

Prevalence of *Eimeria* parasites and sulfachloropyrazine sodium resistance in chicken farms in the Hubei and Henan provinces

Tiantian Geng

Huazhong Agriculture University

Zhenyu Lei

Huazhong Agriculture University

Cheng Ye

Huazhong Agriculture University

Bang Shen

Huazhong Agriculture University

Rui Fang

Huazhong Agriculture University

Min Hu

Huazhong Agriculture University

Junlong Zhao

Huazhong Agriculture University

yanqin zhou (✉ yanqinzhou@mail.hzau.edu.cn)

Huazhong Agriculture University

Research

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Abstract

Background Coccidiosis is an intestinal parasitic disease that causes huge economic losses to the poultry industry globally. At present, the primary control strategy is administration of anticoccidial drugs with feed. However, overuse of anticoccidials, such as sulfachloropyrazine sodium (SC), has resulted in an increase in the emergence of drug resistance.

Methods: We aimed to evaluate coccidiosis prevalence and SC resistance in field isolates to provide reasonable guidance on the use of anticoccidial drugs in the Hubei and Henan provinces. We collected 318 fresh fecal samples from 137 chicken farms. We used internal transcribed spacer 1 (ITS1) sequence of ribosomal DNA to identify the species from 94 samples that were collected from different farms and to assess drug resistance.

Results: As shown by genus-specific PCR results, the positivity rate of *Eimeria* was 97.17 % (309/318), and the most common species were *E. mitis* (66.67%), *E. tenella* (46.86%), and *E. necatrix* (41.51%). Animal experiment demonstrated that 25 strains were completely resistant to SC, among which 16 were from Henan and nine were from Hubei. Twenty-four strains were partially resistant, among which 8 and 16 strains were identified from Hubei and Henan, respectively.

Conclusions: In summary, these data indicated that chicken coccidia is ubiquitous and SC resistance is widespread, in the Hubei and Henan provinces. The results provide important insights into the control of chicken coccidiosis in this region.

Background

Coccidiosis, a protozoan disease, is often a mixed infection of *Eimeria* spp. [1]. Furthermore, nine different *Eimeria* spp. are described [1], and seven species of *Eimeria* are mainly recognized worldwide [2]. Each species of *Eimeria* infects chicken with absolute host specificity [1], and the virulence of coccidia varies with the type of coccidiosis [1], *E. necatrix* is recognized as the most pathogenic species [3].

Coccidiosis causes huge economic losses to the global poultry industry [4]. According to incomplete statistics, the annual economic burden of the prevention and treatment of chicken coccidiosis in the world exceeds 3 billion USD [4]. In the past 30 years, the poultry industry in Asia has developed extremely rapidly, and China as Asia's largest economy has become the third largest chicken-producing country in the world [5]; the annual burden of chicken coccidiosis exceeds 73 million USD [5], accounting for approximately 30% of all chicken disease burden [5].

As such, it is extremely important to understand the detection and control of pathogens. The traditional pathogen classification of avian coccidiosis depends on features such as morphology and life history. At present, PCR is used for the classification and identification of parasites and for studying genetic evolution. In the case of coccidiosis caused by *Eimeria*, the 5' end of the ITS1 region is widely used for species identification [6, 7]. Lew et al. amplified and cloned ITS-1 of five species of *Eimeria*, namely *E.*

tenella, *E. necatrix*, *E. acervulina*, *E. brunetti*, and *E. mitis*, isolated from Australia using PCR [6]. Hamidinejat et al. identified *Eimeria* spp. in commercial broilers using PCR, based on ITS1 regions of rDNA and showed that the most prevalent species in Khuzestan was *E. tenella* [8].

So far, the control of chicken coccidiosis still relies on the addition of coccidiostats to feed [9]. Coccidiostats mainly include chemical synthetic anticoccidial drugs and polyether ionophore antibiotic anticoccidial drugs [10]. Sulfonamides are an important and commonly used synthetic chemical drugs. They mainly achieve anticoccidial effect by inhibiting the growth and development of coccidia; they have no direct anticoccidial effect. Aminobenzoic acid is one of the most important raw materials in the synthesis of dihydrofolate. The chemical structure of sulfonamide is similar to that of p-aminobenzoic acid. The latter can compete with aminobenzoic acid for dihydrofolate synthetase, thereby hindering the formation of dihydrofolate, affecting the synthesis of nucleic acids, inhibiting the growth and reproduction of coccidia, and achieving anticoccidial effects [11, 12].

In this study, we collected fresh fecal samples from chicken farms in different areas of Hubei and Henan provinces, investigated anticoccidial drugs in chicken farms, and analyzed SC resistance of *Eimeria*. The results of this study have great significance for the prevention and treatment of avian coccidiosis in the region.

Methods

Fecal sample collection

Data on age, variety of birds, and the method of breeding, size of the chickens on the farm, the occurrence of coccidiosis, and the history of drug use were collected across the Hubei and Henan provinces. Fecal samples were collected from 137 different local chickens' farms (commercial broiler production farms and backyard flocks) between June 2017 and October 2018 (Additional file 1: Table S1). No less than 500 g of sample was collected from each farm.

Eimeria oocysts obtained from the fecal samples were purified by saturated sodium nitrate flotation technique and sporulated using standard procedures [13]. The saturated sodium chloride floatation method was performed as follows. First, the fecal sample was mashed and mixed with a right quantity of water to achieve suspension; the suspension was filtered through a 180 diameter mesh, and the obtained filtrate was centrifuged at 2000 to 2500 rpm/min. The supernatant was discarded; the obtained precipitate was mixed with saturated saline solution and centrifuged at 3000 rpm/min; Subsequently, we collected the supernatant, which was diluted five times with purified water and centrifuged at 3000–3500 rpm. The obtained precipitate was contained oocysts of *Eimeria* spp.

DNA extraction

For the extraction of coccidia sample DNA, the sporulated oocysts were washed three times with deionized water, and subsequently, the oocyst wall was vortexed three times using Lysing Beads-Matrix D

lysate (MP Biomedicals, Shanghai, China) in a rapid sample preparation device to release sporozoites and 20 µl (20 mg/ml) of proteinase K (Genomic DNA Kit, TIANGEN, Beijing, China) was added. Subsequently, the mixture was incubated at 56 °C for 10 h, and DNA was extracted by using a Genomic DNA Kit (TIANGEN) according to the manufacturer's instructions.

Species identification of Eimeria

To confirm the species identity of these Eimeria isolates, seven pairs of species-specific primers (for *E. acervulina*, *E. brunetti*, *E. mitis*, *E. necatrix*, *E. maxima*, *E. praecox*, and *E. tenella*) that amplify ITS1 region were used. Sequences of the PCR primer pairs used were summarized in Table 1 [14, 15, 16] and the following PCR program was used: initial denaturation at 94 °C for 4.5 min, followed by 25 cycles of denaturation for 50 s at 94 °C, annealing for 40 s at primer-dependent temperatures, and extension for 60 s at 72 °C, and final extension for 2 min at 72 °C. The annealing temperatures were 53 °C for *E. brunetti* and 62 °C for *E. acervulina*, *E. necatrix*, *E. maxima*; for *E. tenella*, *E. mitis*, and *E. praecox*, DNA was subjected to initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 53 °C, and extension for 45 s at 72 °C, and final extension for 10 min at 72 °C.

Table 1
Sequences of the ITS1-PCR primer pairs

Species	Primer name	sequence 5' to 3'	Expected amplicon size (bp)	Annealing(°C)
E. acervulina	EAF ^a	GGCTTGGATGATGTTTGCTG	321	62
	EAR ^a	CGAACGCAATAACACACGCT		
E. brunetti	EBF ^a	GATCAGTTTGAGCAAACCTTCG	311	53
	EBR ^a	TGGTCTTCCGTACGTCGGAT		
E. necatrix	ENF ^a	TACATCCCAATCTTTGAATCG	384	62
	ENR ^a	GGCATACTAGCTTCGAGCAAC		
E. tenella	ETF ^a	AATTTAGTCCATCGCAACCCT	279	53
	ETR ^a	CGAGCGCTCTGCATACGACA		
E. maxima	EMAF ^b	GTGGGACTGTGGTGATGGGG	205	62
	EMAR ^b	ACCAGCATGCGCTCACAACCC		
E. mitis	EMIF ^b	TATTTCTGTGTCGTCTCGC	327	53
	EMIR ^b	GTATGCAAGAGAGAATCGGGA		
E. praecox	EPF ^c	CATCGGAATGGCTTTTTGAAAGCG	215	53
	EPR ^c	GCATGCGCTAACACCTCCCCTT		
^a Primers designed by Schnitzler et al. 1998.				
^b Primers designed by Schnitzler et al. 1999.				
^c Primers designed by Haug et al. 2007.				

Drug resistance test

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. Sulfachloropyrazine sodium (SC) soluble powder (Lilly, Shanghai, China, batch no: 1611038) was provided by the Shanghai Veterinary Research Institute, Chinese Academy of Agriculture Sciences. The selected isolates were orally administered to specific pathogens free (SPF)

chickens (14-day-old). After 7 days of inoculation, the chickens were sacrificed, the oocysts were obtained from the intestinal contents by protease digestion method [17], the oocysts in the previous step were transferred to 2% w/v potassium dichromate in a 50 ml centrifuge tube, and placed in an incubator 28.6 °C for 4–7 days. If sporulation was identified in > 80% of the oocysts by microscopic examination, they were stored in 2.5% potassium dichromate at 4 °C [18].

Ninety-four isolates were randomly selected to evaluate drug effectiveness. Nine hundred and forty SPF chickens (14-day-old) were weighed and randomly divided into 94 groups; each group included 8–12 chickens, 4–6 of which were treated with 2 g/kg SC in feed. Every chicken was inoculated with 1 ml of 5×10^4 sporulated oocysts. The blank control group consisted consisting of 10 chickens, which were only fed deionized water. Chickens were weighed at 21 days of age, and subsequently, the lesional scores were recorded. From each post-infection group between 5–7 days, single droppings of chicken feces were collected each day to evaluate the relative number of oocysts per gram of feces (OPG) [17]. The coccidial lesions present in the chickens were scored on a 4-point scale (0–4 points), as described previously [19]. OPG values were determined using the McMaster's method [20]. The relative oocyst production rate (OPR) is equal to the ratio of the oocysts produced by the infected-treated group versus those produced by the infected-untreated group. Subsequently, the anticoccidial index (ACI) was calculated to assess drug effectiveness [21].

Results

Identification of *Eimeria* species in field isolate

A total of 318 samples were collected from chicken farms in the Hubei and Henan provinces (Fig. 1). Each isolate was amplified with seven pairs of species-specific primers (Fig. 2). The specific identification results of each sample are shown in Table S2-1 and Table S2-2. We found that *E. mitis* (66.67%), *E. tenella* (46.86%), and *E. necatrix* (41.51%) were the most prevalent species in the Hubei and Henan provinces (Table 2). In the Henan province, *E. mitis* (78.57%), *E. tenella* (39.01%), and *E. necatrix* (36.26%) ($n = 182$) were the most common species, while in the Hubei province ($n = 136$) the most prevalent species were *E. tenella* (57.35%), *E. mitis* (50.74%), and *E. necatrix* (48.53%) (Table 2).

Table 2

The relative pathogenicity and occurrence (number and ranking) of *Eimeria* species detected on poultry farms sampled in Henan and Hubei Provinces. na = not applicable. Rankings 1–7 indicates most to least common

<i>Eimeria</i> species	Hubei (n = 136)		Henan (n = 182)		Henan and Hubei Provinces (n = 318)	
	No. positive (%)	Rank	No. positive (%)	Rank	No. positive (%)	Rank
Any <i>Eimeria</i> species	133(97.79)	na	176(96.70)	na	309(97.17)	na
<i>Eimeria necatrix</i>	66(48.53)	3	66(36.26)	3	132(41.51)	3
<i>Eimeria brunetti</i>	13(9.56)	7	8(4.40)	6	21(6.60)	7
<i>Eimeria tenella</i>	78(57.35)	1	71(39.01)	2	149(46.86)	2
<i>Eimeria acervulina</i>	43(31.62)	4	28(15.38)	5	71(22.33)	5
<i>Eimeria maxima</i>	21(15.44)	6	1(0.55)	7	22(6.92)	6
<i>Eimeria mitis</i>	69(50.74)	2	143(78.57)	1	212(66.67)	1
<i>Eimeria praecox</i>	43(31.62)	4	63(34.62)	4	106(33.33)	4

Geographically, the distribution of *E. mitis* and *E. tenella* in the Henan and Hubei provinces was more widespread than that of the other species. *E. necatrix* was mainly distributed in the northern (Anyang, Puyang, Hebi, and Xinxiang) and central (Zhengzhou and Xuchang) Henan and in the northeastern (Suizhou, Xiaogan, Huanggang, and Wuhan) and southwestern (Enshi Tujia and Miao Autonomous Prefecture and Yichang) Hubei (Fig. 3a). *E. tenella* was not detected in Kaifeng, Jiaozuo, Nanyang, and Shangqiu of the Henan Province (Fig. 3b) and Xiangyang of the Hubei province (Fig. 3c). Only the chicken feces samples from Yichang and Wuhan in the Hubei province did not show *E. mitis* (Fig. 3c).

Additionally, we found that *E. maxima* was detected only in Sanmenxia of the Henan Province (Fig. 3b) and Wuhan, Huanggang, Suizhou, Xiaogan, Yichang and Enshi Tujia, and Miao Autonomous Prefecture in the Hubei province (Fig. 3c).

Mixed-species infections were common in the Hubei and Henan Provinces (Fig. 4a). The single *Eimeria* infection rate was lower in the Hubei province (15.44%) than in the Henan province (34.07%) (Fig. 4b), while the proportion of isolates mixed infected with two (Hubei versus Henan: 36.76% versus 26.92%), three (Hubei versus Henan: 29.41% versus 26.37%), and four (Hubei versus Henan: 12.50% versus 4.40%) species of *Eimeria* isolates was higher in Hubei than in Henan and the emergence of mixed coccidial infections involving six species of *Eimeria* appeared only in the Hubei province (2.21%) (Fig. 4b),

especially in Suizhou and Huanggang (Fig. 4a). Therefore, it could be preliminary indicated that the Hubei province showed a higher proportion of mixed infections than the Henan province did.

Mixed-species infections were common irrespective of the type of the chicken; indigenous chicken was infected with one (indigenous chicken versus layers and broilers: 47.73% versus 22.67% and 12.24%) or two (indigenous chicken versus layers and broilers: 36.36% versus 29.33% and 34.69%) *Eimeria* spp. higher than layers and broilers. While indigenous chicken was infected with three (indigenous chicken versus layers and broilers: 6.82% versus 32.44% and 24.49%) or four (indigenous chicken versus layers and broilers: 4.55% versus 8.44% and 8.16%) *Eimeria* spp. lower than layers and broilers; the emergence of mixed infections involving six *Eimeria* spp. appeared in the layers (1.33%). It could be preliminary concluded that indigenous chicken showed a lower degree of mixed infections than layers and broilers (Fig. 4c).

Sulfachloropyrazine sodium (SC) sensitivity of *Eimeria*

In this study, among the 94 isolates selected (Table S3), 53 were from the Henan province and 41 were from the Hubei province. We found that 25 of the isolates—16 Henan and nine from Hubei—had an ACI of less than 160, indicating that these strains were fully resistant. ACI of 24 strains—16 Henan and eight from Hubei—was 160–180, indicating that these strains were partially resistant. The ACI of the other strains was > 180, indicating that these strains were not resistant (Table 3).

Table 3
Summary information of Anticoccidial index (ACI)

District	Henan ^a	Hubei ^b	Total
ACI < 160	16	9	25
160 ≤ ACI < 180	16	8	24
ACI ≥ 180	21	24	45
Total	53	41	94
^a Henan, Henan Province, China; ^b Hubei, Hubei Province, China			

Geographically, we found that the sulfa-resistant *Eimeria* spp. were distributed in the central (Zhengzhou, Kaifeng, Xuchang, Luohe, and Zhumadian) and western (Sanmenxia, Luoyang, and Jiyuan) parts of Henan (Fig. 5). In Hubei, it was distributed in the northwest (Shiyan and Shennongjia Forest Area) and southeast (Xiantao, Xianning, and Huangshi).

Four urban areas in Henan (Hebi, Xinxiang, Shangqiu, and Nanyang) account for more than 50% sensitivity to sulfa drugs (ACI > 180). In Hubei, nine cities have a sensitivity of more than 50% to sulfa drugs, including Xiangyang, Suizhou, Huanggang, Wuhan, Ezhou, Yichang, Jingmen, Tianmen, and Jingzhou (Fig. 5).

Discussion

In this study, 97.17% of the samples tested positive to for *Eimeria*. The high prevalence of *Eimeria* in field samples was relatively consistent with the percentages recorded previously in France (95%) [22]; however, these values are higher than those recorded in the Anhui Province in China (87.7%) [5], north India (81.3%) [23], and Korea (78.7%) [24]. This could be possibly explained by differences in detection methods and geographical conditions. In addition, most of the samples come from small chicken farms, which have high stocking densities and are commonly populated with specialized, genetically homogeneous breeds that are possibly conducive to disease transmission.

The individual prevalence rates of the four characterized *Eimeria* species were higher than those of the species reported in the Anhui Province, China from seven flocks (present study versus previous study: *E. acervulina*, 22.33% versus 2.67%; *E. mitis*, 66.67% versus 55.83%; *E. praecox*, 33.33% versus 0%) [5]; the prevalence of other *Eimeria* species was lower than that reported by Huang et al. [5] (present study versus previous study: *E. maxima*, 6.92% versus 54.67%; *E. tenella*, 46.86% versus 80.67%; *E. brunetti*, 6.60% versus 44.67%; *E. necatrix*, 41.51% versus 68.00%). The three most prevalent species were *E. mitis* (78.57%), *E. tenella* (39.01%), *E. necatrix* (36.26%) in Henan (n = 182) and *E. tenella* (57.35%), *E. mitis* (50.74%), and *E. necatrix* (48.53%) in Hubei (n = 136); as reported by previous studies, the three most prevalent species *E. tenella* (67.30%), *E. mitis* (58.90%), and *E. acervulina* (45.80%) in North India were and *E. tenella* (57.50%), *E. mitis* (29.90%), and *E. necatrix* (14.90%) Southern India [25]. This may be because of differences in geographical conditions.

The positivity rate of *Eimeria* was 97.79% (133/136) in Hubei and 96.70% (176/182) in Henan province (Table 2); however, *Eimeria* infections in Hubei were more complex than those in Henan (Fig. 4b). Indigenous chickens showed a lower relative abundance of *Eimeria* species than the others (Fig. 4c). The reasons for this phenomenon must be investigated more comprehensively in the future.

The first strain of sulfa drug-resistant coccidia was found in the United States of America in 1948; since then, research focused identifying coccidial resistant strains in the world has not stopped. Currently, the annual financial burden of coccidiosis control in China is high. Therefore, investigation the status of sulfa drug resistance in Hubei and Henan provinces is highly significant. This is the first study that systematically evaluated the complete SC resistance status of *Eimeria* in Hubei and Henan provinces so far. Considering that there are eighteen municipalities in Henan and seventeen municipalities in the Hubei province were sampled, it is of utmost importance to evaluate the true distribution of different *Eimeria* species. The results showed that the field isolates showing drug resistance in the Henan province were higher than those in the Hubei province; therefore, it could be preliminarily indicated that SC resistance of chicken coccidia in Henan was more common than that in the Hubei province. Possible explanation for this is that most of the samples come from semi-intensive small chicken farms in the Henan province and the use of anticoccidial drugs was not standardized by technical guidance. Surprisingly, by contrast, the results were different from those reported in Anhui Province in China by Huang et al. [5], and many of the strains were not resistant ($ACI \geq 180$), most likely because reducing the use of sulfonamides resulted

in expanding the population of sulfa drug-sensitive coccidia strains; this may explain why the phenomenon of drug resistance was relatively improved in the Hubei and Henan provinces.

Conclusions

In conclusion, firstly, this study completed an epidemiological survey of coccidiosis and filled the knowledge gap of the distribution of coccidiosis in the Hubei and Henan provinces. The findings suggest that chicken coccidiosis is widespread (97.17%) in the Hubei and Henan provinces. Secondly, it is the first systematic investigation of the SC resistance status (52.13%, ACI < 180), the partially improved results demonstrate that the poultry farms can reasonably use SC to replace other anticoccidial drugs.

Abbreviations

SC: sulfachloropyrazine sodium; ITS1: Internal transcribed spacer 1; OPG: Oocysts per gram of feces; SPF: Specific pathogens free; ACI: Anti-coccidial index; OPR: Relative oocyst production rate.

Declarations

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and Additional file 1.

Authors' contributions

YQZ conceived and designed the study. TTG wrote the manuscript with input from other coauthors. TTG, ZYL, and CY performed the experiments and analyzed the data. All authors read and approved the final manuscript.

Competing interests

Not applicable.

Consent for publication

Not applicable.

Ethics approval

All animals were maintained under standard conditions according to the regulations specified by the Administration of Affairs Concerning Experimental Animals. Animal experiments were approved by the ethical committee of Huazhong Agricultural University (approval number: 42000400002483).

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Figures



Figure 1

Geographical locations of the 137 farms in Central China, from which fecal samples (numbers in parentheses) were collected from chickens. Fecal samples were collected from different local chicken farms (▲). The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

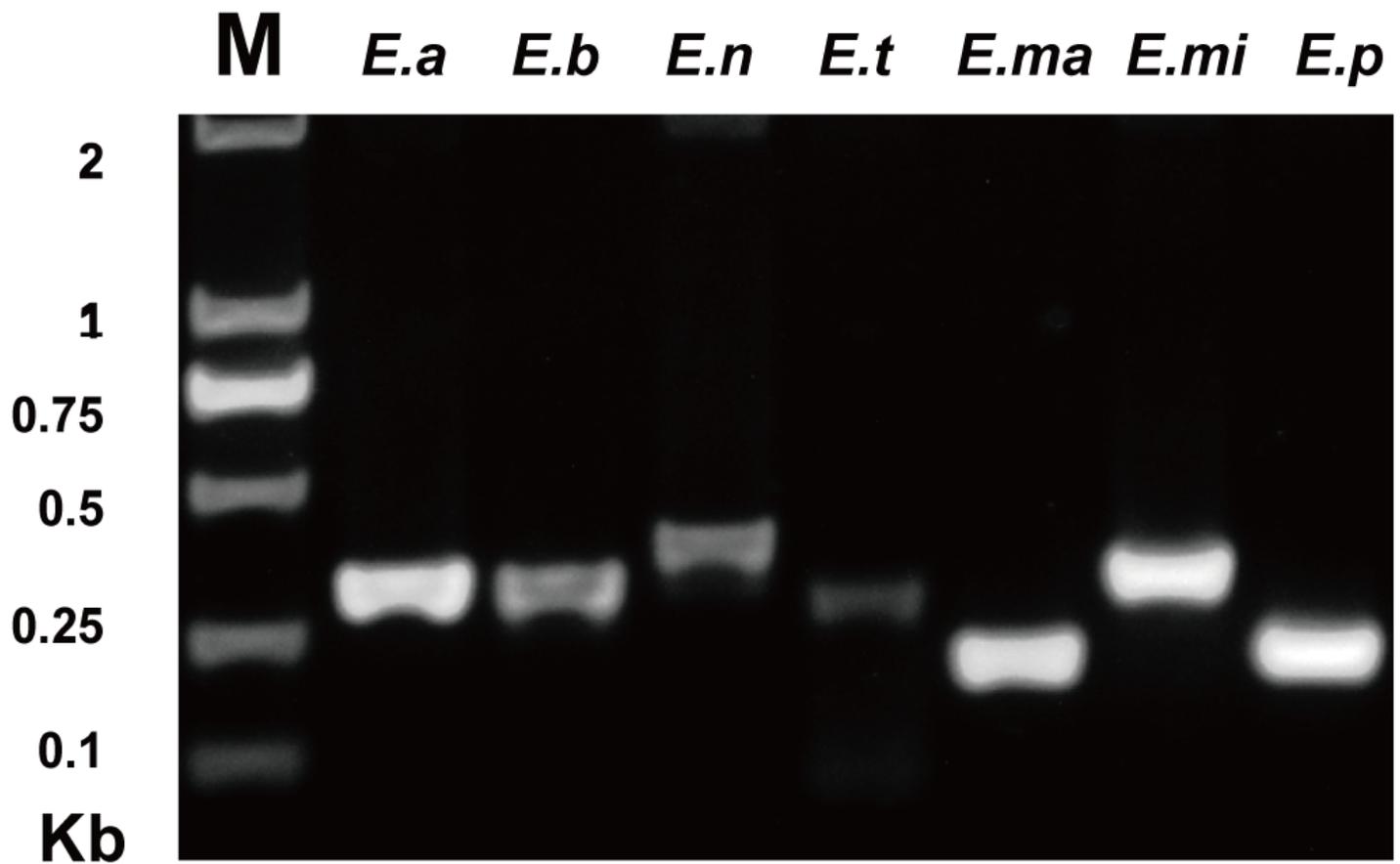


Figure 2

ITS1-specific primers for the identification of *Eimeria* species. Abbreviations: *E.a*, *Eimeria acervulina*; *E.b*, *Eimeria brunetti*; *E.n*, *Eimeria necatrix*; *E.t*, *Eimeria tenella*; *E.ma*, *Eimeria maxima*; *E.mi*, *Eimeria mitis*; *Ep*, *Eimeria praecox*.

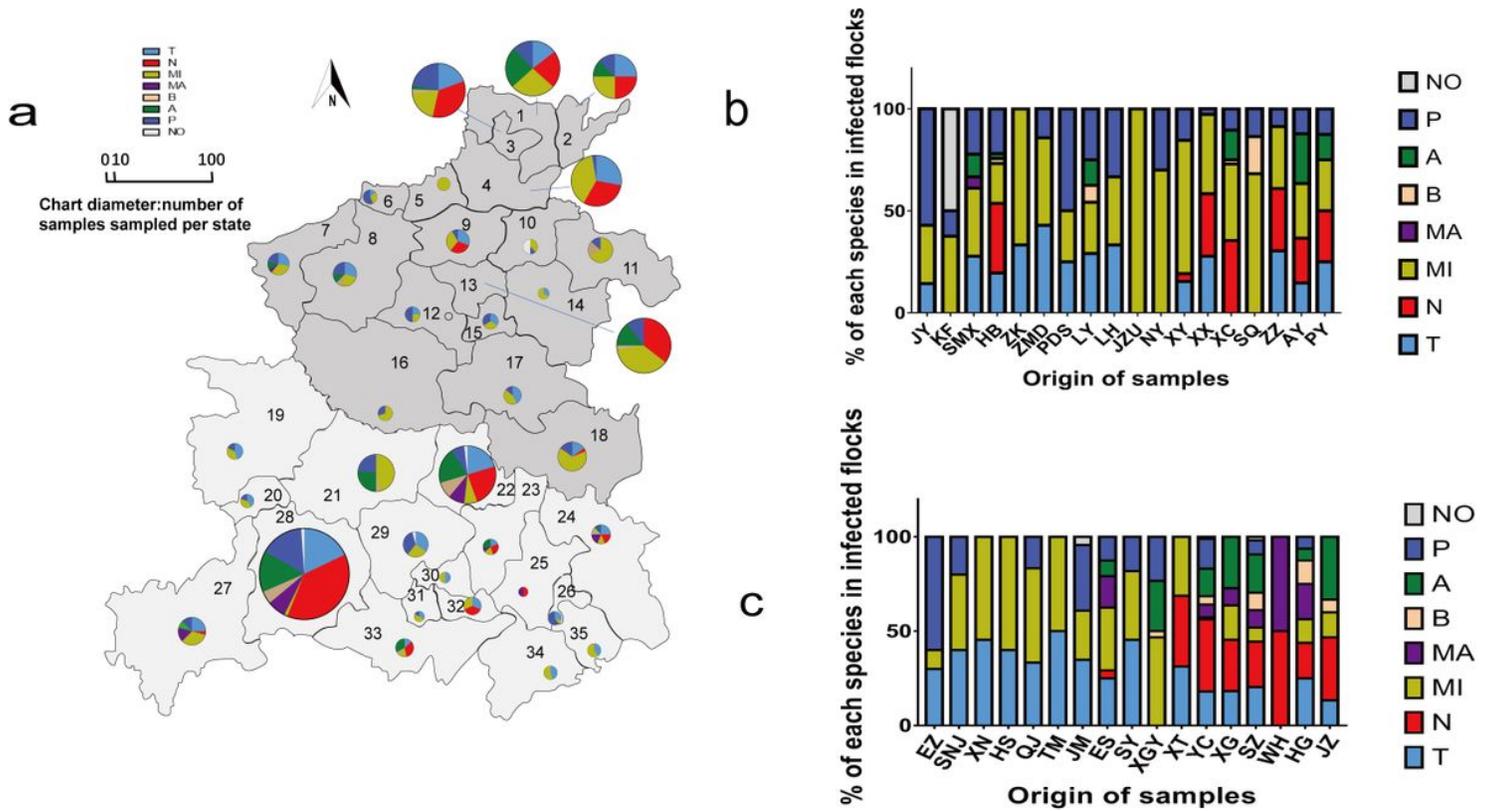


Figure 3

3 Each isolate identified the type of *Eimeria* species in Henan and Hubei Provinces. a Map indicating the number and species of *Eimeria* sampled from the Henan and Hubei Provinces, China. 1, Anyang (AY); 2, Puyang (PY); 3, Hebi (HB); 4, Xinxiang (XX); 5, Jiaozuo (JZU); 6, Jiyuan (JY); 7, Sanmenxia (SMX); 8, Luoyang (LY); 9, Zhengzhou (ZZ); 10, Kaifeng (KF); 11, Shangqiu (SQ); 12, Pingdingshan (PDS); 13, Xuchang (XC); 14, Zhoukou (ZK); 15, Luohe (LH); 16, Nanyang (NY); 17, Zhumadian (ZMD); 18, Xinyang (XY); 19, Shiyang (SY); 20, Shennongjia Forest District (SNJ); 21, Xiangyang (XGY); 22, Suizhou (SZ); 23, Xiaogan (XG); 24, Huanggang (HG); 25, Wuhan (WH); 26, Ezhou (EZ); 27, Enshi Tujia and Miao Autonomous Prefecture (ES); 28, Yichang (YC); 29, Jingmen (JM); 30, Tianmen (TM); 31, Qianjiang (QJ); 32, Xiantao (XT); 33, Jingzhou (JZ); 34, Xianning (XN); and 35, Huangshi (HS). b Percentage of each *Eimeria* species present in infected fecal samples from each area in the Henan province, China. c Percentage of each *Eimeria* species present in infected fecal samples from each area in the Hubei province, China. Abbreviations: A, *Eimeria acervulina*; B, *Eimeria brunetti*; N, *Eimeria necatrix*; T, *Eimeria tenella*; MA, *Eimeria maxima*; MI, *Eimeria mitis*; P, *Eimeria praecox*; NO, no data. The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

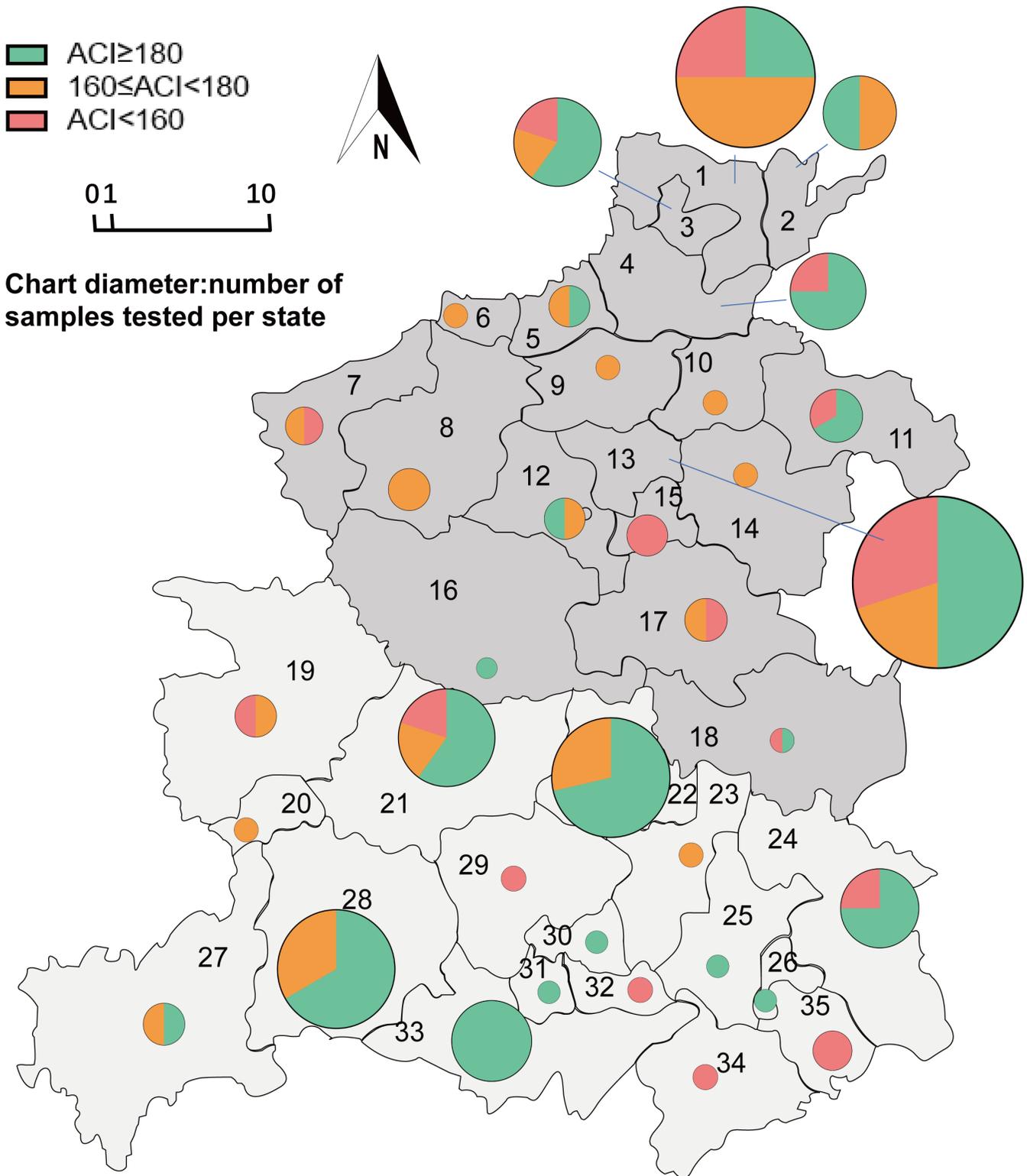


Figure 5

Map indicating the number and type of ACI selected from Henan and Hubei Provinces, China. 1, Anyang; 2, Puyang; 3, Hebi; 4, Xinxiang; 5, Jiaozuo; 6, Jiyuan; 7, Sanmenxia; 8, Luoyang; 9, Zhengzhou; 10, Kaifeng; 11, Shangqiu; 12, Pingdingshan; 13, Xuchang; 14, Zhoukou; 15, Luohe; 16, Nanyang; 17, Zhumadian; 18, Xinyang; 19, Shiyan; 20, Shennongjia Forest District; 21, Xiangyang; 22, Suizhou; 23, Xiaogan; 24, Huanggang; 25, Wuhan; 26, Ezhou; 27, Enshi Tujia and Miao Autonomous Prefecture; 28, Yichang; 29,

Jingmen; 30, Tianmen; 31, Qianjiang; 32, Xiantao; 33, Jingzhou; 34, Xianning; and 35, Huangshi. The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

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