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Short Communication

Morphology and Molecular Identification of the *Eimeria* spp. in Domestic Rabbits

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MS performed PCR. HL and ZH identified *Eimeria* spp. and wrote the article. Data were analyzed by HL and XY.

Key words
Rabbit, *Eimeria*.

ABSTRACT

Although *Eimeria* diseases common in rabbits in China, no information was available on it in Heilongjang Province. In this study, at least eight *Eimeria* species were detected in domestic rabbits in Harbin in Heilongjang Province. The diagnosis of *Eimeria* species has been based on the morphology unsporulated oocysts as well as analysis of PCR product obtained by specific primers.

Rabbits are mainly infected by monoxenous genus *Eimeria* although they could be infected by the coccidia including the genera Cryptosporidium, Besnoitia, Sarcocystis, Toxoplasma, and *Eimeria* (Pakandl, 2009). There are 11 species of rabbit *Eimeria*, which is in agreement with most previous findings, named as *Eimeria stiedai*, *E. intestinalis*, *E. flavescens*, *E. irresidua*, *E. magna*, *E. media*, *E. piriformis*, *E. coecicola*, *E. exigua*, *E. perforans*, and *E. vejdovskyi*. *E. stiedai* develops in the epithelium of bile ducts and others in the intestinal tract (Pakandl, 2009; Oliveira *et al*., 2011). Among 11 valid Eimeria species, *E. stiedai*, *E. intestinalis*, and *E. flavescens* are highly pathogenic in rabbits, especially before the age of 3 months (Pakandl, 2009; Yan *et al.*, 2013; Kvicerova *et al.*, 2008). Most of these *Eimeria* species affect the rabbit production, reduces growth rate and feed conversion, and increases mortality according to their level of pathogenicity (Pakandl, 2009).

Traditional identification of rabbit 11 *Eimeria* spp. was based on their morphological features, such as oocyst size, oocyst residuum (presence or absence), micropyle (conspicuous or inconspicuous), prepatent and patent period, and sporulation time (Kvicerova *et al*., 2008; Oliveira *et al*., 2011; Pakandl, 2009). Recently, various molecular differentiation assays, using distinct targets such as ITS1, ITS1-5.8S rRNA-ITS2 and 18S rRNA, have been proposed to identify the rabbit *Eimeria* species (Kvicerova *et al.*, 2008; Oliveira *et al.*, 2011; Yan *et al.*, 2013; Johnson *et al.*, 1991).

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In the present study, fecal samples of domestic rabbits suspected of being infected with *Eimeria* species were analyzed based on unsporulated oocyst morphology traits and the molecular method simultaneously.

Materials and methods

Fecal samples were collected from a rabbit farm in Harbin, Northeast of China. All samples were stored at 4°C until analysis. The oocysts were isolated using sedimentation and floatation techniques (Jing *et al.*, 2012). The species were first identified based on the sizes and some other biological features including shape, form index and micropyle of the oocyst (Kvicerova *et al.*, 2008).

DNA extraction was performed according to the methods described by Hou *et al.* (2011). Eleven primer-pairs for PCR were designed to amplify the target fragments following the protocol described by Oliveria (Oliveira *et al.*, 2011). Procedures for avoiding contamination were strictly followed, and negative (no-DNA) controls were included in every experiment. Amplifications were performed in 25µl reaction mixtures consisting of the following: 100 pmol of each primer, 200 µM each deoxynucleoside triphosphate (dNTP), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 2.5 U of Taq DNA polymerase (Takara, Japan) in the appropriate buffer. Reactions were run in a Takara thermocycler using a step-cycle program. Cycling conditions comprised an initial denaturation of DNA at 94°C for 5 min, followed by 35 cycles of 94° for 30 s, each annealing temperature for 30 s, and 72° for 1 min, and a final 10 min 72°C extension step. The PCR products were recovered using the TaKaRa Agarose Gel DNA Purification Kit Ver.2.0 (TaKaRa) and sequenced directly by Invitrogen Company.

Table I.- Morphological characteristics of the rabbit-associated *Eimeria* species.

Species	Occysts shape	Occysts size (μm)	Index	Micropyle
E. vejdovskyi (or *E. coecicola*)	elongate-ovoid	29.11×15.55	1.87	Conspicuous
E. intestinalis (or *E. piriformis*)	piriform	26.72×16.96	1.58	Conspicuous; on the narrow polar
E. flavescens	piriform	24.82×16.05	1.55	Conspicuous; on the widest polar
E. magna	ovoid	33.24×17.92	1.85	Conspicuous; outside the oocyst wall
E. media	ellipsoid-ovoid	30.62×18.35	1.67	Conspicuous
E. stiedai	ellipsoid	30.14×18.32	1.65	Conspicuous
E. perforans	ellipsoid-subrectangular	20.26×12.09	1.68	inconspicuous
E. exigua	spherical-subspherical	13.66×12.82	1.07	inconspicuous

Table II.- The nucleotide sequence alignment results of *Eimeria* spp. in BLAST search and the Genbank Accession numbers.

Accession No.	Identities	Expect	The nucleotides and sequence numbers		
E. vejdovskyi HRB (1-164)	99%(163/164)	1e-47	A(107)	deletion	deletion
HM768891.1 (172-337)			G(278)	C(281)	T(282)
E. piriformis-HRB)(1-289)	100%(289/289)	2e-148	\	\	\
HM768889.1 (102-390)			\	\	\
E. flavescens-HRB(1-199)	99%(198/199)	7e-97	A(110)	\	\
HM768883.1 (196-394)			T(305)	\	\
E. magna-HRB (1-213)	99%(211/213)	6e-103	A(15)	T(193)	
JQ071391.1 (173-385)			G(187)	C(365)	
E. media-HRB (1-152)	100%(152/152)	2e-72	\	\	\
JQ071392.1 (259-410)			\	\	\
E. stiedai-HRB (1-217)	99% (215/217)	4e-105	A(101)	A (165)	\
JQ328190.1 (127-343)			T(227)	G(291)	\

Results and discussion

There were at least eight species detected in this study, including *Eimeria vejdovskyi* (or *E. coecicola*), *E. intestinalis* (or *E. piriformis*), *E. flavescens*, *E. magna*, *E. media*, *E. stiedai*, *E. perforans*, and *E. exigua* (Fig. 1, Table I).

Six *Eimeria* spp. species were detected by PCR method, and they were *E. vejdovskyi* (164bp), *E. piriformis* (289bp), *E. flavescens* (199bp), *E. magna* (213bp), *E. media* (152bp), and *E. stiedai* (217bp). The sequences obtained in this study by amplifying the Eimeria occysts ITS1 was submitted to a BLAST search for similarity (www.ncbi.nlm.nih.gov/blast), and the identities were 99% to 100% (Table II).

In the present study, at least eight *Eimeria* spp. were based on unsporulatd oocysts morphology characters, but only six species were detected by PCR assays. The unsporulated morphology characteristics between *E. vejdovskyi* and *E. coecicola*, *E. piriformis* and *E. intestinalis* were similar, so it was impossible to differentiate them only on the basis of unsporulatd morphology features. Other *Eimeria* spp. had either different oocyst size and oocyst shape, or micropyle, and could been differentiated from each other by those unsporulated biology traits excepting the *E. perforans* and *E. exigua*.

The classic diagnostic method of the rabbit *Eimeria* spp. is based on morphology which needs to sporulate examining for days before inspecting. The molecular method accurate and sensitive was time-consuming. This problem could be resolved by detecting the *Eimeria* spp. infecting rabbits based on their unsporulated morphology characteristics such as oocyst shape, oocyst size, and micropyle. Although it was not accurate to distinguish *E. vejdovskyi* from *E. coecicola*, *E. piriformis* from *E. intestinalis*, or even others species, this method could be used to identify most of the Eimeria species in infected rabbits. The highly pathogenic species

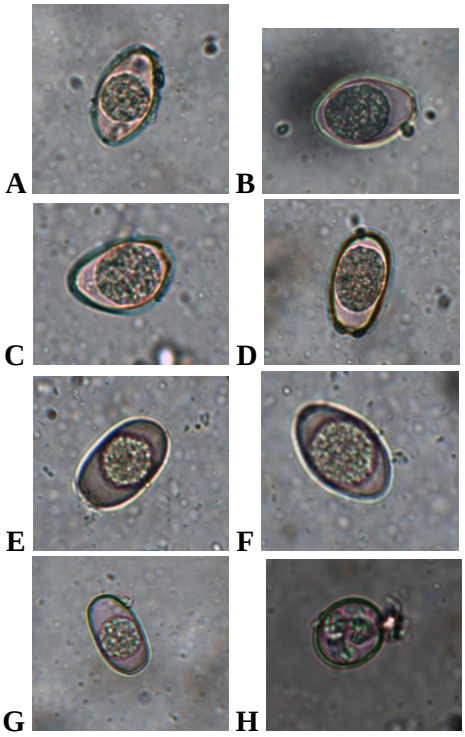

Fig. 1. The *Eimeria* species detected in the rabbits in this study (×400). A, *Eimeria vejdovskyi* (or *E. coecicola*); B, *E. intestinalis* (or *E. piriformis*); C, *E. flavescens;* D, *E. magna;* E, *E. media*; F, *E. stiedai*; G, *E. perforans*; H, *E. exigua.*

(*E. stedai*, *E. flavescens*, and *E. intestinalis)* and wild pathogenic species (*E. manga*, *E. media*, and *E. piriformis*) can be distinguished based on the unsporulated morphological features if permitted to take the *Eimeria* spp oocyst with piriform shape as intestinalis. Enough information for controlling the disease was inferred timely based on these features because there were not many drugs to be selected for treatment (Jing *et al*., 2012), even though all *Eimeria* spp. were differentiated accurately. Moreover, most drugs have similar action on most *Eimeria* species. Therefore, diagnosing the *Eimeria* infection based on the unsporulated oocyst morphology was practical for small rabbit farms for controlling and preventing the rabbit Eimeria diseases.

In this study, the *E. perforans* and *E. exigua* were identified based on the morphological characteristics, but could not be detected by the PCR methods

To our knowledge, this is the first report on prevalence of *E. vejdovskyi* in China. Jing *et al.* (2012) reported that 8 species prevailed in the Northeast China except *E. vejdovskyi*, *E. stiedai*, and *E. exigua*. The reason for this might be because they did not collect the samples in Heilongjiang province, one main area of Northeast China.

In conclusion, we found that at least eight *Eimeria* species prevailed in Harbin, and had developed a practical method to diagnose the rabbit Eimeria disease timely for small rabbit farms.

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Conflict of interest statement

Authors have no conflict of interest to declare.

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