Antimicrobial Residue Detection in Chicken Yolk Samples Following Administration to Egg-Producing Chickens and Effects of Residue Detection on Competitive Exclusion Culture (PREEMPT) Establishment

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Competitive exclusion (CE) cultures may offer alternatives to antimicrobial agents for disease prophylaxis in poultry. To avoid potential transfer of antibiotic resistance, safe and effective CE cultures must, by necessity, be highly sensitive to antimicrobial residues. The following studies evaluated the effect of maternal administration of selected antibiotics on the establishment of a licensed CE culture, PREEMPT. Selected antibiotics were administered to actively laying hens for a period of 7 days (experiment 1) or 9 days (experiment 2) in drinking water [sulfadimethoxine (0.05%), enrofloxacin (0.005%), and tylosin tartrate (0.05%)] or feed (sulfadimethoxine with ormetoprim, 250 ppm). In experiment 1, fertile eggs were collected daily and subjected to bioassay for detectable antimicrobial residues in yolk. Antimicrobial residues were not detected during the 7 days of treatment or the subsequent 3 days following cessation of treatment in the control, sulfadimethoxine, sulfadimethoxine with ormetoprim, or tylosin treatment groups. However, detectable residues were observed in eggs derived from enrofloxacin-treated hens on days 6 and 7 during antibiotic administration and also on days 2 and 3 post-antibiotic administration. In experiment 2, antimicrobial residues were also only detected in yolks from hens treated with enrofloxacin. Residue detection occurred on days 2−6 of antibiotic administration, on day 9 of antibiotic administration, on days 1−3 post-antibiotic administration, and also on day 7 post-antibiotic administration. A subset of eggs from each experimental group, corresponding to days 2−6 of antibiotic administration, days 4−6 post-antibiotic administration, and days 14−16 post-antibiotic administration, were pooled for incubation, and chicks hatched from these pools of fertile eggs were treated with PREEMPT at hatch. When 48-h cecal propionate concentrations were used as an index of culture establishment, reduced ($P < 0.05$) efficacy was observed only in chicks derived from enrofloxacin-treated hens at either collection period. Although several antibiotics do not appear to produce detectable egg residues or interfere with CE culture establishment, these data suggest that chicks derived from enrofloxacin-treated hens may not be candidates for safe and effective CE culture treatment.

Keywords: *Chicken; competitive exclusion; chicks; propionate; antibiotics*

INTRODUCTION

The administration of competitive exclusion (CE) cultures to neonatal poultry as a means of controlling infection or colonization of the gastrointestinal (GI) tract by bacterial food-borne pathogens, including *Salmonella* and *Campylobacter*, is currently experiencing increased acceptance by the commercial poultry industry. Reasons for increasing CE culture application to neonatal poultry stem from two primary areas: increased restrictions on the application and use of antibiotics in poultry and increased regulatory pressure on the microbial quality of poultry meat, actions that are now mandated and enforced by federal regulatory agencies. For poultry producers to comply with these current standards, it will be necessary to adopt management practices that will ultimately lead to an overall reduction of the food-borne pathogen load on processed poultry meat. The application of CE cultures to neonatal poultry in hatcheries may prove to be one efficacious option for producers.

The concept of CE was first described by Nurmi and co-workers (Nurmi and Rantala, 1973). Since this original study, several laboratories have developed and used CE cultures to reduce enteric pathogen colonization or infection in commercial-type poultry (Loyd et al., 1977; Snoeyenbos et al., 1978; Barnes et al., 1980; Pivnick et al., 1981). Recently, one CE culture originally isolated from the cecal microflora of healthy adult chickens, PREEMPT, received U.S. Food and Drug Administration approval for distribution and use in the United States to reduce *Salmonella* intestinal colonization in commercial meat-type or egg-laying chicken

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flocks. PREEMPT is a defined CE product that is produced using continuous flow fermentation methodologies. Previous analyses of the culture composition have identified 29 different obligate and facultative anaerobic bacteria (Nisbet et al., 1995). The administration of PREEMPT to neonatal chickens on day-of-hatch has been associated with reductions in cecal colonization or organ invasion following experimental or natural *Salmonella* challenge under both laboratory and field conditions (Corrier et al., 1994, 1995a,b, 1998).

Although a great deal of research has been conducted to develop and test the efficacy of CE cultures, little work has been conducted to evaluate the parameters of culture establishment in the gut of neonatal poultry. Environmental conditions such as temperature and photointensitiy have recently been evaluated by our laboratory for effects on CE culture establishment (Caldwell et al., 1999a,b). Antibiotic administration and the presence of antibiotic residues in the yolk material in neonatal poultry represent an additional factor that could have tremendous impacts on CE culture associated bacterial establishment. Research conducted by Humbert et al. (1991) suggests that certain drugs, even when administered at relatively low concentrations, have antagonistic effects on CE culture establishment. Other laboratories have reported on the incorporation of antibiotic residues in eggs, especially within the yolk material, as residues appear to be concentrated in yolk material during the process of egg formation (Roudaut et al., 1990; Yoshimura et al., 1991; Omija et al., 1994; Donoghue et al., 1996). Yolk material during embryogenesis and early neonatal life is deposited into the midileum of the GI tract, a site just cranial to the distal ileum and cecum where CE cultures establish, so it is conceivable that antibiotic residues present in yolk material could interfere with CE culture associated bacterial establishment. The purpose of the present investigation was to evaluate the effects of the administration of selected antibiotics routinely used by the commercial poultry industry to actively laying breeder hens and measure the effects that such administration may have on the ability of CE cultures to establish in the gut of their progeny.

EXPERIMENTAL PROCEDURES

Maternal Antibiotic Administration. Single-comb White Leghorn (SWCL) hens at ~30 weeks of age were digitally palpated for optimal reproductive activity and placed in natural breeding floor pens with pine shavings under a lighting schedule that consisted of 18 h of light and 6 h of dark daily. Feed that met or exceeded NRC requirements for poultry and water were provided ad libitum for the duration of the experiment. Approximately 30 reproductively active SCWL hens and 5 reproductively active SCWL males were placed in each of five independent but similar floor pens pen for the present study.

Several antibiotics that are commonly administered to breeding flocks in the United States were evaluated for their ability to produce detectable residues in egg yolk samples and to interfere with PREEMPT establishment in day-of-hatch chicks. In the present study, hens in independent pens were administered either enrofloxacin (0.005%), tylosin tartrate (0.053%), or sulfadimethoxine (0.05%). All concentrations represent final concentrations in the drinking water provided to hens during the time period of antibiotic administration. Additionally, sulfadimethoxine with ormetoprim was administered to a separate experimental group at a final concentration of 250 ppm in the feed. A control pen that did not receive antibiotics in either the drinking water or feed was also included in the present study. The duration of antibiotic administration was limited to either 7 or 10 days in experiments 1 and 2, respectively. Eggs from individual pens containing independent experimental groups were collected daily for bioassay for antibiotic residue detection or incubation for hatching progeny.

CE Culture Administration. PREEMPT is a CE culture originally isolated from the cecal microflora of healthy adult chickens. This defined CE product is produced using continuous flow fermentation methodologies. Previous analyses of the culture composition have identified 29 strains of obligate and facultative anaerobic bacteria (Nisbet et al., 1995). Unless stated in the experimental design (please see below), a single manufacturer's recommended dose of PREEMPT was administered to all chicks in experiment 2 on day-of-hatch by oral gavage.

Antibiotic Residue Detection. *Klebsiella pneumoniae* (ATCC 10031) and *Bacillus cereus* (ATCC 11778), which are strains of aerobic bacteria known for sensitivity to several antibiotics used by the poultry industry, were selected to screen antibiotic activity in neonatal yolk sacs and blood serum samples. Briefly, *K. pneumoniae* or *B. cereus* was grown in tryptic soy broth (TSB) at 37 °C for 15 h. Bacteria were concentrated by centrifugation and washed once in sterile H_2O by centrifugation (1900*g* for 15 min). Following removal of wash supernatant, 1 mL of sterile H_2O was added and mixed to produce a bacterial suspension. This bacterial suspension was sequentially added to 3 mL of sterile H_2O to achieve an optical density of 0.6 at a wavelength of 625 nm in a spectrophotometer. This suspension was further diluted 1000-fold and added to antibiotic medium 1 agar (Difco) at a ratio of 1 part bacterial suspension to 9 parts agar at selected temperatures (*K. pneumoniae* at ≤58 °C and *B. cereus* at ≤55 °C). Following solidification of plates (22 °C), stainless steel penicylinders were placed upon the agar, creating a firm seal. Neonatal yolk sac and serum samples were diluted 1:1 with sterile water for placement in the penicylinders (200 μL/cylinder). Plates were incubated at room temperature (22 °C) for 3 h and then incubated at 37 °C for selected times (*K. pneumoniae*, 15 h; and *B. cereus*, 7 h). Zones of inhibition of bacterial growth were measured using calipers, and data were recorded following removal of plates from the incubator.

Analysis of Cecal Contents for VFA Establishment. The presence of a selected profile of volatile fatty acids in the chick cecum following PREEMPT administration has been associated with protection against experimental and natural *Salmonella* challenge (Corrier et al., 1998). In these investigations, PREEMPT establishment is associated with elevated levels of propionate in chick cecal contents. In the present study, to evaluate the effects of selected antibiotic administration on PREEMPT establishment, 20 chicks in each treatment group were sacrificed by cervical dislocation and ~0.2 g of cecal contents from each chick was added to a polypropylene tube containing 1.8 mL of reagent grade, deoinized (d) H_2O. Propionic acid concentration of cecal contents present in each tube was determined by gas−liquid chromatography as previously described (Barnes et al., 1980).

Experimental Design. Egg collection for detection of antibiotic residues in eggs laid by hens in all experimental groups in individual pens was performed daily. From the entire daily egg production from each of the five pens, five eggs (or ~20% of daily production) were removed for antibiotic residue bioassay and the remaining eggs were held at 13 °C until incubation was initiated. Bioassay for antibiotic residue detection began prior to the first day of antibiotic administration, continued daily throughout both experiments, and was terminated 10−14 days following the final day of antibiotic administration.

Eggs chosen for incubation from independent experimental groups in experiment 2 were based on detection of antibiotic residues and the subsequent disappearance of residues during the withdrawal period following antibiotic administration. Eggs from independent experimental groups on days when antibiotic residues were detected on consecutive days were pooled and incubated. Additionally, beginning on the second

Table 1. Experiment 1: Detection of Antibiotic Residues in the Yolk Material of Eggs Collected from Laying Hens Administered Enrofloxacin for 7 Days in Drinking Water[a]

day of antibiotic administration	control	enrofloxacin
0	0/12	0/12
1	0/12	0/12
2	0/12	0/12
3	0/12	0/12
4	0/12	0/12
5	0/12	0/12
6	0/12	4/12 (11 mm)
7	0/12	7/11 (11 mm)
+1	0/12	0/12
+2	0/12	3/14 (10 mm)
+3	0/12	2/12 (10 mm)
+4	0/12	0/12

[a] Other antibiotics administered maternally in experiment 1 did not result in detectable levels of antibiotic residues in egg yolk samples. Data represent number of positive residue detections per total number of eggs sampled in control or enrofloxacin administered groups of laying hens. Data in parentheses represent mean diameters of zones of inhibition in yolk samples in which antibiotic residues were detected.

day of the disappearance of detectable antibiotic residues, eggs from consecutive days were pooled and incubated. Eggs from the control group of hens from identical days of experiment 2 were similarly pooled and incubated. On day-of-hatch, all chicks from individual experimental groups received a single dose of PREEMPT by oral gavage immediately after being removed from the hatching cabinet. Chicks were then placed in separate floor rearing pens on clean pine shaving litter material and were reared for a total of 48 h. At 48 h post PREEMPT administration, cecal contents from 20 chicks in each treatment group were collected and individually evaluated for propionate concentration as described above.

Statistical Analysis. Propionate values were analyzed using the General Linear Model procedure for analysis of variance (SAS Institute, 1988). Statistically different means ($P < 0.05$) were further separated using Duncan's multiple-range test (SAS Institute, 1988).

RESULTS AND DISCUSSION

During both experiments 1 and 2, for which selected antibiotics routinely administered to commercial breeding or laying flocks were evaluated for the ability to produce detectable antibiotic residues in yolk sac samples, only enrofloxacin administration at 0.005% in drinking water was associated with antibiotic residue detection in the yolk material of collected eggs (Tables 1 and 2). In experiment 1, residues were detected in the yolk material of collected eggs in the enrofloxacin-administered group on days 6 and 7 of antibiotic administration and also on days 2 and 3 post-antibiotic administration. Detectable antibiotic residues in other experimental groups were not observed.

During experiment 2, detection of antibiotic residues associated with enrofloxacin administration were identified on days 2−6 of antibiotic administration, day 9 of antibiotic administration, days 1−3 post-antibiotic administration, and also day 7 post-antibiotic administration. Similar to experiment 1, detectable antibiotic residues in other experimental groups were not observed in experiment 2. When the progeny from individual experimental groups were administered PREEMPT on day-of-hatch, only enrofloxacin administration was associated with depressed ($P < 0.05$) mean cecal propionate concentrations (Table 3). Interestingly, this enrofloxacin administration-associated depression in cecal

Table 2. Experiment 2: Detection of Antibiotic Residues in the Yolk Material of Eggs Collected from Laying Hens Administered Enrofloxacin for 9 Days in Drinking Water[a]

day of antibiotic administration	control	enrofloxacin
0	0/5	0/5
1	0/5	0/5
2	0/5	5/5 (22 mm)
3	0/5	5/5 (22 mm)
4	0/5	5/5 (28 mm)
5	0/5	5/5 (18 mm)
6	0/5	4/4 (25 mm)
7	0/5	0/5
8	0/5	0/5
9	0/5	5/5 (22 mm)
+1	0/5	5/5 (22 mm)
+2	0/5	5/5 (29 mm)
+3	0/5	5/5 (20 mm)
+4	0/5	0/5
+5	0/5	0/5
+6	0/5	0/5
+7	0/5	5/5 (9 mm)

[a] Other antibiotics administered maternally in experiment 2 did not result in detectable levels of antibiotic residues in egg yolk samples. Data represent number of positive residue detections per total number of eggs sampled in control or enrofloxacin administered groups of laying hens. Data in parentheses represent mean diameters of zones of inhibition in yolk samples in which antibiotic residues were detected.

Table 3. Effect of Maternal Antibiotic Administration on Cecal Propionate Levels in Progeny Administered PREEMPT on Day-of-Hatch in Experiment 2[a]

exptl group	days 2−6 of ABTX admin	days 4−6 post ABTX admin	days 14−16 post ABTX admin
control	8.65 ± 0.56^{B}	7.17 ± 0.76^{AB}	13.81 ± 1.54^{A}
enrofloxacin	4.02 ± 0.34^{C}	4.76 ± 0.55^{C}	12.07 ± 1.07^{A}
tylosin tartrate	11.52 ± 1.17^{A}	8.93 ± 1.14^{A}	NT[b]
sulfadimethoxine	9.26 ± 0.53^{B}	NT	NT
sulfadimethoxine + ormetoprim	8.94 ± 0.77^{B}	$6.57 \pm 0.60B^{C}$	NT

[a] Data represent mean ± standard error of propionate values (μmol/g) in cecal contents in each experimental group. Means with no common superscript are statistically different ($P < 0.05$). [b] NT, not tested in the present experiment.

propionate levels ($P < 0.05$) following PREEMPT administration to progeny was evident even when antibiotic residues were not detected in egg yolk samples. When evaluated in an extended withdrawal time period, cecal propionate levels in progeny from hens administered enrofloxacin were indistinguishable ($P > 0.05$) from controls ~10 days following the final day of antibiotic residue detection. Although other antibiotics evaluated in the present investigation were not associated with depressions in cecal propionate levels in progeny that were administered PREEMPT, the administration of tylosin tartrate to laying hens in the present study was actually associated with an elevation of cecal propionate levels in progeny. A major focus of our laboratories at present is to identify methods of improving CE culture establishment and efficacy in neonatal poultry. The selective use of antibiotics or other compounds to improve the mechanisms of CE culture establishment is presently being investigated. Although the present findings suggest that tylosin tartrate may be a good candidate for such selective use, additional investigation of the present observations is needed.

Data from the present investigation suggest that when several antibiotics commonly used for treatment

of bacterial disease conditions in commercial poultry flocks were evaluated, only the maternal administration of one of the four antibiotics evaluated, enrofloxacin, was associated with detectable antibiotic residues in yolk samples. Similarly, only the maternal administration of enrofloxacin was associated with negative parameters of CE culture establishment in day-of-hatch chicks. Interestingly, maternal enrofloxacin administration was associated with depressed cecal propionate levels in progeny chicks receiving PREEMPT administration even when residues were present in concentrations too low to be detected by the residue detection bioassay used in the present investigation. Cecal propionate levels in progeny hatched from eggs laid by hens administered enrofloxacin became indistinguishable from controls after approximately 10 days following the final day of antibiotic residue detection. Collectively, these data suggest that CE cultures may be exquisitely sensitive to certain antibiotics currently used in commercial poultry flocks and that careful consideration should be given to choosing individual antibiotics for use in commercial table egg or breeding flocks, if CE culture use is being considered.

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