PCR identification and prevalence of *Eimeria* species in commercial turkey flocks of the Midwestern United States

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ABSTRACT The present study used a PCR approach to characterize prevalence of coccidial species in fecal samples obtained from 40 individual Midwestern turkey flocks to characterize distribution of species in commercial flocks. Each sample was screened for 6 prominent *Eimeria* species using species-specific primers and was supplemented with a primary nested-PCR approach for amplification of mitochondrial cytochrome *c* oxidase subunit gene I where initial sample DNA concentrations were low. All samples were positive for at least one species of *Eimeria*, while most presented 2 (20/40) or 3 (14/40) species in total. Prevalence across farms was primarily dominated by *E. meleagrimitis* (97.50%), *E. adenoeides* (95%), and *E. gallopavonis* (40%). Of the samples positive for *E. adenoeides* and *E. meleagrimitis*, almost half (17/40) contained additional species. Data presented here offer insight into *Eimeria* species currently challenging the Midwestern US turkey industry and potential need to evaluate flocks for species prior to implementing vaccination programs.

Key words: turkey, coccidiosis, PCR, *Eimeria adenoeides*, *Eimeria meleagrimitis*, *Eimeria gallopavonis*

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INTRODUCTION

Coccidiosis continues to be a pervasive enteric disease of significant economic importance within the poultry industry. While often regarded more problematic in broilers, coccidial infection is an ongoing concern in other poultry such as layers and turkeys. In a recent survey intended to supplement the 2020 USAHA Transmissible Diseases of Poultry and Avian Species report, US industry professionals and veterinarians ranked coccidiosis fifth in the top 10 disease concerns in turkeys, demonstrating increased concern given a 2019 rank of eighth (USAHA Committee on Poultry and Other Avian Species, 2019; Clark and Froebel, 2020). At present, there are 6 well-characterized, taxonomically validated *Eimeria* spp. known to cause coccidiosis in turkeys: *E. adenoeides, E. dispersa, E. gallopavonis, E. innocua, E. meleagridis*, and *E. meleagrimitis* (Imai and Barta, 2019). While coccidial infections in turkeys often fail to results in the manifestation of clinical signs, inhibitory effects on growth performance are evident (Chapman, 2008; Vrba and Pakandl, 2014). Furthermore, due to oocyst survivability and persistence within the environment, anticoccidial prophylactic and therapeutic management practices are crucial for disease control and industry profitability (Reyna et al., 1983; Chapman et al., 2013).

Currently available coccidia control strategies include live commercial or autogenous oocyst vaccination and rotation or shuttle programs that cycle anticoccidial medications such as synthetic chemicals or ionophores in feed (Chapman, 1997; Noack et al., 2019). Notably, in today's commercial poultry market, raised without antibiotics (**RWA**) and no antibiotics ever (**NAE**) management styles continue to become more prevalent, and further narrow the list of direct-fed anticoccidial options considered acceptable (Cervantes, 2015). While selection between anticoccidial drugs or RWA/NAE approved natural alternatives such as phytonutrients are at the discretion of the producer or flock veterinarian, just one live oocyst vaccination, Immucox-T, is currently approved and commercially available for US turkey flocks. Moreover, Immucox-T is comprised only of *E. adenoeides* and *E. meleagrimitis* oocysts. Considering the prevalence of an additional four species *Eimeria* in turkeys, this partial specificity of protection may be inadequate. In fact, little or often no cross-immunity between turkey *Eimeria* species has been demonstrated several times over (Moore and Brown, 1951, 1952; Hawkins, 1952; Moore et al., 1954; Vrba and Pakandl, 2014; Imai and Barta, 2019).

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Given potentially limited protection afforded by vaccines with abbreviated species inclusion, it is important to reexamine the distribution and frequency of *Eimeria* species detected in US turkey flocks. This information could help evaluate potential need for inclusion of additional species in future commercially available live oocyst vaccines or to make informed decisions about species inclusions in flock-specific autogenous vaccines in order to enhance disease protection. Original characterization of *Eimeria* species relied on phenotypic characteristics such as host specificity, intestinal localization and lesions, oocyst morphology, cross-immunity, prepatent period, and pathogenicity (Joyner and Long, 1974; El-Sherry et al., 2015). While these parameters are still useful today, overlap between species can lead to inconclusive or incorrect identification, especially in cases of mixed species infection (McDougald and Jeffers, 1976; Long and Joyner, 1984). Supplementation of these criteria with molecular methods such polymerase chain reaction (**PCR**) or DNA sequencing would, therefore, enhance the accuracy of species assignment (Chapman, 2014; Imai and Barta, 2019). In the current study, a total of 40 field samples were obtained from various Midwestern US turkey flocks to characterize prevalence and diversity of *Eimeria* species through a species-specific PCR approach (Hafeez et al., 2015; Imai and Barta, 2019).

MATERIALS AND METHODS

Obtaining Samples

Fecal samples evaluated in this study were obtained as part of an *Eimeria* detection and diagnostic sensitivity service provided by the Ohio State University's Poultry Enteric Health Research Laboratory (**PEHRL**). Participating Midwest producers were provided information on recommended sample collection procedures which highlighted the need for fresh fecal droppings and minimal litter contamination. Producers were instructed to ship samples to PERHL overnight on cold ice packs, and received samples were stored at 4°C to be processed within 24 to 48 h. Producers provided the age of the flock from which samples were collected, which in the current study varied from 6 d to 11 wk. All samples represented in this work originated from Iowa, Minnesota, and Arkansas and were collected between 2019 and 2021. No information regarding flock anticoccidial programs was provided for any samples.

Oocyst Detection and Processing

Fecal samples were weighed and diluted 1:2 (w:v) with 2.0% potassium dichromate (**PDC**; Sigma-Aldrich, St. Louis, MO) into Erlenmeyer flasks. A small volume from each sample was diluted 10-fold in saturated NaCl to quantify total oocysts by McMaster's chamber float method. A minimum threshold of 60,000 total oocysts was set to determine whether samples would be retained for sporulation to assure sufficient volume of sporulated, infective oocysts for dosing and cycling in nonmedicated poults. Sufficient oocyst viability is necessary to induce coccidial infection and subsequent oocyst shedding for DNA extraction and PCR analysis. Acceptable samples were covered with perforated aluminum foil to allow air exchange and placed on a rotary platform shaker at 100 rpm at room temperature (**RT**) for approximately 1 wk to promote sporulation.

Animal Housing and Handling

Aviagen turkey poults were used to propagate *Eimeria* samples received through the PERHL diagnostic services. All propagations were conducted at the Ohio Agricultural Research and Development Center poultry research facility in Wooster, OH. Poults were reared in batteries or wire floor pens with a thin layer of fresh pine shavings (n = 4 poults/pen; 4 replicate pens per sample) with ad libitum access to water and feed. Ambient temperature and lighting schedules were maintained within age-appropriate ranges throughout all experiments. Several methods were used to minimize cross-contamination which included footbaths containing ammonia to limit the spread of *Eimeria*, insect control such as fly bait, fly tape, and closed doors between surrounding barn spaces and outdoors, and using different gloves for collection respective to farm sample. All protocols were approved by the Ohio State University's Institutional Animal Care and Use Committees (**IACUC**) under protocol number 2019A000000138.

Sample Propagation and Processing

As previously mentioned, after approximately 1 wk of sporulation, each farm-specific fecal sample was diluted in 0.9% saline to reach a final concentration of 100 sporulated oocysts/mL for oocyst propagation. At 9 days of age, poults (n = 16 poults/sample) were inoculated with 1 mL of oocysts from a designated farm via oral gavage. From d 4 to 7 postinoculation, feces were collected twice daily into cumulative jars respective to inoculum from designated farm, and were preserved in 0.9% saline at 1:2 (w:v). Amplified oocysts within jars were kept refrigerated at 4°C over the course of the collection period.

Within 48 h following the collection period, collected samples were cleaned of debris and concentrated with a saturated NaCl float and wash protocol (Imai and Barta, 2019; Snyder et al., 2021a). Undiluted samples were centrifuged in 50-mL conical tubes at 1,250 × *g* for 10 min to pellet oocysts along with other debris, and supernatants discarded. Pellets were resuspended 1:4 (v:v) in saturated NaCl, centrifuged at 1,250 × *g* for 15 min, and supernatants, which contained oocysts, were collected into fresh 50 mL tubes or 750 mL centrifuge jars depending on final volume. Supernatants were diluted 1:10 (v:v) in diH_2O, centrifuged at 1,250 × *g* for 10 to 15 min, and supernatants discarded for a total of three washes with diH_2O. Finally, pellets were

resuspended in a small volume of 2.0% PDC and stored at 4°C until DNA extraction.

DNA Extraction

Approximately 2 mL of previously described cleaned and concentrated oocysts were added to 2 mL screw-top microcentrifuge tubes and centrifuged at 21,000 × *g* for 5 min at RT. Original supernatants containing PDC were discarded and diH_2O was used to resuspend pellets for a total of 3 washes. Pellets were then suspended in 100 μL of DNAzol (Invitrogen, Waltham, MA) and approximately 0.3 g of 0.5 mm glass beads (BioSpec Products, Bartlesville, OK) were added to cover the pellet and aid in mechanical lysis (Hafeez et al., 2015). Tubes were then loaded into a horizontal bead beater (MiniBeadBeater-16; BioSpec Products) and processed for 60 s. An additional 900 μL of DNAzol was added and tubes placed on a rotary platform shaker at approximately 120 rpm at RT for 1 h. Halfway through incubation, tubes were loaded into the bead beater for 30 s and returned to the rotary shaker for the remaining time. Following incubation, tubes were centrifuged at 13,000 × *g* for 15min at 4°C. Supernatants were collected into new 2.0 mL microcentrifuge tubes, 500 μL of 100% molecular grade EtOH (Thermo Fisher Scientific, Waltham, MA) was added, and tubes were inverted by hand for 1 min. Tubes were centrifuged at 7,000 × *g* for 15 min at 4°C, supernatant aspirated, and tubes set in a fume hood to dry. After approximately 15 min of air drying, 200 μL of 10 mM Tris-buffer (pH 8.0), 200 μL of AL buffer (Qiagen GmbH, Hilden, Germany) and 200 μL of 100% EtOH were added to each tube and vortexed. Tube solutions were then transferred into spin columns (Epoch Life Sciences, Missouri City, TX) and centrifuged at 21,000 × *g* for 2 min at 21°C. Supernatant in collection tubes were disposed, 500 μL of AW1 buffer (Qiagen GmbH) was added to spin columns, and tubes centrifuged at 21,000 × *g* for 1 min at 21°C. After discarding collection tube supernatant, 500 μL of AW2 buffer (Qiagen GmbH) was added to spin columns and tubes centrifuged at 21,000 × *g* for 4 min at 21°C. Spin columns were then transferred to new 1.5 mL microcentrifuge tubes, 20 μL of nuclease free water (Sigma-Aldrich, St. Louis, MO) was added directly onto the filter component and left to incubate at RT for 5 min. Final DNA product was collected by centrifugation at 11,000 × *g* for 1min at 21°C, and DNA concentration was estimated using a Take3 Micro-volume plate and Synergy HTX, multimode microplate reader (BioTek Instruments Inc., Winooski, VT).

PCR Detection

Species-specific PCR amplification was performed in a CFX Connect Real-Time System (BioRad, Hercules, CA), and reactions consisted of 100 ng of template DNA, and PCR master mix made from 10X (Magnesium-free) standard buffer, 25 mM $MgCl_2$, 10 mM dNTPs, 5 U/μL Taq polymerase (New England Biolabs, Ipswich, MA), sterile deionized water, and 20 μM of both forward and reverse primers (Eurofins Genomics, Louisville, KY) specific for turkey *Eimeria* species (Table 1). PCR amplification conditions and expected product sizes were based on Imai and Barta (2019) and are summarized in Table 2. Each PCR assay contained a positive template control respective to targeted species and 2 negative controls that consisted of *E. maxima* template DNA and diH_2O in place of template DNA. PCR products were electrophoresed in a horizontal chamber filled with Tris-borate-EDTA (**TBE**) buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) at 80 V for 50 m on either 1.0 or 1.5% TBE agarose gels with ethidium bromide. Gels were visualized by UV transillumination, and product sizes estimated by comparison with 100 bp or 1 kb DNA ladders (New England Biolabs).

If initial DNA concentrations were low (<25 ng/μL) post-extraction, samples were subjected to nested PCR prior to species-specific detection to enhance sensitivity for low abundance *Eimeria* that may have been present (Hafeez et al., 2015; Imai and Barta, 2019). Mitochondrial cytochrome *c* oxidase subunit I (**mtCOI**), a conserved region of the mitochondrial genome in all *Eimeria* spp., was targeted for amplification with genus-specific primers (COI_UNI_199F — 5'-ATGATYTTCTTTGTAGTTATGCC-3'; mtRNA20_UNI_R — 5'-GTATGGATTTCACGGTCAA-3'). For nested PCR, samples were run at 94°C for 2 min, followed by 29 cycles of denaturation at 94°C for 80 s, annealing at

Table 1. *Eimeria* species-specific PCR primers.

Species	Primer Name	Sequence (5'—3')
E. adenoeides	E.ad.CO1_427F	CCAACCTCAGTAGATCTAATTGTA
	E.ad.CO1_1186R	GTGGAAGTGAGCAATGACA
E. meleagrimitis	E.mel.CO1_474F	CTCAAGTTTCCTATCCTCAG
	E.mel.CO1_1028R	GCGTACCAGATATCTAAGGAG
E. gallopavonis	E.gal.CO1_292F	AGAGTGAATTGTGTATCACTATTAT
	E.gal.CO1_1153R	GAGATAATACGAAATGGAAGTGG
E. meleagridis	E.md.CO1_431F	CCTCAGTAGATTTAATTGTC
	E.md.CO1_1443R	TTAGAAGATTAGGGAATATAA
E. dispersa	E.disp.CO1_577F	ACAGCTATTATGTTAATTGGT
	E.disp.CO1_1028R	GCATACCAAGTATCTAATGAA
E. innocua	E.inn.COI.396F	TCCATTAAGTACATCCCTG
	E.inn.COI.604R	GAAGTGTACCAATTAACATAATG

Table 2. PCR conditions used with *Eimeria* species-specific PCR primers.

Species	Initial Denaturation	Denaturation*	Annealing*	Extension*	Final Extension	Amplicon size (base pairs)
E. adenoeides	94°C	94°C	53°C	72°C	72°C	713
	2 min	45 s	45 s	45 s	10 min	
E. meleagrimitis	94°C	94°C	53°C	72°C	72°C	554
	2 min	45 s	45 s	45 s	10 min	
E. gallopavonis	95°C	95°C	60°C	72°C	72°C	861
	3 min	30 s	30 s	1 min	5 min	
E. meleagridis	95°C	95°C	55°C	72°C	72°C	1,012
	3 min	30 s	30 s	1 min 15 s	5 min	
E. dispersa	95°C	95°C	59°C	72°C	72°C	451
	3 min	30 s	30 s	1 min	5 min	
E. innocua	95°C	95°C	57°C	72°C	72°C	209
	3 min	30 s	30 s	30 s	5 min	

*For each *Eimeria* species amplified, denaturation, annealing, and extension steps were repeated for 34 cycles.

51°C for 80 s, and extension at 72°C for 80 s, and a final extension at 72°C for 10 min. Amplicons were then diluted 10-fold in sterile deionized water and used as template DNA for species-specific PCR.

Statistical Analysis

Eimeria species-specific primers used in these studies were previously confirmed by Hafeez et al. (2015) and Imai and Barta (2019). All extracted DNA samples were tested as single samples without replicates in PCR analysis. Statistical differences between species prevalence were determined using Chi-squared analysis ($\chi^2 > 3.841$). Data are represented as total number of positive PCR results and percentage of farms with positive PCR results relative to total farms tested.

RESULTS

A complete summary of farms, including flock age and resulting *Eimeria* speciation is presented in Table 3 and was used as the basis for subsequent analyses. Of the 6 species of *Eimeria* targeted by PCR across 40 farms, *E. adenoeides* and *E. meleagrimitis* were detected in 95 and 97.50% of farms, respectively, and were also the most significantly prevalent species as determined by Chi-squared analysis ($\chi^2 > 3.841$; Table 4). *E. gallopavonis* was detected in 40% of all farms tested and was significantly more prevalent ($\chi^2 > 3.841$) than *E. meleagridis, E. dispersa*, and *E. innocua* which were detected in 15, 0, and 2.50% of farms, respectively (Table 4). Of the latter three species, *E. meleagridis* was significantly more prevalent than both *E. dispersa* and *innocua* ($\chi^2 > 3.841$; Table 4). All farms tested were positive for at least one species of *Eimeria*, and most had between 2 (50% of farms) and 3 (35% of farms) total species detected. The highest cumulative species abundance totaled 4 and was observed in 10% of farms, while the lowest cumulative species abundance was one and was observed in only 5% of farms (Table 5). The 2 farms with only one species detected were positive for either an *E. adenoeides* or *E. meleagrimitis* infection, however, 92.50% of farms were positive for both *E. adenoeides* and *meleagrimitis* with or without consideration of additional species (Table 6). Due to the potential relationship between Immucox-T vaccination and prevalence of *E. adenoeides* and *E. meleagrimitis*, co-infection with these 2 species in combination with any of the other 4 species described was further examined. As presented in Table 6, of farms infected with *E. adenoeides* and *meleagrimitis*, 37.50% were also infected with *E. gallopavonis*, 12.5% with *E. meleagridis*, and 2.50% with *E. innocua*. In total, 32.50% of farm samples infected with *E. adenoeides* and *meleagrimitis* were infected with one additional species, and 10% were positive for 2 additional species.

DISCUSSION

Despite great literary redundancy regarding the economic impact of coccidiosis in the poultry industry, there is a remarkable lack of literature evaluating the distribution of *Eimeria* species considered problematic in present-day US turkeys. Furthermore, to the authors' knowledge this is one of the few reports characterizing the prevalence of *Eimeria* species across multiple field samples from commercial turkey flocks via PCR identification (Imai and Barta, 2019). In 2020, United States turkey production produced 7.32 billion pounds of turkey and was valued at $5.19 billion. Midwestern states, making up 7 of the 13 states reported, were responsible for $2.62 billion of that profit (U.S. Department of Agriculture, 2021). These initial data provide a baseline for continued study of the geographic distribution and impact of *Eimeria* species in US turkeys. As mentioned, historic characterization of *Eimeria* relied predominately on morphological diagnoses, and it was not until recently that molecular and biological characterization of turkey *Eimeria* was advanced enough to permit more detailed analysis (Cook et al., 2010; Ogedengbe et al., 2011; El-Sherry et al., 2013; Hafeez et al., 2015; Imai and Barta, 2019). Having utilized these technologies, findings presented here could grant the industry valuable insight to turkey health and supplement anticoccidial control strategies to limit monetary losses associated with coccidiosis.

Table 3. *Eimeria* species detection by farm. Farms are summarized by identification number, provided flock age at time of sample collection, and *Eimeria* species detected by PCR.

Farm ID	Flock age	MEL	AD	GALL	MD	INN	DISP	Total *Eimeria* spp. detected (per farm)
F1	28 d	+	+	-	-	-	-	2
F2	30 d	+	+	-	-	-	-	2
F3	28 d	+	-	-	-	-	-	1
F4*	17 d	+	+	+	-	-	-	3
F5*	21 d	+	+	+	-	-	-	3
F6*	20 d	+	+	+	+	-	-	4
F7*	27 d	+	+	+	-	-	-	3
F8*	35 d	+	+	-	-	-	-	2
F9*	3 wk	+	+	+	+	-	-	4
F10	25 d	+	+	-	-	-	-	2
F11	28 d	-	+	-	-	-	-	1
F12	30 d	+	+	-	-	-	-	2
F13	32 d	+	+	-	-	-	-	2
F14	28 d	+	+	-	-	-	-	2
F15	4 wk	+	+	+	-	-	-	3
F16	4 wk	+	+	+	-	-	-	3
F17	32 d	+	+	-	-	-	-	2
F18*	4 wk	+	+	+	-	-	-	3
F19*	3 wk	+	+	-	-	-	-	2
F20*	6 d	+	+	-	-	-	-	2
F21*	14 d	+	+	-	-	-	-	2
F22*	21 d	+	+	-	-	-	-	2
F23*	nd	+	+	+	-	+	-	4
F24*	36 d	+	+	+	-	-	-	3
F25*	36 d	+	+	-	-	-	-	2
F26*	14 d	+	+	+	-	-	-	3
F27*	5 wk	+	+	-	-	-	-	2
F28*	21 d	+	+	+	-	-	-	3
F29	28 d	+	+	-	-	-	-	2
F30	20 d	+	+	-	-	-	-	2
F31	28 d	+	+	-	-	-	-	2
F32	27 d	+	+	-	-	-	-	2
F33	nd	+	+	-	-	-	-	2
F34	nd	+	+	-	-	-	-	2
F35	4 wk	+	+	+	-	-	-	3
F36	4 wk	+	+	+	-	-	-	3
F37*	45 d	+	+	-	+	-	-	3
F38*	11 wk	+	+	+	+	-	-	4
F39*	9 wk	+	-	+	+	-	-	3
F40*	34 d	+	+	-	+	-	-	3
Total positive (per species)		39	38	16	6	1	0	

d, day; wk, week; nd, no age provided.

Abbreviations: AD, *Eimeria adenoeides*; DISP, *Eimeria dispersa*; GALL, *Eimeria gallopavonis*; MD, *Eimeria meleagridis*; MEL, *Eimeria meleagrimitis*; INN, *Eimeria innocua*.

*Subjected to primary PCR (mitochondrial cytochrome *c* oxidase subunit I amplification).

Based on PCR analyses, at least 4 of the 6 previously highlighted species of turkey *Eimeria* were notably prominent across the Midwest. The majority of samples amplified with PCR were positive for 2 (20/40 farms) or 3 (14/40 farms) *Eimeria* species, while a small number (4/40 farms) were positive for 4 species (Table 4). The most commonly identified of these were *E. adenoeides* and *E. meleagrimitis*, observed simultaneously in 37 of 40 farms. While no information was provided regarding anticoccidial programs in place when samples were obtained, high prevalence of these 2 species could likely be due, in part, to immunization with Immucox-T in

Table 4. Species prevalence. Total number and percentage of farms positive for any of the six *Eimeria* species after PCR analysis.

Eimeria species	Total positive farms	%Farms
E. meleagrimitis	39[a]	97.50%
E. adenoeides	38[a]	95.00%
E. gallopavonis	16[b]	40.00%
E. meleagridis	6[c]	15.00%
E. innocua	1[d]	2.50%
E. dispersa	0[d]	0.00%

n = 40 farms.

[a,b,c,d]Superscripts indicate significant differences as determined by Chi-squared ($\chi^2 > 3.841$, $P < 0.05$).

Table 5. Total species prevalence. Total number and percentage of farms positive for zero through six species of *Eimeria* following PCR detection.

Total species detected	Total positive farms	%Farms
0	0	0.00%
1	2	5.00%
2	20	50.00%
3	14	35.00%
4	4	10.00%
5	0	0.00%
6	0	0.00%

n = 40 farms.

Table 6. Multispecies detection. The most prevalent species detected by PCR were *E. adenoeides* and *E. meleagrimitis*. The number and percentage of farms positive for *E. adenoeides* and *E. meleagrimitis* alone or in combination with other species.

Combination of species detected	Total farms	%Farms
AD only	1	2.50%
MEL only	1	2.50%
AD + MEL (with or without other *spp.*)	37	92.50%
AD + MEL + GALL	15	37.50%
AD + MEL + MD	5	12.50%
AD + MEL + DISP	0	0.00%
AD + MEL + INN	1	2.50%
AD + MEL + 1 other sp.	13	32.50%
AD + MEL + 2 other spp.	4	10.00%

n = 40 farms.

Aabbreviations: AD: *Eimeria adenoeides*; DISP: *Eimeria dispersa*; GALL: *Eimeria gallopavonis*; MD: *Eimeria meleagridis*; MEL: *Eimeria meleagrimitis*; INN: *Eimeria innocua*.

some flocks which is composed specifically of these 2 species. Perhaps more important to consider, however, were the number of farms (18/40 farms) that were positive for additional species of *Eimeria* not included in Immucox-T. Nearly half of all farms (16/40 farms) tested positive for *E. gallopavonis*, which in addition to *E. adenoeides, E. meleagrimitis*, and *E. dispersa*, are considered pathogenic and economically important species associated with mortality in young poults and diminished growth performance in older birds (Chapman, 2008; Cook et al., 2010; El-Sherry et al., 2017). In an *Eimeria* distribution analysis of Canadian flocks, of 26 flocks positive for *Eimeria* following nested PCR, 50, 88, and 62% were positive for *E. adenoeides, E. meleagrimitis*, and *E. gallopavonis*, respectively, (Imai and Barta, 2019) while detection of these species in the present study were 95, 97.50, and 40%, respectively. Contrary to observations documented by Imai and Barta (2019), no *E. dispersa* was detected in any of the 40 farms tested, and prevalence of *E. meleagridis* and *E. innocua* were similarly low by comparison. These contrasting results may reflect geographic differences between Canadian and US turkey *Eimeria* ubiquity, management strategies, or be a result of limited sample size, distribution of species in the reconstituted doses, and species fecundity during propagation in the present study. An added factor that may have contributed to these discrepancies is more frequent use of primary (nested) PCR on samples from the Canadian flocks (Imai and Barta, 2019) than used here which would have increased the detection of species present at very low levels. This notion is supported by the fact that 14 of 21 samples subjected to nested PCR here were positive for additional species beyond *E. adenoeides* and *E. meleagrimitis* and represented 78% (14/18) of all samples of this nature (Table 3). Therefore, in future uses of PCR for the analysis of species distribution, all samples should be subjected to nested PCR for detection of species present at very low levels in order to produce truly accurate results. Additionally, a more well-suited PCR negative control would strengthen these results. In the current study, *Eimeria maxima*, a chicken specific *Eimeria* species was used to evaluate whether turkey *Eimeria* primers were cross-reactive with non-turkey *Eimeria* species. However, a more adequate negative control would consist of using a non-target turkey *Eimeria* species.

Species detection in this study demonstrated variable distribution and combinations of coccidial infection across Midwest flocks. In agreement with conclusions by Imai and Barta (2019), PCR results challenge the notion that Immucox-T is capable of adequately protecting flocks against pathogenic *Eimeria* infections in today's turkey production systems, especially in flocks where multiple species were detected. A recent comprehensive in vivo cross-species challenge study evaluated this quandary and reported no significant detection of adaptive cross-immunity among any of the 6 *Eimeria* species in turkeys but did observe limited nonspecific innate protection against *Eimeria* species that localize in the same region of the intestine. For instance, co-infection with *E. adenoeides* and *E. meleagridis, or E. gallopavonis*, which all localize in or around the ceca, resulted 48% reduction in oocyst shedding as compared to cross-infection with heterologous species (Imai, 2018). Based on these findings, it was concluded that under proper management conditions, Immucox-T would likely illicit a robust protective immune response against *E. adenoeides* and *E. meleagrimitis* homologous strains, but only partial and limited nonspecific protection against infection with other species. It is worth noting that no presently available coccidiosis vaccine marketed for turkey production in the United States contains all *Eimeria* species known to challenge respective hosts (Poplstein and Vrba, 2011; Imai, 2018). However, given reports of *E. gallopavonis* and *E. dispersa* pathogenicity (Wehr et al., 1962; Chapman, 2008; El-Sherry et al., 2017), and more notably, prevalence of *E. gallopavonis* detected herein, species inclusion in current commercial live coccidiosis vaccination strategies in turkey's calls for re-evaluation.

Anticoccidial control methods, whether immunization or feed additive, are prone to variable or diminished efficacy over time. Drug and even multidrug resistance are a prominent problem in flocks raised on anticoccidial compounds due to continued usage. Rotation and shuttle programs alternate medications based on mechanism of action in an attempt to control development of resistance, but based on observed multidrug resistance, these strategies delay the onset of resistance rather than prevent it (Martin et al., 1997; Chapman, 2001; Bafundo et al., 2008). In broiler production, anticoccidial compounds are also supplemented with immunization of drug sensitive *Eimeria* oocysts to mitigate drug resistance and reseed litter with sensitive coccidial strains (Chapman and Jeffers, 2014; Snyder et al., 2021b). This strategy is practical in broiler flocks as there is currently a wider selection of commercially available vaccines that include various *Eimeria* species, which as previously mentioned, is truncated in turkeys. In addition

to a commercial vaccine, US turkey producers can also implement autogenous vaccines per consultation with an attending flock veterinarian. Autogenous anticoccidial vaccines are manufactured from oocysts isolated from a particular flock or complex in order to immunize that same flock and are especially effective where *Eimeria* immunovariants, which vary geographically and may offer little cross-protection between one another, are present (Danforth, 1998; Allen and Fetterer, 2002). Further supplementing these available coccidial control methods with species identification per a given location would aid not only in development of more efficacious vaccine technologies, but also provide valuable information regarding flock health.

These preliminary results highlight successful characterization of prominent *Eimeria* species. However, a more comprehensive geographic evaluation of *Eimeria* distribution in the US is warranted, as such a small population of Midwestern flocks cannot accurately represent coccidial prevalence in turkeys across the country. The PCR approach presented here (Hafeez et al., 2015; Imai and Barta, 2019) serves as a reliable and consistent means to further examine *Eimeria* distribution in turkey flocks country-wide. This and future characterization of *Eimeria* distribution across US turkey flocks would represent a valuable asset in future evaluation and management of bird health and anticoccidial control strategies.

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DISCLOSURES

The authors declare no conflict of interest financial, personal, or otherwise.

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