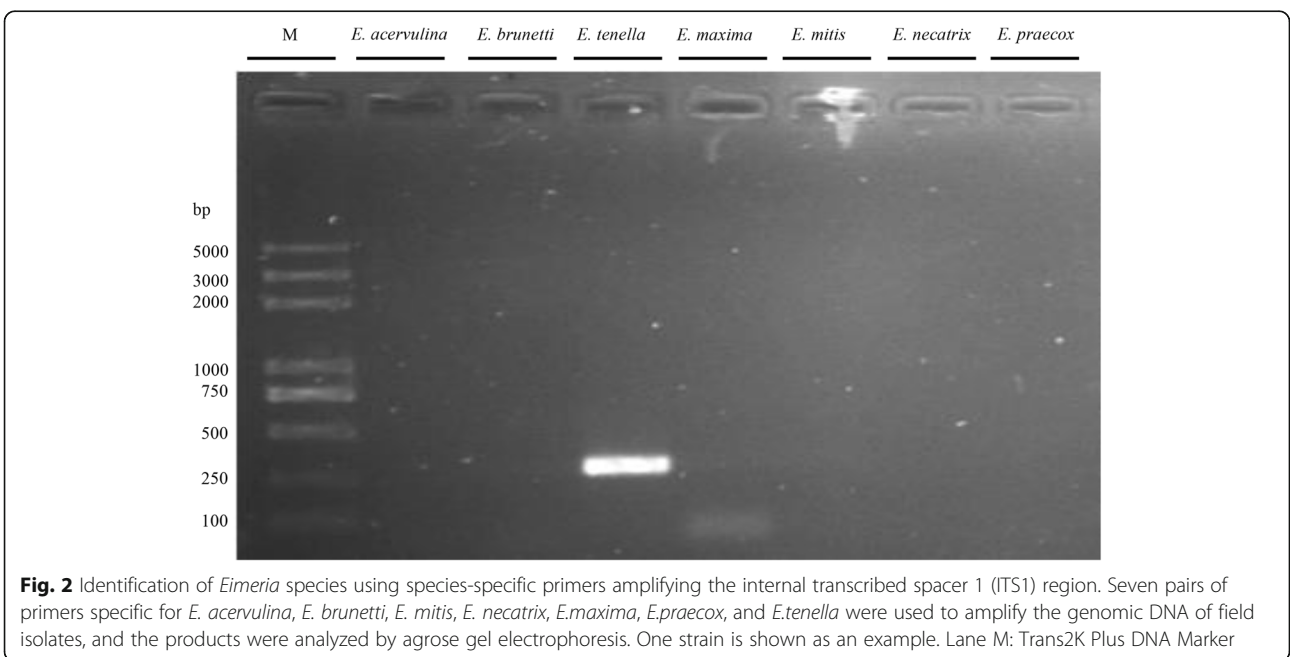
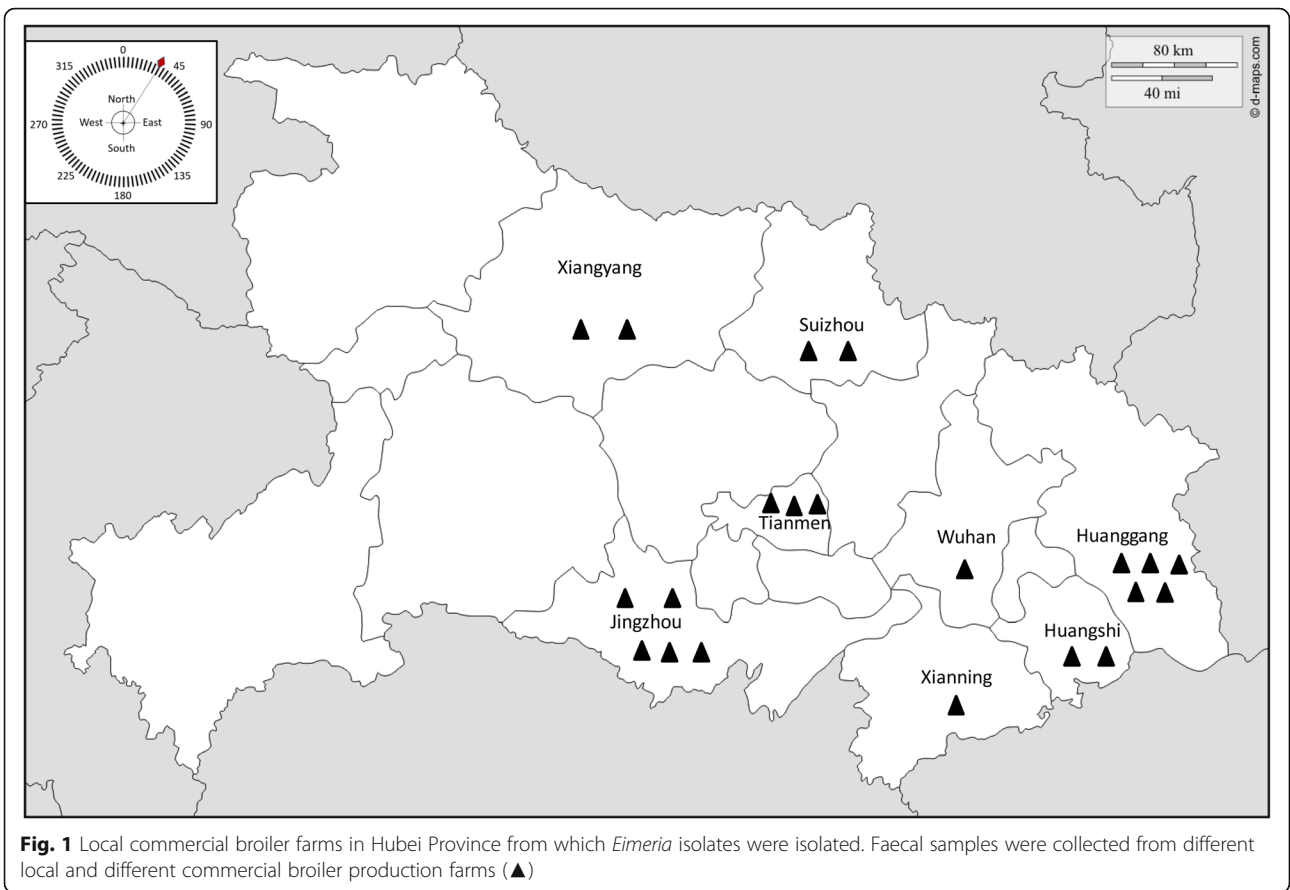


Table 1 Pairwise comparison between the ITS-1 sequences of different *Eimeria tenella* isolates

Seq->	TM1	TM2	TM3	SZ1	SZ2	HS	JZ1	JZ2	JX	XN	XS1	XS2	XS3	XS4	XS5	SS1	SS2	SS3	XY1	XY2	DY	
TM1																						
TM2	94.8																					
TM3	95.6	94.8																				
SZ1	96.8	95.6	97.6																			
SZ2	96.0	95.6	97.2	96.8																		
HS	94.8	96.4	94.8	95.6	95.2																	
JZ1	97.6	95.2	96.8	98.0	97.2	96.4																
JZ2	94.9	93.7	94.5	94.9	94.9	94.9	95.7															
JX	96.0	94.4	95.2	96.0	96.8	95.6	97.6	95.3														
XN	95.6	95.2	96.0	96.0	96.0	94.5	96.8	94.9	95.7													
XS1	94.4	96.4	96.0	96.4	95.6	96.0	95.6	94.5	94.4	94.9												
XS2	97.2	95.6	97.2	98.4	97.6	96.4	99.6	95.7	97.6	97.2	96.0											
XS3	97.2	95.2	97.2	97.2	97.2	95.6	98.4	95.7	96.4	96.8	95.6	98.8										
XS4	96.8	96.0	96.8	97.2	98.4	96.0	98.4	96.4	97.2	97.2	96.4	98.8	98.0									
XS5	96.8	94.4	96.0	96.8	96.4	95.2	98.8	95.7	96.4	96.0	96.0	98.4	97.6	97.6								
SS1	92.5	94.0	94.8	93.6	93.6	94.8	94	92.6	93.7	93.7	96.0	94.4	94.0	94.0	94.1							
SS2	94.1	95.2	95.6	95.2	95.2	95.6	95.2	94.9	94.8	94.9	96.8	95.6	95.2	96.0	94.5	97.2						
SS3	96.8	95.6	98.3	97.6	97.2	94.8	97.2	94.5	96.4	96.0	96.0	97.6	96.8	96.8	96.0	94.4	95.6					
XY1	96.8	95.6	97.2	98.0	96.4	95.6	98	94.5	96.0	96.0	95.6	98.4	97.2	97.2	96.8	94.0	94.8	98.0				
XY2	95.2	96.4	96.4	96.0	98.4	96.0	96.4	94.1	96.0	95.2	96.4	96.8	96.0	97.2	96.0	95.2	96.4	96.8	95.6			
DY	93.3	94.0	95.2	94.0	94.4	95.2	94.4	94.1	94.0	93.7	96.0	94.8	94.4	95.2	94.5	97.6	98.0	94.4	93.6	96.0		

Toxoplasma gondii (GenBank: L49390) was accomplished by MEGA v6.0 using neighbor-joining method with default setting [26], and nodal support values were indicated (%). The phylogenetic tree demonstrated the four *E. tenella* field isolates had close relationship with *E. tenella* UK (Fig. 3).

RAPD analysis

To optimize the primers for RAPD analysis, 70 decamer were screened using *E. tenella* DNA samples. After the

initial screening, seven primers were found to discriminate the *E. tenella* species. They resulted in two to eight amplicons ranging from 250–1,500 bp using the field isolates as templates. The amplification patterns of the field isolates generated by the S2133 primer are shown in Fig. 4. NTSYS-pc software was used to convert the amplification results into data to assess the phylogenetic relationships among the *E. tenella* field isolates (Fig. 5). Briefly, the unweighted pair-group method with arithmetic

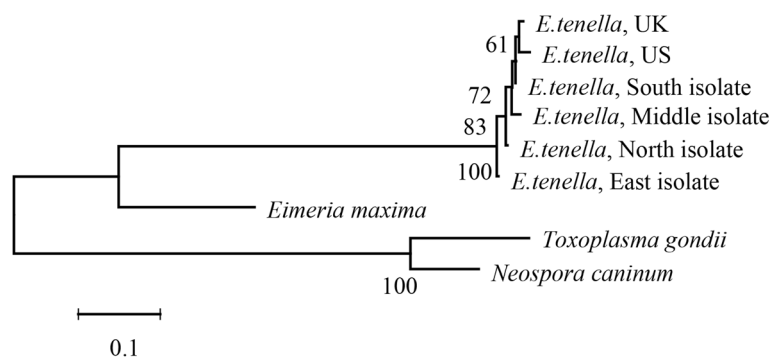


Fig. 3 Phylogenetic relationships of *E. tenella* north (Suizhou isolate), *E. tenella* east (Huanggang isolate), *E. tenella* south (Jingzhou isolate) and *E. tenella* middle (Tianmen isolate) with *E. tenella* UK (GenBank: LN609779), *E. tenella* US (GenBank: LN609784), *E. maxima* (GenBank: AF065095), *Neospora caninum* (GenBank: AF029702) and *Toxoplasma gondii* (GenBank: L49390); based on ITS1 sequences by neighbor-joining analysis. Nodal support values are indicated (%). The scale-bar indicates sequence substitution per site

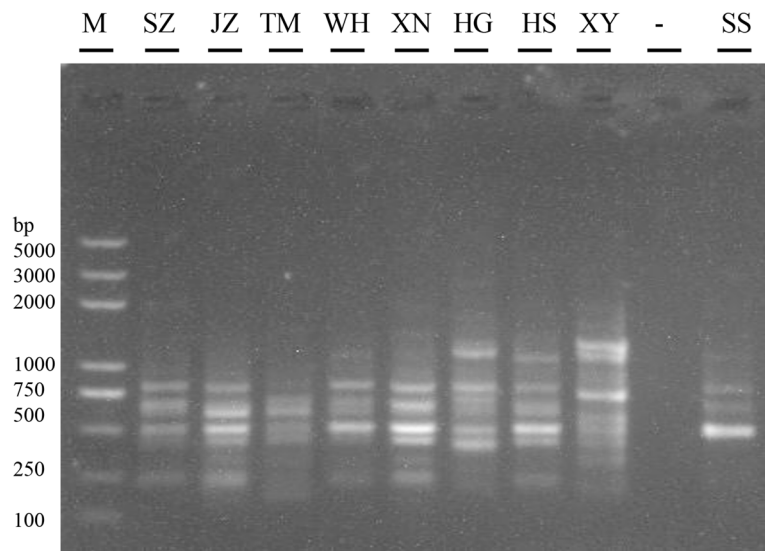


Fig. 4 Random amplified polymorphic fragments obtained with decamer primers S2133 using DNA samples of different field isolates. Abbreviations: SZ, Suizhou; JZ, Jingzhou; TM, Tianmen; WH, Wuhan; XN, Xianning; XS, Xishui; HS, Huangshi; XY, Xiangyang; SS, Shashi; "-", Negative control; M: Trans2K Plus DNA Marker

averages (UPGMA) dendrogram of the RAPD data was calculated and analysed using SAHN program of the NTSYS-pc software. The RAPD data were generated from the DNA fingerprints of the eight *E. tenella* isolates using seven random primers, and the results indicated eight *E. tenella* isolates were separated into two branch clusters mainly based on geographical distribution, the first cluster included SZ, XY, TM and WH, and the second cluster included JZ, SS, XS and HG. The phylogenetic branch lengths ranged from 0.49 to 0.75, and the clusters may be caused by cross-regional transportation of animals.

Sequence diversity of the cytochrome B gene

Amplicons of the *E. tenella* *cytb* gene (1,268 bp) derived from the field isolates were sequenced and aligned with that of the *E. tenella* Houghton strain (GenBank: HQ173891.1). The result revealed that the *cytb* gene

sequences from our field isolates (GenBank: KY117213–KY117232) have 99.9% similarity with the *cytb* gene from the *E. tenella* Houghton strain. Polymorphisms were observed at three positions (position 22, C-A; position 49, A-G; position 838, T-C); these are listed in Table 2. These nucleotide changes do result in amino acid alterations in the CytB protein.

Sequence diversity of the MIC-2 and SAG genes

Amplification of the MIC-2 gene from the *E. tenella* field isolates produced a specific 1,647-bp band. This fragment from each isolate was sequenced and aligned with the *E. tenella* MIC-2 gene (GenBank: AF111702.1). The results indicated that there is no polymorphism between field isolates (GenBank: KY117173 to KY117192) and the reference strain. Amplification of SAG gene yielded a single 1,101-bp band in all field isolates. Sequence alignment with the *E. tenella* surface antigen gene (GenBank: M21088.1) indicated that all of the field isolates (GenBank: KY117193–KY117212) contained two nucleotide substitutions at positions 209 (A-G) and 901 (C-G), but these nucleotide substitutions did not cause amino acid changes.

EtCat ATPase gene

PCR amplification of the *EtCat* ATPase gene from the field isolates produced one single band of 494 bp on agarose gels. Sequencing analysis indicate that the sequences of *Et.Cat* ATPase gene from all field isolates (GenBank: KY126385 to KY126404) are also identical to the *E. tenella* mitochondrial hypothetical protein (GenBank: KF670727.1). The amino acid sequence of *Et.Cat*

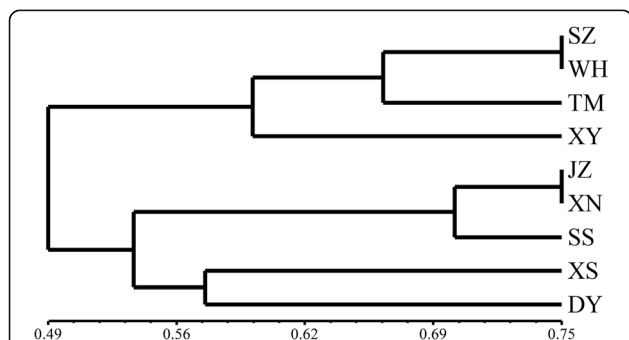


Fig. 5 Clustering of *E. tenella* field isolates following RAPD analysis. Abbreviations: SZ, Suizhou; JZ, Jingzhou; TM, Tianmen; WH, Wuhan; XN, Xianning; XS, Xishui; HS, Huangshi; XY, Xiangyang; SS, Shashi

Table 2 Mutations in the CytB gene of *Eimeria tenella* field isolates

Strains	Mutations
DY	C-A(22 th) A-G(49 th) T-C(838 th)
XS1	C-A (22 th)
XS2	C-A (22 th)
XS3	0
XS4	0
HS	0
JZ1	C-A/C(22 th) A-G/A(49 th) T-C(838 th)
JZ2	C-A/C(22 th) A-G/A(49 th) T-C(838 th)
SS1	C-A/C(22 th) A-G/A(49 th) T-C(838 th)
SS2	C-A/C(22 th) A-G/A(49 th) T-C(838 th)
SS3	C-A(22 th) A-G(49 th) T-C(838 th)
SZ1	C-A/C(22 th) A-G/A(49 th) T-C(838 th)
SZ2	C-A/C(22 th) A-G/A(49 th) T-C(838 th)
TM1	C-A(22 th) A-G(49 th) T-C(838 th)
TM2	C-A(22 th) A-G(49 th) T-C(838 th)
TM3	C-A(22 th) A-G(49 th) T-C(838 th)
WH	C-A(22 th) A-G(49 th) T-C/T(838 th)
XN	C-A(22 th) A-G(49 th) T-C(838 th)
XY1	C-A(22 th) A-G(49 th) T-C(838 th)
XY2	C-A(22 th) A-G(49 th) T-C(838 th)

ATPase shared 87% similarity with that of the *E. acervulina* cation transporter ATPase gene (GenBank: EU590120.1), 80% similarity with *E. maxima* Cation-transporting ATPase (GenBank: XP_013337038.1), and 34% similarity with the cation-transporting ATPase gene (GenBank: EPR61390.1) of *T. gondii* (Fig. 6).

Drug susceptibility of *E. tenella* isolates from Hubei Province

To test the status of drug resistance in the field, four isolates representing different geographical origins were selected and their sensitivity to commercially available drugs were tested. Diclazuril had an ACI higher than 180 in the field isolates from northern, eastern and middle regions of Hubei Province. The ACI of Diclazuril on the strain from southern part of Hubei is 168.8. These results suggest that all field isolates from these regions are sensitive to Diclazuril. The data for decoquinate drug tests showed that *E. tenella* field isolates from northern, southern and middle Hubei had ACI below 160, suggesting decoquinate tolerance. While for the strain from eastern Hubei, decoquinate had an ACI value of 175.8, indicating that this isolates is still sensitive to decoquinate. For maduramicin, and all tested isolates had ACI values below 160, which indicate that they were resistant to this drug (Table 3).

Discussion

Chicken coccidiosis caused by *Eimeria* species leads to severe economic losses to the poultry industry worldwide [27, 28]. It is listed as one of the top five most devastating diseases in poultry. The morbidity of coccidiosis is estimated to be 50–70% and the disease is a major threat to 15–50 day-old chickens [29–31].

In this study, we purified *Eimeria* oocysts from chicken faecal samples collected from eight regions (Suizhou, Xiangyang, Huanggang, Huangshi, Xianning, Wuhan, Jingzhou, Tianmen) in Hubei Province of China. Subsequently all the isolates were identified to be *E. tenella* using species-specific PCR amplification of the ITS1 rDNA region.

The PCR-based RAPD technique, which was originally developed in the 1990s, can be used to genotype organisms and identify unknown sequence polymorphisms among genetically diverse isolates [32]. Here, we used random primers designed by Sangon Biotech for RAPD analysis of the *E. tenella* field isolates collected from different regions of Hubei, China. The result indicated that there are genetic differences among the *E. tenella* field isolates in this region and the differences may correlate with geographical origin of the isolates.

Many studies have investigated the development of resistance mechanisms against anti-coccidian drugs in *Eimeria* parasites and proposed various hypotheses [33]. However, the most popular opinion for the development of drug resistance is the endogenous mutations occurring in coccidian parasite [34]. Because of continuous and prolonged use of anticoccidial drugs, mutations have been observed among coccidian population during this continuous selection process. These mutations maybe involved in the development of drug resistance [35].

In other apicomplexan species, genetic mutations are known to be associated with drug resistance. The correlation between the acquisition of mutations and drug resistance has been verified, for example, a point mutation at position 268 of the *cytb* gene was reported to induce atovaquone resistance in *Plasmodium falciparum* [36]. In the present study, genetic mutations also existed in resistant field isolates, whether the mutations are related with drug resistance of field isolates or not need further verification.

In addition to RAPD, we also examined the genetic diversity of field isolates by checking the sequence polymorphisms in MIC-2, SAG, *EtCat* ATPase and *cytb* genes. Sequence analysis indicated that the MIC-2, SAG, *EtCat* ATPase genes are highly conserved among *E. tenella* isolates. For the *cytb* gene, the alignments showed that some isolates contain polymorphisms leading to missense mutations. These changes occurred at positions eight, 17 and 280 of the CytB protein.



Table 3 The drug susceptibility of *E. tenella* field isolates

Groups	Field isolates	Weight gain (g)	Weight gain of NNC ^a (%)	Survival rate (%)	Lesion score	OPG (10 ⁵)	Anticoccidial index
1 ^a	North isolate ^d	76	100	100	1.2	0.813	183
2 ^a	East isolate ^e	77	101.3	100	1.0	0.658	186.3
3 ^a	South isolate ^f	69	90.8	90	1.2	1.44	168.8
4 ^a	Middle isolate ^g	77	101.3	100	1.1	0.792	185.3
5 ^b	North isolate ^d	54	71.1	100	2.6	1.06	140.1
6 ^b	East isolate ^e	66	86.8	100	0.6	0.598	175.8
7 ^b	South isolate ^f	47	61.8	100	1.6	1.27	140.8
8 ^b	Middle isolate ^g	62	81.6	70	3.2	1.3	129.6
9 ^c	North isolate ^d	53	69.7	100	2.8	0.938	136.7
10 ^c	East isolate ^e	55	73.3	100	2.1	0.957	147.3
11 ^c	South isolate ^f	51	67.1	90	2.4	1.16	128.1
12 ^c	Middle isolate ^g	50	65.8	90	3.1	1.25	119.8
13	North control	39	51.3	90	3.5	6.25	108.8
14	East control	63	82.9	80	3.4	5.98	123.5
15	South control	46	60.5	90	3.4	5.52	114.1
16	Middle control	44	57.9	80	3.3	5.66	98.6
17 ^h	NNC control	76	100	100	0	0	200

^aDiclazuril treatment groups

^bDecoquinatate treatment groups

^cMaduramycin treatment groups

^dSuizhou isolate

^eHuanggang isolate

^fJingzhou isolate

^gTianmen isolate

^{d-g} isolates control: infected dose per chicken for 5 × 10⁴ sporulated oocysts, non-treatment control (groups, 13–16)

^hNNC control: non-infected, non-treatment control (group,17)

Conclusions

In this study, we isolated and analyzed *E. tenella* field isolates from eight regions of Hubei Province, P. R. China. RAPD analysis and multiple sequence alignments of SAG, MIC-2, *cytb* and *EtCat* ATPase revealed genetic diversity among field isolates. The drug sensitivity tests indicated that maduramycin and decoquinate resistance are widely present in Hubei, but diclazuril is still effective towards *E. tenella* isolates in this region. These results are important for the selection of strategies to control chicken coccidiosis in this region.

Additional file

Additional file 1: Table S1. Local commercial broiler farms in Hubei province from which *Eimeria* isolates were isolated. **Table S2.** Primers used in RAPD analysis by Sangon Biotech, Shanghai. **Table S3.** Primers used for PCR amplification of indicated genes. **Table S4.** The design of drug-sensitivity tests. **Table S5.** Identification results of *Eimeria* species using species specific primers amplifying the internal transcribed spacer 1 (ITS1) region. (DOC 126 kb)

Abbreviations

RAPD: Random amplified polymorphic DNA; ITS: Internal transcribed spacer 1; SAG: Surface antigen; MIC-2: Micronemal protein 2; *cytb*: Cytochrome *b*; *EtCat* ATPase: *Eimeria tenella* cation-transport ATPase; RT: Reverse transcription; OPG: Oocysts per gram of faeces; SPF: Specific pathogens free; ACI: Anti-coccidial index

Acknowledgements

We would like to thank professor Shijun Li for providing space for animal experiments.

Funding

This study was supported by the national key research and development program (2016YFD0501303) and the Fundamental Research Funds for the Central Universities (Grant No. 2662015PY048).

Availability of data and materials

All data generated or analyzed during this study are included in the article. The sequences are submitted to the GenBank database under accession numbers KY117132–KY117152, KY117173–KY117232 and KY126385–KY126404.

Authors' contributions

JLZ and RF conceived and designed the study. LT and YLL wrote the manuscript with input from other coauthors. LT, XY, WQL, QYK, and MNM performed the experiments and analyzed the data. YQZ and BS assisted in study design and manuscript editing. All authors read and approved the final manuscript.

Competing interests

Not applicable.

Consent for publication

Not applicable.

Ethics approval

All animal experiments were performed under the instructions of Laboratory Animals Centre of Hubei province in P. R. China and approved by the ethical committee of Huazhong Agricultural University, according to the Regulations of the Care and Use of Laboratory Animals in China.

Received: 6 September 2016 Accepted: 27 February 2017

Published online: 09 March 2017

References

- McDonald V, Shirley MW. Past and future: vaccination against *Eimeria*. *Parasitology*. 2009;136(12):1477–89.
- Blake DP, Tomley FM. Securing poultry production from the ever-present *Eimeria* challenge. *Trends Parasitol*. 2014;30(1):12–9.
- Blake DP, Clark EL, Macdonald SE, Thenmozhi V, Kundu K, Garg R, et al. Population, genetic, and antigenic diversity of the apicomplexan *Eimeria tenella* and their relevance to vaccine development. *Proc Natl Acad Sci USA*. 2015;112(38):E5343–5350.
- Beck HP, Blake D, Darde ML, Felger I, Pedraza-Diaz S, Regidor-Cerrillo J, et al. Molecular approaches to diversity of populations of apicomplexan parasites. *Int J Parasitol*. 2009;39(2):175–89.
- Lew AE, Anderson GR, Minchin CM, Jeston PJ, Jorgensen WK. Inter- and intra-strain variation and PCR detection of the internal transcribed spacer 1 (ITS-1) sequences of Australian isolates of *Eimeria* species from chickens. *Vet Parasitol*. 2003;112(1–2):33–50.
- Clark EL, Macdonald SE, Thenmozhi V, Kundu K, Garg R, Kumar S, et al. Cryptic *Eimeria* genotypes are common across the southern but not northern hemisphere. *Int J Parasitol*. 2016;46(9):537–44.
- Schwarz RS, Jenkins MC, Klopp S, Miska KB. Genomic analysis of *Eimeria* spp. populations in relation to performance levels of broiler chicken farms in Arkansas and North Carolina. *J Parasitol*. 2009;95(4):871–80.
- Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res*. 1990;18(22):6531–5.
- Johnston DA, Fernando MA. *Eimeria* spp. of the domestic fowl: analysis of genetic variability between species and strains using DNA polymorphisms amplified by arbitrary primers and denaturing gradient-gel electrophoresis. *Parasitol Res*. 1995;81(2):91–7.
- Fernandez S, Costa AC, Katsuyama AM, Madeira AM, Gruber A. A survey of the inter- and intraspecific RAPD markers of *Eimeria* spp. of the domestic fowl and the development of reliable diagnostic tools. *Parasitol Res*. 2003; 89(6):437–45.
- Reid AJ, Blake DP, Ansari HR, Billington K, Browne HP, Bryant J, et al. Genomic analysis of the causative agents of coccidiosis in domestic chickens. *Genome Res*. 2014;24(10):1676–85.
- Shumard RF, Callender ME. Anticoccidial drugs: screening methods. *Exp Parasitol*. 1970;28(1):13–24.
- Peek HW, Landman WJ. Coccidiosis in poultry: anticoccidial products, vaccines and other prevention strategies. *Vet Q*. 2011;31(3):143–61.
- Chapman HD, Jeffers TK, Williams RB. Forty years of monensin for the control of coccidiosis in poultry. *Poult Sci*. 2010;89(9):1788–801.
- Guo FC, Suo X, Zhang GZ, Shen JZ. Efficacy of decoquinate against drug sensitive laboratory strains of *Eimeria tenella* and field isolates of *Eimeria* spp. in broiler chickens in China. *Vet Parasitol*. 2007;147(3–4):239–45.
- Peek HW, Landman WJ. Resistance to anticoccidial drugs of Dutch avian *Eimeria* spp. field isolates originating from 1996, 1999 and 2001. *Avian Pathol*. 2003;32(4):391–401.
- Tomley FM, Bumstead JM, Billington KJ, Dunn PP. Molecular cloning and characterization of a novel acidic microneme protein (Etmic-2) from the apicomplexan protozoan parasite, *Eimeria tenella*. *Mol Biochem Parasitol*. 1996;79(2):195–206.
- Tabares E, Ferguson D, Clark J, Soon PE, Wan KL, Tomley F. *Eimeria tenella* sporozoites and merozoites differentially express glycosylphosphatidylinositol-anchored variant surface proteins. *Mol Biochem Parasitol*. 2004;135(1):123–32.
- Upton SJ, McAllister CT, Brillhart DB, Duszynski DW, Wash CD. Cross-transmission studies with *Eimeria arizonensis*-like oocysts (Apicomplexa) in New World rodents of the genera *Baiomys*, *Neotoma*, *Onychomys*, *Peromyscus*, and *Reithrodontomys* (Muridae). *J Parasitol*. 1992;78(3):406–13.
- Shirley MW. *Eimeria* spp. from the chicken: occurrence, identification and genetics. *Acta Vet Hung*. 1997;45(3):331–47.
- Duszynski DW, Wilber PG. A guideline for the preparation of species descriptions in the Eimeriidae. *J Parasitol*. 1997;83(2):333–6.
- Jiang L, Zhao Q, Zhu S, Han H, Dong H, Huang B. Establishment of *Eimeria tenella* (local isolate) in chicken embryos. *Parasite*. 2012;19:285–9.
- Schnitzler BE, Thebo PL, Tomley FM, Ugglia A, Shirley MW. PCR identification of chicken *Eimeria*: a simplified read-out. *Avian Pathol*. 1999;28(1):89–93.

24. Schnitzler BE, Thebo PL, Mattsson JG, Tomley FM, Shirley MW. Development of a diagnostic PCR assay for the detection and discrimination of four pathogenic *Eimeria* species of the chicken. *Avian Pathol.* 1998;27(5):490–7.
25. Johnson J, Reid WM. Anticoccidial drugs: lesion scoring techniques in battery and floor-pen experiments with chickens. *Exp Parasitol.* 1970;28(1):30–6.
26. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol.* 2011;28(10):2731–9.
27. Clark EL, Tomley FM, Blake DP: Are *Eimeria* genetically diverse, and does it matter?. *Trends in Parasitol.* 2016;33(3):231–41.
28. Fornace KM, Clark EL, Macdonald SE, Namangala B, Karimuribo E, Awuni JA, et al. Occurrence of *Eimeria* species parasites on small-scale commercial chicken farms in Africa and indication of economic profitability. *PLoS One.* 2013;8(12):e84254.
29. Wang M. *Veterinary parasitology.* Beijing: China Agricultural Press; 2003.
30. Jiang JS. *Aminal protozoology.* Beijing: China Agricultural University Press; 2000.
31. Suo X, Cai J. *Poultry coccidiosis.* Beijing: China Agricultural Press; 2004.
32. Procunier JD, Fernando MA, Barta JR. Species and strain differentiation of *Eimeria* spp. of the domestic fowl using DNA polymorphisms amplified by arbitrary primers. *Parasitol Res.* 1993;79(2):98–102.
33. Jeffers TK. Avian coccidiosis. *British Poultry Science.* 1978;50:125.
34. Jeffers TK. Genetic transfer of anticoccidial drug resistance in *Eimeria tenella*. *J Parasitol.* 1974;60(6):900–4.
35. Weppelman RM, Battaglia JA, Wang CC. *Eimeria tenella*: the selection and frequency of drug-resistant mutants. *Exp Parasitol.* 1977;42(1):56–66.
36. Teo BH, Lansdell P, Smith V, Blaze M, Nolder D, Beshir KB, et al. Delayed onset of symptoms and atovaquone-proguanil chemoprophylaxis breakthrough by *Plasmodium malariae* in the absence of mutation at codon 268 of pmcytb. *PLoS Negl Trop Dis.* 2015;9(10):e0004068.

Submit your next manuscript to BioMed Central and we will help you at every step:

- We accept pre-submission inquiries
- Our selector tool helps you to find the most relevant journal
- We provide round the clock customer support
- Convenient online submission
- Thorough peer review
- Inclusion in PubMed and all major indexing services
- Maximum visibility for your research

Submit your manuscript at
www.biomedcentral.com/submit

