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A Novel Procedure For Total Nucleic Acid Extraction from Small Numbers of *Eimeria* Species Oocysts

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SUMMARY: A series of experiments were performed in an attempt to extract genomic DNA from a small number of Eimerian oocysts. Sonication, ammonia, ethanol and lysozyme were all found to be unsuitable for the digestion of *Eimeria* oocysts. The chemicals and enzyme given were not capable of either disruption or digestion of oocysts for nucleic acid extraction. They had the capability of penetrating the oocyst wall but could not break-up the oocyst wall. It is impossible to obtain nucleic acid from *Eimeria* oocysts if the wall is not broken-up. In this study oocyst disruption was achieved using a simple but highly effective treatment regime involving sodium hypochlorite treatment, osmotic shock and proteinase K digestion. Following the disruption of the oocyst walls, a commercially available nucleic acid purification kit (Wizard® DNA Purification Kit, Promega) can be used to prepare high quality nucleic acid.

Key Words: Apicomplexa, *Eimeria*, nucleic acid extraction, PCR

Az Sayıdaki *Eimeria* Ookistlerinden Toplam Nükleik Asit Eldesi İçin Yeni Bir Metod

ÖZET: Az sayıdaki *Eimeria* ookistinden DNA ektraksiyonu için bir seri deneme yapıldı. Sonikasyon, amonyum, ethanol ve lizozim kullanılan denemeler *Eimeria* ookistlerini parçalamak için uygun olmadıkları tespit edildi. Bu kimyasalların, nükleik asit ektraksiyonu için ookistleri parçalama ya da sindirebilme özelliklerinin olmadığı belirlendi. Bazılarının ookist duvarından geçebildikleri ancak ookist duvarını parçalayamadıkları belilendi. Ookist duvarı dağılmadan içerisindeki DNA'nın elde edilmesi imkansızdır. Ookist duvarının iç katmanı proteinden yapılmış olmakla beraber Proteinaz K sindirimi etkisiz kalmaktadır. Ookist duvarının, dış faktörlere karşı koruyucu etki gösteren özel bir yapıya sahip olabileceği kabul edilmektedir. Bu makale, *Eimeria* ookistlerinden yüksek kalitede total nükleik asit eldesi için uygun olan basit prosedurü açıklamaktadır. Bu prosedürde, oldukça basit fakat etkili sodyum hipoklorit, ozmotik şok ve proteinaz K sindirimi işlemlerini içeren bir uygulama ile ookist duvarının parçalaması sağlanmıştır. Ookist parçalanmasını takiben ticari olarak satılan nükleik asit ekstraksiton kitleri de (Wizard® DNA Purification Kit, Promega) yüksek kalitede nükleik asit eldesi için kullanılabilmektedir.

Anahtar Sözcükler: Apicomplexa, *Eimeria*, Nükleik Asit Eldesi, PZR

INTRODUCTION

Coccidiosis is a condition caused by the intracellular parasitic protozoa *Eimeria* spp. These parasites cause disease of medical and economic importance in a wide range of hosts including man and domestic livestock (5).

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While an animal may harbour coccidia parasites, the symptoms of disease may not be apparent throughout the infection cycle leading to a chronic subclinical condition. In this condition, animals harbouring *Eimeria* parasites normally shed low numbers of oocysts in their faeces providing a continuous source of infection for other animals. Acute, clinical disease of coccidiosis is characterised by a reduced feed efficiency in the infected host animal, leading to cost of medication and decreased animal productivity (5). In addition the presence of *Eimeria* parasites in the animal intestine has been correlated with increased susceptibility to secondary infection, especially bacterial diseases (7, 14).

Effective diagnostic tools would be useful for the detection of pathogenic subclinical *Eimeria* infections in domestic livestock through faecal monitoring. Such technology could be implemented in a basic control strategy where animals harbouring pathogenic subclinical infections could be isolated from uninfected animals preventing disease transmission. Diagnostic tools developed to date, whilst providing adequate detection of subclinical *Eimeria* infection, have failed to provide a distinction between pathogenic and non-pathogenic *Eimeria* species (3, 8). Molecular biological techniques, particularly the PCR, have proved useful for the specific detection of pathogenic microorganisms, providing a means of accurate identification for a wide range of important diseases. There is PCR based species identification only for chicken *Eimeria* species in coccidiosis (8). The application of this technology for the detection of *Eimeria* has been limited by the remarkable resilience of the *Eimeria* oocyst wall (4, 10, 11). Methods developed for the isolation of *Eimeria* nucleic acids involve either *in vitro* excystation or mechanical, chemical or enzymatic disruption of the oocyst wall (1, 8, 9). *In vitro* excystation has proved to be time consuming and laborious (9) while mechanical oocyst disruption methods including crushing or grinding with glass or zirconium beads are known to result in damage to large DNA molecules and therefore produce a low yield of nucleic acids (1). Some other applications have been used successfully in order to extract DNA from *Cryptosporidium* oocysts such as freeze-thaw or direct digestion in the alkaline lysis buffer (2, 12). However, the oocysts wall of *Eimeria* spp. are known to be highly refractory to the actions of both chemical and enzymatic disruption procedures (4, 9) and consequently these methods have also been of little value as a first step towards successful small-scale nucleic acid purification (1).

The innate resilience of the *Eimeria* oocyst wall is explained by the composition of the inner and outer layers of the oocyst wall. The outer layer consists of a lipid component comprising long chain alcohols, phosholipids, sterols and triglycerides which provide no obvious target for enzymatic hydrolysis whilst conferring a high degree of mechanical integrity to the oocyst (4, 10). The inner layer, consisting of glycoprotein (10) provides a high degree of resistance to extremes of temperature and pH (4, 13).

MATERIAL AND METHODS

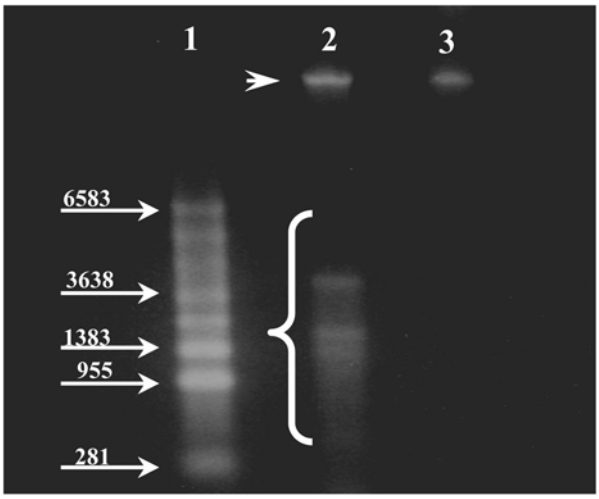
For this study, a standard parasitological technique (6) was used to collect oocysts from the faeces of six groups of lambs infected (monoaxenically) with six different species of *Eimeria*. Purified oocysts were stored in 2 % (w/v) potassium dichromate prior to nucleic acid preparation. The oocysts were sterilised by sodium hypochlorite treatment (4 % available chlorine, 1 h, 4 ^{o}C) which also aids in digestion of the outer layer of the oocyst wall (15) and makes the oocysts susceptible to protease digestion.

For large-scale oocyst nucleic acid extraction 10µl of an oocyst suspension (5×10^5 oocysts/µl) was combined with 35 µl saturated sodium chloride solution and incubated at 55 ^{o}C for one hour with gentle mixing every 15 min. Following this incubation, the oocyst suspension was prepared for protease digestion through the addition of 300 µl TE buffer (10 mM Tris-HCl, 0.1 mM EDTA pH 8), 3 µl of 10 % (w/v) SDS and 10 µl of proteinase K (20 mg/ml). After a one hour incubation at 37 °C, oocyst DNA was extracted through the use of a commercially available nucleic acid purification kit (Wizard® DNA Purification Kit, Promega) following the manufacturers instructions.

RESULTS

Using this method approximately 3µg of total nucleic acid was extracted from 5×10^5 oocysts and a small sample of this nucleic acid preparation was electrophoresed using a technique permitting simultaneous resolution of both DNA and RNA (Fig. 1). The presence of both DNA and RNA, demonstrated by RNAse digestion shows that this method is useful for the extraction of either DNA or RNA from *Eimeria* spp. oocysts.

Figure 1. Total nucleic acids extracted from 5.000.000 E. weybridgensis oocysts before and after treatment with RNAse.



Lane 1: RNA marker (Promega); **Lane 2:** 1 mg total nucleic acid extracted from *E. weybridgensis* oocysts; **Lane 3:** 1 mg total nucleic acid extraction from *E. weybridgensis* oocysts following 30 min treatment with RNAseA (2,5 µg/ml) at 37 ^{0}C.

1.4% (w/v) phosphate buffered agarose gel. Arrows adjacent to Lane 1 indicate molecular weights of marker species (bp). Arrow adjacent to Lane 2 indicates high molecular weight DNA refactory to RNAse treatment with genomic DNA. Bracket indicates RNA, including characteristic rRNA bands.

For more practical applications such as PCR-based detection, it was examined the use of the outlined procedure for the extraction of DNA from a number of *Eimeria* spp. oocysts which might be found in samples of faecal material. Using DNA isolated from approximately 1×10^4 oocysts as template,

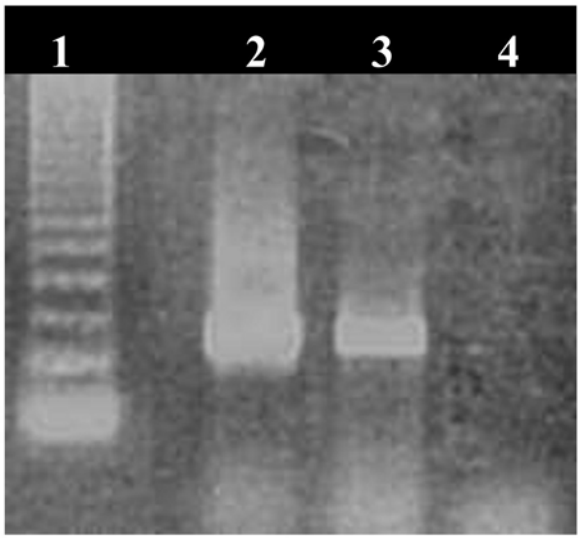
the PCR was used to detect *E. crandallis* using specific PCR primers as below (1).

Ecr1F; 5'-TAGGTTGTTGTAGCGTTGCG-3' and

Ecr1R; 5'-CTACCCTATCATCCTTGGCG-3'

The PCR, using primers specific for *E. crandallis* DNA, gave a positive result both for DNA extracted through the large scale procedure and for DNA extracted using the small scale procedure. By diluting the DNA template extracted from approximately 1×10^4 oocysts (Fig. 2), it was found that PCR could be used to detect DNA extracted from only 10 *E. crandallis* oocysts (Fig. 3).

Figure 2. PCR amplification of *E. crandallis* DNA using primers Ecr1F and Ecr1R.



Lane 1: 125 bp DNA ladder (Sigma); **Lane 2:** Template DNA equivalent to approximately 50.000 *E. crandallis* oocysts; **Lane 3:** Template DNA equivalent to approximately 1.000 *E. crandallis* oocysts; **Lane 4:** Negative control (no template DNA)

1.4% (w/v) agarose gel stained with ethidium bromide. The expected 338 bp PCR product was observed in lanes 2 and 3.

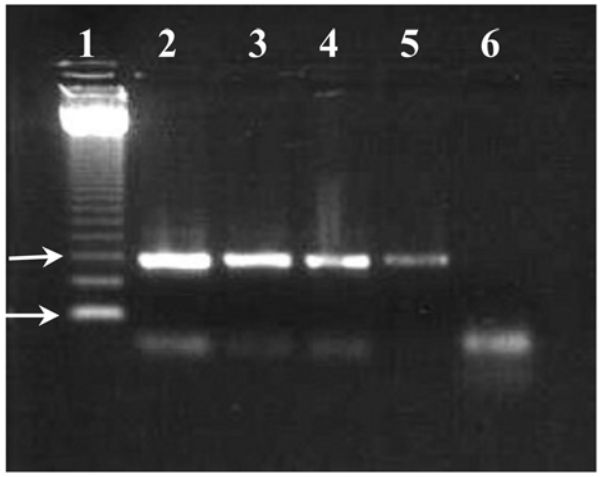
DISCUSSION

Clearly, using the outlined DNA extraction procedure in combination with a sensitive PCR-detection assay, it should be possible to detect both clinical and subclinical *Eimeria* spp. infections in contaminated faeces.

Species specific primers for *E. crandallis* and *E. ovinoidalis* were used for PCR amplification (1). In addition, specific PCR products were amplified from template genomic DNA from both species.

Hot phenol DNA extraction was used to extract DNA (1, 11). A total of 53 PCR reactions were performed using DNA extracted by the hot phenol method without success. PCR amplification was unsuccessful. Other researchers have reported the successful use of this method (1, 11) but in the present study the results were found to be unrepeatable.

Figure 3. PCR amplification of *E. crandallis* DNA extracted using osmotic shock and proteinase K.



Lane 1: 125 bp DNA ladder (Sigma); **Lane 2:** PCR with DNA extracted from 10.000 *E. crandallis* oocysts; **Lane 3:** PCR with DNA extracted from 1.000 *E. crandallis* oocysts; **Lane 4:** PCR with DNA extracted from 100 *E. crandallis* oocysts; **Lane 5:** PCR with DNA extracted from 10 *E. crandallis* oocysts; **Lane 6:** Negative control (no template DNA)
1.4% (w/v) agarose gel stained with ethidium bromide.

Microscopically the contents of the oocysts (sporocysts and sporozoites) in the sample were often destroyed but the oocyst walls remained intact after hot phenol exposure and the oocyst content was retained. The oocysts in the sample were collected from protein phase after phenol extraction.

Glass beads have proved effective for the disruption of *Eimeria* large numbers of oocysts. They were not been used in this study. Instead zirconium beads which have been employed as an alternative to glass beads were used. The results were disappointing and even 10,000 oocysts did not produce positive PCR amplification after extraction.

Some other applications have been used successfully in order to extract DNA from *Cryptosporidium* oocysts such as freeze-thaw or direct digestion in the alkaline lysis buffer (2, 12). However, the oocysts wall of *Eimeria* spp. are known to be highly refractory to the actions of both chemical and enzymatic disruption procedures (4, 9) and consequently these methods have also been of little value as a first step towards successful small-scale nucleic acid purification (1).

Genomic DNA extraction was only achieved with osmotic shock followed by Proteinase K digestion (Figure 1). In addition, the methods presented in this study may be useful in the development of molecular biology techniques suitable for use with *Eimeria* spp. and related apicomplexans.

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