Available online at www.sciencedirect.com
ScienceDirect
The Veterinary Journal 173 (2007) 184–189
The Veterinary Journal
www.elsevier.com/locate/tvjl

Cloning and nucleotide sequencing of the second internal transcribed spacer of ribosomal DNA for three species of *Eimeria* from chickens in Taiwan ☆

Y.Y. Lien [a], S.C. Sheu [b], H.J. Liu [a], S.C. Chen [a], M.Y. Tsai [a], S.C. Luo [c], K.C. Wu [c], S.S. Liu [a], H.Y. Su [c,*]

[a] *Department of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung 91201, Taiwan*
[b] *Department of Food Science, National Pingtung University of Science and Technology, Pingtung 91201, Taiwan*
[c] *Graduate Institute of Biotechnology, National Pingtung University of Science and Technology, Pingtung 91201, Taiwan*

Abstract

Coccidiosis of chickens caused by protozoan parasites of the genus *Eimeria* (Coccidia: Eimeriidae) is an enteric disease that results in great economic losses throughout the world, including Taiwan. Using polymerase chain reaction (PCR) with primers specific for the second internal transcribed spacer (ITS-2) of ribosomal DNA (rDNA), three species of *Eimeria*, *E. tenella*, *E. maxima*, and *E. acervulina* have been successfully characterised from chickens in Taiwan. The sizes of PCR products from various isolates representing these three species were between 370 and 580 base pairs (bp). After cloning and sequencing of the PCR products, high nucleotide sequence identity (96.8–100%) was observed within a species. In addition, ITS-2 nucleotide sequences for *E. tenella* had higher homology (98.5–99.3%) than *E. maxima* (81.6–96.5%) when compared with appropriate sequences deposited in GenBank. To our knowledge, this is the first report of a 412-bp ITS-2 sequence for *E. acervulina* from chickens.

Keywords: Coccidiosis; Eimeria; Ribosomal DNA; Nucleotide sequencing; Internal transcribed spacer

1. Introduction

Coccidiosis in chickens is a worldwide disease caused by protozoan parasites of the genus *Eimeria*. It is responsible for significant economic losses to the livestock and poultry industries (Fernandez et al., 2003). Currently, seven species of *Eimeria*, *E. tenella*, *E. acervulina*, *E. maxima*, *E. necatrix*, *E. brunetti*, *E. praecox* and *E. mitis* are recognised as pathogenic to chickens (McDougald and Reid, 1997). Identification of the species has important implications for the diagnosis and control of the disease, as well as for studying their epidemiology and population biology (Woods et al., 2000b). However, conventional methods of identification, based on physiological and morphological features of the sporulated oocysts, require skilled personnel and have limitations due to overlap of characteristics among different species (Long and Joyner, 1984). For example, identification of *E. maxima* and *E. brunetti* based on oocyst shape and size may be unreliable as these two features can be the same or very similar between the two species (Woods et al., 2000b).

Molecular approaches for improving the accuracy of *Eimeria* species identification have been developed to overcome the limitations of traditional methods (Woods et al., 2000b). One such molecular approach involves analysing enzyme variation through electrophoresis

☆ The nucleotide sequence data reported in this paper are available in the GenBank database under the Accession No. AY742227.

* Corresponding author. Tel.: +886 8 7740511; fax: +886 8 7740550.
E-mail address: suhy@npust.edu.tw (H.Y. Su).

1090-0233/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tvjl.2005.09.017

and has been reported to be useful for the identification of species, strains and clones of *Eimeria* (Johnston and Fernando, 1997). However, this technique provides a limited number of variable enzymes and low level of polymorphisms and it is difficult to apply to a large number of samples (Fernandez et al., 2003). Another approach involves the random amplified polymorphic DNA (RAPD) technique based on the amplification of anonymous targets and the use of arbitrary primers (Welsh and McClelland, 1990; Williams et al., 1990). This generates fingerprints of multiple bands but does not yield reproducible results due to low specificity during polymerase chain reaction (PCR) (MacPherson et al., 1993).

In eukaryotic cells, multiple copies of the highly conserved ribosomal RNA (rRNA) genes are located in a tandem arranged series in which each gene is separated from the next by spacer DNA regions. A single cluster contains the rRNA genes for 18S, 5.8S, and 28S rRNA molecules that are separated by internal transcribed spacers (ITS-1 and ITS-2). Adjacent clusters are separated by external transcribed spacer (ETS) regions. Sequencing of rDNA regions including the small subunit 5S and ITS have provided genetic markers for the identification of *Eimeria* species (Stucki et al., 1993; Barta et al., 1997; Schnitzler et al., 1998). Some of these genetic markers have been applied to developing diagnostic assays and, in particular, primers specific for ITS-1 have been developed for PCR assays for the detection of *Eimeria* species in faecal and intestinal samples (Schnitzler et al., 1998, 1999). The rDNA ITS-2 has recently been sequenced for *E. tenella* and *E. maxima* (Barta et al., 1998, GenBank Accession Nos. AF026388 and AF027722-AF027726) and also appears to provide species-specific markers (Woods et al., 2000b), but so far no published ITS-2 sequences for the other five species of *Eimeria* from chickens have been reported.

In the present study, we have characterised three species of *Eimeria*, *E. tenella*, *E. maxima*, and *E. acervulina* by their ITS-2 rDNA and report here the first ITS-2 sequence of *E. acervulina* (GenBank Accession No. AY742227).

2. Materials and methods

2.1. Parasites

Parasite populations were obtained from different geographical regions of Taiwan during a disease outbreak in 2001–2003. Faeces or intestinal contents from hosts were processed in 2.5% (w/v) $K_2Cr_2O_7$ at 28 °C for 72 h to allow oocyst sporulation as described by Duszynski and Wilber (1997). Oocysts were isolated by centrifugation in a saturated NaCl solution (Shirley, 1995), washed three times in 30 mL of sterile deionised water, and purified using a sucrose-gradient centrifugation method (Gasser et al., 1987). Based on the morphometry (i.e., size, shape and colour) of sporulated oocysts, pre-patent period, and locations of gross lesions in the intestines (Gasser et al., 2001), *E. tenella*, *E. maxima* and *E. acervulina* were identified (data not shown).

2.2. Parasite propagation

One-day-old Arbor Acres chicks were supplied by a commercial hatchery in Southern Taiwan and raised in a coccidia-free environment with an ad libitum supply of filtered water and feed that was free of anti-coccidial agents and antibiotics. At two weeks of age, the chicks were transferred to wire-mesh cages that were cleaned with water at 80 °C and sterilised at 180 °C for 10 min. The animals were checked for the absence of coccidia by faecal examinations between one day and three weeks of age and immediately before experimental infection. At three weeks of age, the chicks were orally infected with a single oocyst based on the procedures suggested by Fernandez et al. (2003).

Experimental procedures employing animals followed the National Pingtung University of Science and Technology Guidelines for the care and use of animals for research purposes.

2.3. Genomic DNA extraction

The *Eimeria* isolates obtained from infected chickens were identified, purified and propagated as described above. Genomic DNA was isolated from oocysts using the methods reported by Fernandez et al. (2003) with some modifications. In brief, approximately $5–6 \times 10^7$ purified oocysts were cleaned with 5.75% sodium hypochlorite solution at 4 °C for 20 min, washed twice with sterile deionised water and resuspended in lysis buffer (600 mM EDTA, 1.3% N-lauroylsarcosine, 2 mg/mL proteinase K, pH 9.5) at 65 °C for 45 min. The lysate was then mixed with cetyl-trimethyl ammonium bromide (CTAB) buffer [2% (w/v) CTAB, 1.4 M NaCl, 0.2% 2-mercapto-ethanol, 20 mM EDTA, 100 mM Tris–Cl, pH 8.0], incubated at 60 °C for 1 h, and added to an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) to remove proteins. These CTAB-treated mixtures were centrifuged at 13,000*g* for 15 min and an equal volume of chloroform was added to the supernatant which was then re-centrifuged. The supernatant was mixed with 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol to precipitate DNA by centrifugation at 13,000*g* for 10 min. The DNA pellet was washed with 70% ethanol, resuspended in TE (10 mM Tris–Cl, pH 8.0, 0.1 mM EDTA), and quantified and visualised as described elsewhere (Fernandez et al., 2003).