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Developing a DNA hybridization technique for the detection of two pathogenic *Eimeria* species of chicken

(Membangunkan teknik DNA penghibridan untuk mengesan dua spesies *Emeria* yang patogen kepada ayam)

A.K. Adlin Azlina*, A.H. Zainal-Abidin**, Z. Zulkiflie**, M.S. Jangi** and F.M. Tomley***

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Abstract

Poultry coccidiosis is caused by *Eimeria* spp. of the phylum Apicomplexa. Coccidiosis still continues to be an important threat to poultry industry. Diagnosis based on microscopic technique is usually used to differentiate species, however these techniques are still less efficient, insensitive and less specific for the detection of mix infections in chicken. Therefore, a DNA-based test particularly using ribosomal RNA (rRNA) was chosen to identify a molecular marker to enable a faster and more sensitive identification of a particular species.

In this study, the 5S rRNA gene was chosen because of its high degree of conservation, ubiquity and the relative ease with which it can be cloned. It also has a semi-conserved region from which a species-specific primer can be designed. The size of 5S rRNA gene is 120 nucleotides and the spacer is different in size and sequence for each species. From this spacer, primers for each species have been designed. Probes from *E. tenella* and *E. maxima* were developed using the specific primers. Using dot-blot, these probes showed that they can be used to differentiate these two species from the others. Probes designed for two of the species i.e. *E. tenella* and *E. maxima* were found to be specific in detecting the parasites. This study showed that information related to 5S rRNA genes is useful because it can be used to develop molecular markers for future diagnostic work on parasites.

Introduction

Coccidiosis is a disease of animals and birds caused by protozoan parasites known as coccidia *(Eimeria)*. This disease is of major economic importance for the poultry industry worldwide. Infections are controlled in the main by prophylactic in-feed medification or increasingly, by vaccination with live parasites. Traditionally, species of *Eimeria* have been identified by a variety of methods. For example, morphological features and/or morphometry of their oocysts or sporocysts (size, shape, length and width), their patterns of development, the nature of the lesions they produce, their predilection site(s) in the gut, sporulation times and reproductive index, or from the specific host from which they originate (Andrews and Chilton 1999). Conventional methods of identification require highly

*Biotechnology Research Centre, MARDI Headquarters, Serdang, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia
**Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia
***Institute of Animal Health, Compton, United Kingdom.
Authors' full names: Adlin Azlina Abdul Kadir, Zainal-Abidin Abu Hasan, Zulkiflie Zamrod, Mohd Sanusi Jangi and Fiona Tomley
E-mail: inashbob@mardi.my
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trained personnel, since the differences in oocyst morphology are small and not easily seen.

Developments in nucleic acid technology offer a detection system for coccidiosis with both increased speed and sensitivity. DNA hybridization or nucleic acid probes have been utilized for diagnostic purposes (Tenover 1988). Recently, techniques based on the polymerase chain reaction (PCR) amplification of DNA have been used for the diagnosis of some other coccidial parasites of man and animals (Schnitzler et al. 1999). DNA-based tests using ribosomal RNA (rRNA) as molecular marker have been developed to enable faster and more sensitive identification of a particular species. Assays that are sensitive, fast and capable of large-scale testing is needed for routine avian diagnostics. Dot-blot hybridization using nucleic acid probes is a method of testing that can be adapted for large sample numbers without sacrificing speed and accuracy.

Many DNA probes are used to identify infective agents. A large number of protocols have been devised for the identification of parasitic protozoa such as *Leishmania* (Wirth and McMahon 1982; Barker 1989), *Plasmodium* (Delves et al. 1989) and diverse species of *Trypanosomes* (Masiga and Gibson 1990). In general, hybridization procedures require 10^3–10^4 protozoal cells for reliable identification. In the last two or three years the sensitivity of detection has been greatly increased with the introduction of the PCR, for which the DNA of a single parasite may suffice for a positive identification (Schnitzler et al. 1999). This paper presents a very simple method (DNA dot blot hybridization) that flanking regions from the 5S rRNA gene of *E. tenella* and *E. maxima* (Schnitzler and Shirley 1999), which is commonly found in poultry farming, can be used for species identification.

Materials and methods

Parasites

Purified DNAs of two *Eimeria* species: *E. maxima* and *E. tenella*, Houghton isolate, were supplied by Fiona Tomley (Institute for Animal Health, Compton, Newbury, Berkshire RG20 7NN, UK).

Preparation of the probes for hybridization

Oligonucleotide primers were designed from the spacer region of the 5S rRNA gene sequence (GenBank accession no. M86547) (Stucki et al. 1993) which are considered to be specific for *E. tenella* and *E. maxima*. The 5S rRNA from *E. tenella* was amplified by PCR using the primers TenelF (forward: 5'-CCA CAG CGC GTC CTC TCT A-3’ and TenelR (reverse: 5'-CCA CAG CCA CAG AGT AAC A-3’), while the 5S rRNA from *E. maxima* was amplified using the primers MaxiF (forward: 5'-TAC GCG CGC CCT CAA CTG AT-3’) and MaxiR (reverse: 5'-ACA CAC GCA AAC ACA AAC CC-3’). PCR was performed in 20 ml reaction volume with 40 pM of each primer, 0.2 mM deoxyribonucleoside 5'-triphosphates (dNTPs) and 5 units *Taq* DNA polymerase (Promega).

The reactions were run with the following parameters: Denaturation was at 94 °C (60 sec), the annealing temperature was 60 °C (60 sec) and elongation was at 72 °C (60 sec) and 30 cycles were performed. The samples were then cooled at 4 °C until they were removed. The resulting PCR products were 300 bp (*E. tenella*) and 223 bp (*E. maxima*) in size. After PCR, the products were checked on a 1% agarose gel together with a 100 bp molecular weight standard. The fragments of expected size were cut out from the agarose and purified with the QIAquick Gel Extraction Kit (Qiagen) and used as probes for hybridization.

Hybridization and detection

For labelling the probes, the ECL direct nucleic acid labelling and detection system (Amersham) was used. A 200 ng of probe in

20 ml of water was denatured by boiling for 5 min and chilled on ice for 5 min. The DNAs were crosslinked to the membrane using a Stratalinker (Stratagene, La Jolla, CA USA). The hybridization between PCR products fixed on the membrane and the labelled probe was carried out under stringent conditions.

A mixture of 20 µl of horseradish peroxidase (labelling reagent), 20 µl of glutaraldehyde (cross-linker) and denatured DNA were incubated for 10 min at 37 °C. After incubation, the probe was immediately added to one hour prehybridized membranes. Prehybridization was carried out at 48 °C, and these prehybridization membranes were then hybridized overnight at the same temperature. Hybridization solution was provided together with the ECL kit where 0.05 ml of hybridization buffer per cm^2 membrane was used. The probe concentration was at 10 ng/ml of hybridization solution.

After hybridization the membranes were washed twice with 0.3x standard saline citrate (SSC), 4% sodium dodecyl sulfate (SDS) at 55 °C for 10 min, followed by two secondary washes of 5 min in 2x SSC at room temperature. The filter was transferred to a clean plastic box and covered by the detection reagents (Amersham) for 60 s. The filter was then exposed to Hyperfilm (Amersham) for one minute, developed and optimal exposure times were estimated.

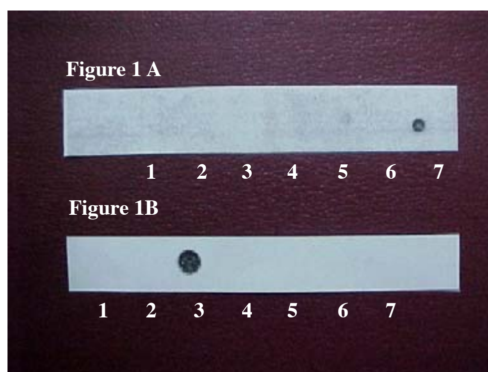

Figure 1. Dot blot using probe from (A) ***E. tenella****. (B)* ***E. maxima*** *(1)* ***E. acervulina*** *(2)* ***E. brunetti*** *(3)* ***E. maxima*** *(4)* ***E. mitis*** *(5)* ***E. necatrix*** *(6)* ***E. praecox*** *and (7)* ***E. tenella***

Results and discussion

The use of ribosomal gene repeats has two advantages in DNA-based diagnosis. They are present in high copy numbers, which improve sensitivity and the spacer regions between ribosomal genes are normally less conserved than the genes themselves, allowing discrimination between species (Stucki et al. 1993). For this reason, primers for *E. tenella* and *E. maxima* were designed from the spacer region. Amplification of PCR was done using these primers. A product of 300 bp was amplified from *E. tenella* and a product of 223 bp for *E. maxima*. Both PCR products were used as probes in dot blot hybridization. To test the species specificity using these probes, plasmids containing these products were hybridized to dot blots of DNA from seven different species of *Eimeria*.

Results showed that the DNA probe amplified using primers derived from 5S rRNA sequence of *E. tenella* was able to specifically hybridize to the DNA of only *E. tenella (Figure 1A)*. Similarly, the probe amplified using primers derived from 5S rRNA sequence of *E. maxima* was shown to specifically hybridize to the DNA of *E. maxima* only *(Figure 1B)*. Using the dot-blot technique, these probes showed that they are specific and can be used to differentiate the two *Eimeria* species from the others.

This work describes a hybridization assay for specific detection of *E. tenella* and *E. maxima*. Traditional methods for diagnosis of coccidial infections are relatively insensitive and do not reliably differentiate between species. In mixed infections, only the major species present is likely to be identified. The assay described here is simple to perform, sensitive and specific for the detection of *E. tenella* and *E. maxima*. Additional work is required to determine the usefulness of this particular

assay as a diagnostic tool, but the potential of hybridization assays for the detection of avian *Eimeria* is obvious.

Conclusion

DNA hybridization techniques are becoming increasingly useful in the clinical setting, eliminating the need for laborious serological techniques and, in some instances, offering increased sensitivity (Henderson and Jackwood 1990). With the development of non-radioactive labels for nucleic acid probes, these techniques have become practical for the diagnostic laboratory. The application described here showed that DNA hybridization techniques can be used to identify coccidiosis infections.

Probes designed for two of the species i.e. *E. tenella* and *E. maxima* were specific in detecting the parasites. The assay developed from this study may be used, experimentally or on a commercial scale, as a mean for routine diagnosis and monitoring of coccidia, particularly avian coccidia. Alternatively, the assay may be applicable to the quality control of species status of monospecific laboratory lines of *Eimeria*. It may further be useful as a complementary tool in the development of future commercial vaccines and diagnostic tests. The information on the 5S rRNA genes are useful as molecular marker for future diagnostic work on parasites.

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Abstrak

Koksidiosis pada ayam adalah penyakit yang disebabkan oleh parasit *Eimeria* daripada filum Apicomplexa. Penyakit ini masih merupakan penyakit utama yang mengakibatkan kerugian besar dalam industri penternakan ayam. Teknik diagnosis berasaskan mikroskopi biasanya digunakan untuk mengenal pasti spesies. Walau bagaimanapun kecekapan dan kespesifikan teknik ini masih rendah terutama untuk mengesan jangkitan berganda daripada beberapa spesies *Eimeria* yang berlaku pada ayam. Oleh itu ujian yang berasaskan DNA khususnya menggunakan gen RNA ribosom (rRNA) telah dipilih untuk mengenal pasti molekul penanda bagi membolehkan pengenalpastian spesies dilakukan dengan lebih cepat dan tepat.

Dalam kajian ini, jujukan gen dan penjarak 5S rRNA dipilih kerana ia mempunyai bilangan salinan yang tinggi dalam genom sel-sel dan jujukannya juga amat terpelihara. Di samping itu, jujukan ini mempunyai kawasan yang separa terpelihara yang membolehkan suatu primer yang spesifik untuk setiap spesies dibina. Didapati saiz gen 5S rRNA ialah 120 nukleotida dan penjaraknya berbeza dari segi saiz dan jujukan bagi setiap spesies. Daripada penjarak ini, primer bagi setiap spesies telah dibina. Proba daripada dua spesies iaitu *E. tenella* dan *E. maxima* telah juga dijana. Dengan menggunakan teknik pedapan titik, proba-proba ini boleh membezakan kedua-dua spesies tersebut daripada spesies *Eimeria* yang lain. Oleh itu, dicadangkan supaya maklumat mengenai gen dan penjarak 5S rRNA daripada spesies *Eimeria* ini digunakan untuk membina proba-proba spesifik sebagai molekul penanda dalam kerja-kerja diagnosis koksidiosis.

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