Veterinary Parasitology 214 (2015) 16–21

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

A molecular survey of *Eimeria* in chickens across Australia

Rosamond M. Godwin[a,b], Jess A.T. Morgan[a,c,*]

[a] Agri-Science Queensland, Department of Agriculture and Fisheries, St. Lucia, QLD 4067, Australia
[b] Poultry CRC, PO Box U242, University of New England, Armidale, NSW 2351, Australia
[c] Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, PO Box 6097, St. Lucia, QLD 4067, Australia

ARTICLE INFO

Article history:
Received 25 June 2015
Received in revised form
18 September 2015
Accepted 26 September 2015

Keywords:
Eimeria
Species survey
Mitochondrial DNA
PCR
Capillary electrophoresis

ABSTRACT

Coccidiosis is a costly enteric disease of chickens caused by protozoan parasites of the genus *Eimeria*. Disease diagnosis and management is complicated since there are multiple *Eimeria* species infecting chickens and mixed species infections are common. Current control measures are only partially effective and this, combined with concerns over vaccine efficacy and increasing drug resistance, demonstrates a need for improved coccidiosis diagnosis and control. Before improvements can be made, it is important to understand the species commonly infecting poultry flocks in both backyard and commercial enterprises. The aim of this project was to conduct a survey and assessment of poultry *Eimeria* across Australia using genetic markers, and create a collection of isolates for each *Eimeria* species. A total of 260 samples (faecal or caecal) was obtained, and survey results showed that *Eimeria* taxa were present in 98% of commercial and 81% of backyard flocks. The distribution of each *Eimeria* species was widespread across Australia, with representatives of all species being found in every state and territory, and the *Eimeria* species predominating in commercial flocks differed from those in backyard flocks. Three operational taxonomic units also occurred frequently in commercial flocks highlighting the need to understand the impact of these uncharacterised species on poultry production. As *Eimeria* infections were also frequent in backyard flocks, there is a potential for backyard flocks to act as reservoirs for disease, especially as the industry moves towards free range production systems. This *Eimeria* collection will be an important genetic resource which is the crucial first step in the development of more sophisticated diagnostic tools and the development of new live vaccines which ultimately will provide savings to the industry in terms of more efficient coccidiosis management.

1. Introduction

Coccidiosis is a worldwide intestinal disease of chickens caused by parasitic *Eimeria*. Infection with *Eimeria* impairs growth, suppresses the immune system and can cause significant mortality with an estimated global cost to the poultry industry in excess of US $3 billion annually (Blake and Tomley, 2014). Current control methods include preventative chemotherapy using anticoccidial drugs, live attenuated vaccines or recombinant vaccines. These approaches are not 100% effective but aim to keep oocyst numbers low and let immunity develop naturally. However in recent years, there have been increasing concerns regarding vaccine efficacy (Arczewska-Włosek and Świątkiewicz, 2014), drug resistance (Peek and Landman, 2011), and chemical residues in food (Kher et al., 2013).

Seven species of chicken *Eimeria* have been identified in Australia, *Eimeria acervulina*, *Eimeria tenella*, *Eimeria necatrix*, *Eimeria maxima*, *Eimeria praecox*, *Eimeria mitis*, and *Eimeria brunetti* (Callow, 1984). Mixed species infections are common with species and strains looking similar but differing in their biology and pathogenicity (Callow, 1984). This complicates the diagnosis and treatment of coccidiosis in poultry. Additionally three genetically distinct variants (operational taxonomic units or OTUs) have also been found to infect chickens (Cantacessi et al., 2008; Godwin and Morgan, 2014; Morris et al., 2007) with OTU-X, Y and Z most closely related to *E. maxima, E. brunetti and E. mitis*, respectively. The impact of these OTUs on poultry production is largely uncharacterised and, as current live vaccines only provide species-specific immunity, there are concerns that the vaccines offer little or no protection against these genetic variants.

Before improvements can be made to *Eimeria* diagnosis and control, it is important to understand the species commonly infecting

* Corresponding author. Present address: DAF, Level 2AW, EcoSciences Precinct, 41 Boggo Rd, Dutton Park QLD 4102, Australia. Fax: +61 7 38444529.
E-mail address: jessica.morgan@uq.edu.au (J.A.T. Morgan).

http://dx.doi.org/10.1016/j.vetpar.2015.09.030
0304-4017/

poultry flocks across Australia both in backyard and commercial enterprises. The aim of this project was to conduct a survey to assess the nationwide status of *Eimeria* species in poultry flocks across Australia. Using genetic markers, the survey was designed to determine the *Eimeria* species present, their geographical distribution and frequency and create a collection of isolates for future research. Backyard and commercial flocks were sampled to enable a comparison among the *Eimeria* species infecting different types of flocks.

2. Materials and methods

2.1. Sampling and genomic DNA extraction

2.1.1. Sampling strategy

Strains of each of the seven recognised *Eimeria* species and three OTUs were used as positive controls during the screening of field samples. The seven recognized species, including two genetic variants of *E. praecox*, were obtained from the Department of Agriculture and Fisheries (DAF) collection of poultry *Eimeria* while the three OTUs, (X, Y and Z) were obtained from Bioproperties Pty., Ltd. (Victoria, Australia).

An opportunistic sampling strategy was employed in this study as there is little known about the prevalence and range of *Eimeria* species infecting poultry flocks in Australia, distances are great, and a flexible sampling approach relying on the cooperation of the commercial sector and members of the public was needed. A total of 260 field samples was obtained from healthy birds from a variety of poultry establishments across Australia between 2010 and 2013. Faecal samples ($n = 231$) were sourced from both commercial broiler farms and backyard flocks. For the purpose of this study, backyard flocks were defined as those owned by small holders in urban or semi-rural areas, with generally fewer than 10 but up to 70 birds of a variety of ages and breeds and kept for personal use (mainly for eggs but also for meat). Commercial flocks were from large scale, predominantly broiler enterprises with 7300–960,000 birds aged <50 days. All the flocks in this study were unvaccinated but in-feed coccidostats were administered to the majority of commercial flocks for coccidiosis control.

Over the duration of the project, sampling of faeces was conducted twice per year in early and late summer with the goal of obtaining at least five samples/state/sampling period. An additional 29 caecal samples were obtained from commercial abattoirs, comprising of 1 sample from Queensland (QLD), 11 from South Australia (SA), 4 from Tasmania (TAS) and 13 from Western Australia (WA). All animal procedures were approved by the Queensland Government Department of Agriculture and Fisheries Animal Ethics committee (approval number SA 2011-02-345).

2.1.2. Sample preparation

At each sampling period, a pooled sample of 50 g of fresh faeces was collected from each of five unvaccinated flocks (either commercial or back-yard) from every Australian state and territory and transported to the laboratory for screening for live oocysts. Industry members were also invited to submit samples from farm outbreaks for screening throughout the term of the project. These samples were submitted as either gut tissues from deceased birds or faecal samples.

Upon arrival at the laboratory, the faeces were thoroughly mixed with a wooden applicator stick. Two 200 mg subsamples were then taken from the vial, placed in 2 mL plastic screw-capped tubes and stored at 4 °C for subsequent DNA screening. The remaining faeces were transferred to 1 L plastic bottles containing 200 mL of 2% potassium dichromate and placed on rollers at 2 revolutions per minute (rpm) for 72 h to sporulate any potential oocysts present. After three days of rolling, 5 mL of the slurry was sub-sampled and screened microscopically for oocysts using a modified Sheather's (Sheather, 1923) sugar flotation solution (Anderson, 1981). Faecal samples that were microscopically positive for *Eimeria* were filtered through a 1 mm mesh to remove any coarse debris then oocysts were cleaned and purified using salt flotation (Jorgensen et al., 1997). Purified oocysts were resuspended in 5 mL of 2% potassium dichromate and stored at 12 °C. Oocysts were counted microscopically using a McMaster chamber (Hodgson, 1970).

Caeca were sampled from randomly selected carcasses on the production line at different commercial poultry abattoirs around Australia. Birds were >40 days old, except for the birds from Tasmania which were 33–34 days old. Whole caeca were dissected from each bird under clean conditions and each caecum was placed in a sterile collection bag and placed at 4 °C for overnight transport to the laboratory. Upon arrival the caeca were placed into a sterile vial and stored at −80 °C until further use.

2.1.3. DNA extraction

DNA was extracted from up to one million purified oocysts following an established method (Morgan et al., 2009). Briefly, oocyst walls were cracked prior to lysis using a bead-beater and 1 mm glass beads then the lysate was extracted using a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA).

DNA was also extracted directly from 200 mg samples of faecal or caecal material using mechanical bead lysis, followed by extraction through a QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) to remove PCR inhibitors (Morgan et al., 2009). This extraction protocol inserts an additional step incorporating an inhibitex tablet (Qiagen, Valencia, CA, USA) to remove inhibitors prior to silica-membrane column purification of the DNA. A negative control was included in every extraction run.

2.1.4. Species identification using capillary-electrophoresis assay (CE-assay)

The presence of OTUs in both commercial and backyard flocks meant that results from microscopic oocyst screening and genetic screening using real-time PCR (Morgan et al., 2009) were inconsistent. Instead a new diagnostic assay based on capillary-electrophoresis (CE) was developed as part of this project for rapid screening of *Eimeria* DNA (Godwin and Morgan, 2014). The CE-assay allowed the simultaneous detection of all seven known species of *Eimeria* plus the 3 OTUs in a single tube. Briefly, the mitochondrial DNA PCR fragment length diagnostic assay uses PCR-coupled capillary electrophoresis and generic primers targeting a non-coding region of mitochondrial DNA, just downstream of the 3′ end of cytochrome oxidase subunit 3 (*cox3*), and amplified length diagnostic fragments (174–197 bp depending on the species) (Table 1). The forward primer was given an M13 extension, so that an additional 6-FAM-labelled M13 forward primer could be included in the reaction for product detection.

For both commercial and backyard flocks, Chi squared homogeneity tests were conducted to determine if the relative proportion of *Eimeria* varied among states and territories. Calculations were performed using Microsoft Excel 2010 (Version 1.2.1 Microsoft Corporation).

2.2. Laboratory amplification of isolates and separation of oocysts

Samples that were found to be positive for *Eimeria* by microscopic examination, with moderate to high infection levels, and collected from locations poorly represented in live oocyst collections, were selected for passaging through chickens to amplify the available number of oocysts (Animal Ethics Approval SA 2011-02-345). Birds were Rhode Island Red x Rhode Island White Bond and obtained at one day old from a minimal disease flock.