Development of a Rapid and Inexpensive Assay for the Nonspecific Detection of Antimicrobial Residues in Chicken Egg Yolks and Neonatal Yolk Sacs

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Competitive exclusion of intestinal pathogens by administration of beneficial and defined cultures of normal intestinal microflora is a safe and effective means of reducing the incidence and severity of chick infections with *Salmonella* and other intestinal pathogens. It is important that competitive exclusion cultures not carry genetic material (e.g., plasmids), which could transfer antibiotic resistance to other microflora, including pathogens. As such, safe and effective competitive exclusion cultures must be sensitive to commonly used antimicrobial agents. By necessity, intentional or accidental exposure of these beneficial microflora to antibiotics will reduce or eliminate the protection provided by competitive exclusion culture establishment. As antibiotic residues can be present from embryonic, hatchling, or maternal administration, a rapid and sensitive assay for the nonspecific detection of residues, which could interfere with competitive exclusion culture establishment, is needed. This study was conducted to develop a rapid and inexpensive bioassay to detect multiple antimicrobial residues in egg yolk and neonatal yolk sacs. Aerobic bacterial strains with known sensitivity to several antibiotics used by the poultry industry were selected and individually compared for sensitivity to enrofloxacin, gentamicin, tetracycline, ceftiofur, and tylosin concentrations in egg yolks. This assay was found to be relatively sensitive for the detection of these antimicrobials, and detection of residues was associated with reduced competitive exclusion culture (PREEMPT) establishment in one experiment. Importantly, this assay can be implemented with minimal training or equipment under commercial hatchery practices and could be used to determine embryo groups, in advance of hatch, that are not suitable candidates for competitive exclusion treatment in the hatchery.

Keywords: *Antibiotic assay; antimicrobial residues; competitive exclusion; yolk*

INTRODUCTION

Since the concept of competitive exclusion (CE) was originally described by Nurmi et al. (1973), 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 flocks. PREEMPT is a defined CE product that 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., 1996). Under both laboratory and field conditions, 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 (Corrier et al., 1994, 1995a,b, 1998).

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Although a great deal of research has been conducted to develop and test the efficacy of CE cultures, very little experimentation has been initialized to evaluate the parameters of culture establishment in the gut of neonatal chicks. Environmental conditions such as temperature and photointensity have recently been evaluated by our laboratory for effects on CE culture establishment (Caldwell et al., 1999a–c). One factor that could have a tremendous impact on CE culture establishment could be the presence or absence of antibiotic residues in neonatal chicks that result from antibiotic administration by either in ovo, day-of-hatch, or maternal routes of administration. Humbert et al. (1991) have suggested that certain drugs, even when administered at relatively low concentrations, could have antagonistic effects on CE culture establishment. Other investigators (Bolder et al., 1995) suggest that this interference is limited to certain antibiotics and that all antibiotics do not interfere with CE culture establishment. Several laboratories have reported the incorporation of antibiotic residues in eggs, especially within the yolk material, as residues of some drugs 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). Because yolk material during embryogenesis and early neonatal life is deposited into the mid-ileum of the gastrointestinal tract, a site just cranial to the distal

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ileum and cecum where CE cultures are believed to establish, it is conceivable that antibiotic residues present in yolk material could interfere with CE culture associated bacterial establishment. The purpose of this experiment was to develop a rapid and inexpensive bioassay to detect multiple antimicrobial residues in egg yolk and neonatal yolk sacs that could be predictive of groups of chicks that are not candidates for competitive exclusion treatment at the hatchery.

EXPERIMENTAL PROCEDURES

Plate and Sample Preparation. *Klebsiella pneumoniae* (ATCC 10031), *Bacillus cereus* (ATCC 11778), and *Staphylococcus aureus* (ATCC 25923), which are aerobic bacteria strains known for sensitivity to several antibiotics used by the poultry industry, were selected to screen antibiotic activity in egg yolks and neonatal yolk sacs. Briefly, *Klebsiella*, *Bacillus*, or *Staphylococcus* 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 the wash supernate, 1 mL of sterile H_2O was added and mixed to produce a bacterial suspension. This bacterial suspension was titrated to 3 mL of sterile H_2O to achieve an optical density of 0.6 at 626 nm. 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, *S. aureus* 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. Egg yolk 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* and *S. aureus*, 15 h; *B. cereus*, 7 h). Zones of bacterial growth of inhibition were measured using calipers, and data were recorded following removal of plates from the incubator.

Dose Titration for Bacterial Growth Inhibition. A dose titration curve for bacterial growth inhibition was established for five antibiotics commonly used in poultry breeding programs (enrofloxacin, gentamicin, tetracycline, ceftiofur, and tylosin), for each of the indicator bacteria, by spiking egg yolks from untreated laying hens with known concentrations of antibiotics. Each concentration of each antibiotic was independently assayed five times. Serial dilutions were made using a solution emulsion (1 part yolk and 1 part 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* and *S. aureus*, 15 h; *B. cereus*, 7 h). Zones of bacterial growth of inhibition were measured using calipers, and data were recorded following removal of plates from the incubator. Mean zones of inhibition for each bacterial isolate and each antibiotic are reported in Figures 1−5.

Simultaneous Assay for Residues and Evaluation of PREEMPT Efficacy. Commercial day-of-hatch broilers with unknown maternal antibiotic history were obtained from three different commercial hatcheries (sources A−C) and delivered to Texas A&M University's poultry farm. All birds were allowed time to recover from traveling (2 h) before PREEMPT administration by gavage (0.25 mL containing 1 dose as per the manufacturer's directions) to each of 30 chicks in all groups. Also, on the day of hatch, a subset of 20 chicks from each source was killed by cervical dislocation and yolk sacs were dissected and subjected to the antibiotic residue detection assay described above. Within 3 h of treatment, each treatment group was placed in an individual pen with clean pine shavings and ad libitum provision of feed, formulated to meet or exceed NRC recommendations (National Research Council, 1984) and water. Chicks were maintained at an appropriate rearing temperature with provision of supplemental brooder lamps for the duration of the 48 h of the experiment. Forty-eight hours after chick placement, chicks were killed by groups, and the ceca were immediately removed by dissection for collection of cecal material. Approximately 15 chicks were randomly selected for cecal content collection. For each sample, 0.17−0.22 g of cecal material was collected in 12 × 75 mm polypropylene tubes containing 1.8 mL of dH_2O. Samples were immediately snap frozen on dry ice and maintained at −70 °C for transport and until assay for propionate content. Propionate was determined by gas chromatography, using previously published methods (Nisbet et al., 1996), and propionate concentrations were adjusted to a per gram basis based on collected weights of cecal content. The results of this experiment are illustrated in Table 1.

Table 1. Evaluation of Antimicrobial Residues in Neonatal Yolk Sacs from Three Different Commercial Hatcheries versus 48-h Cecal Propionate Concentrations

source	propionate[a] (μmol/g)	*K. pneumoniae* zone of inhibition (mm)	*B. cereus* zone of inhibition (mm)
A	12.237[a]	0	0
B	3.953[b]	12.8	2.9
C	4.788[b]	11.3	5.8

[a] Means within a column with no common superscript differ significantly ($P < 0.05$).

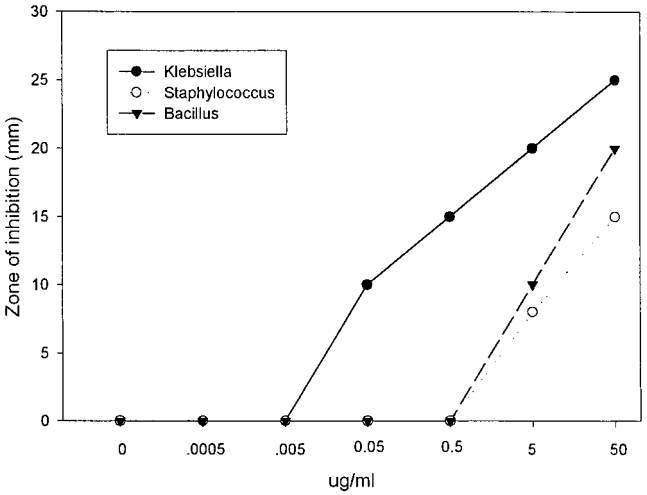

Figure 1. Detection of enrofloxacin in artificially contaminated egg yolk samples by three different bacteria in a bacterial growth inhibition assay.

Statistical Analysis. Mean propionate data and measured zones of bacterial growth inhibition from treatment groups in experiment 2 were analyzed using analysis of variance (SAS Insitute, 1987). Significant differences ($P \leq 0.05$) were further separated using Duncan's multiple-range test (Duncan, 1955).

RESULTS AND DISCUSSION

When enrofloxacin was added to egg yolk material and subjected to residue bioassay using *K. pneumoniae*, *S. aureus*, or *B. cereus*, *Klebsiella* growth was inhibited 100-fold less by enrofloxacin (≤50 ng/mL) than either the *Staphylococcus* (≤5 μg/mL) or *Bacillus* (≤5 μg/mL) (Figure 1). Gentamicin was detected at concentrations of ≤200 μg/mL by the *Klebsiella* or the *Staphylococcus*, with either of these organisms apparently detecting 10-fold lower concentrations than the *Bacillus* (≤2000 μg/mL; Figure 2). Growth of each of these three bacteria was inhibited by ≤5 μg/mL of tetracycline (Figure 3). Ceftiofur was detected by *Klebsiella* at concentrations of ≤200 ng/mL, or 10-fold lower concentrations than the *Staphylococcus* (≤2 μg/mL) or 100-fold lower concentrations than the *Bacillus* (≤20 μg/mL; Figure 4). Thus, for these antibiotics, enrofloxacin, gentamicin, tetracycline, or ceftiofur, the *Klebsiella* we evaluated was the

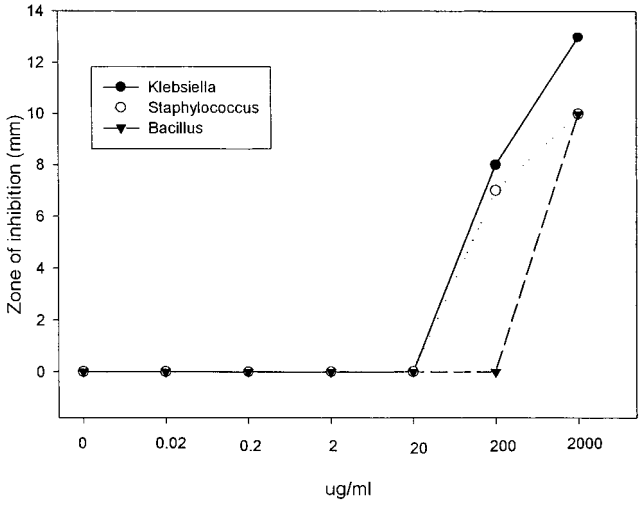

Figure 2. Detection of gentamicin in artificially contaminated egg yolk samples by three different bacteria in a bacterial growth inhibition assay.

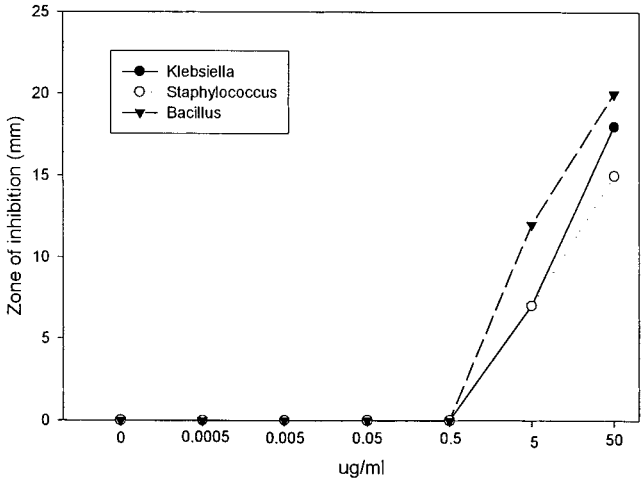

Figure 3. Detection of tetracycline in artificially contaminated egg yolk samples by three different bacteria in a bacterial growth inhibition assay.

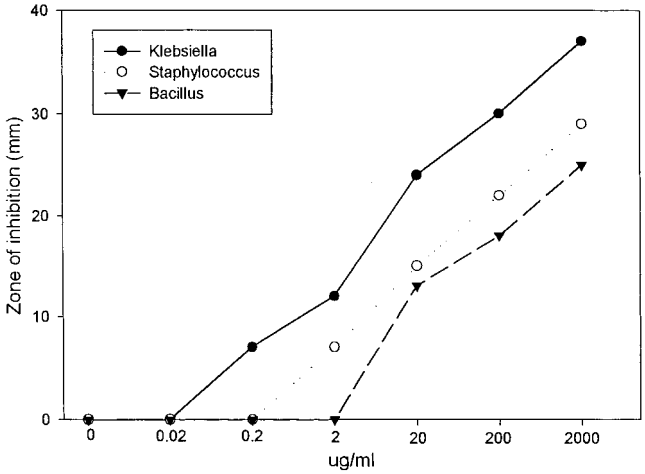

Figure 4. Detection of ceftiofur in artificially contaminated egg yolk samples by three different bacteria in a bacterial growth inhibition assay.

most sensitive organism for detection in this assay. However, the *Bacillus* was more sensitive, 10-fold lower, to concentration of tylosin (≤20 μg/mL) than either the *K. pneumoniae* (≤200 μg/mL) or the *Staphylococcus*

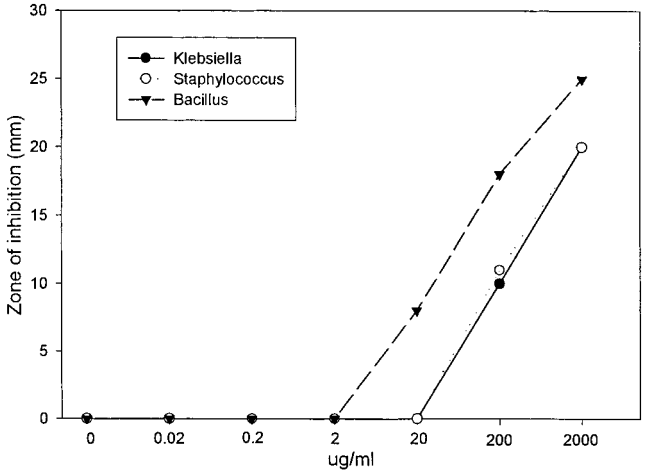

Figure 5. Detection of tylosin in artificially contaminated egg yolk samples by three different bacteria in a bacterial growth inhibition assay.

(≤200 μg/mL; Figure 5). These preliminary data suggest that this simple bacterial growth inhibition assay, based on the diffusion of antibiotic residues into the bacteria-seeded agar, can effectively measure residue levels as described. Furthermore, for the five antibiotics evaluated, in no case did the *Staphylococcus* used in these experiments provide a more sensitive indication to the presence of antibiotics in the egg yolk.

To preliminarily evaluate the possibility that this bioassay might detect antibiotic residues in the yolk sacs of commercial broilers and to determine if these residues were inversely associated with 48-h cecal propionate concentrations, a known indicator of PREEMPT establishment (Nisbet et al., 1996), an experiment testing the assay for residues and evaluation of PREEMPT efficacy was implemented. In this experiment, egg yolk sac samples from a subset of chicks obtained from three separate hatchery sources, on the day-of-hatch, were subjected to either the *Klebsiella* or the *Bacillus* bioassay, as described above. Another subset of chicks, from each hatchery source, was treated with PREEMPT, as described above. Cecal propionate concentrations from samples obtained 48 h after PREEMPT indicated strong hatchery source-related differences in concentrations of propionate (Table 1). Whereas chicks from hatchery source A developed mean cecal propionate concentrations of 12.2 μmol/g, equivalent to levels associated with protection (Nisbet et al., 1996), chicks from either source B or source C developed significantly ($P < 0.05$) lower levels of propionate. Interestingly, no antibiotic residues were detected in yolk sac samples from hatchery source A, whereas strong indications of antimicrobial residues were detected in similar samples obtained from source B and source C chicks.

Although these data are only preliminary, they do suggest that actual occurring antimicrobial residues can be detected by bacterial growth inhibition assay and that detection of antimicrobial residues may indicate chicks in which PREEMPT may not perform at optimal levels. As most of the beneficial microflora in PREEMPT are sensitive to many commonly used antibiotics, it is perhaps not surprising that antimicrobial agents may interfere with the establishment of this, or any other, competitive exclusion culture that does not carry resistance factors to antibiotics.

Because therapeutic antimicrobial treatments of breeder hens are sometimes required, it is also antici-

pated that newly hatched chicks will carry some low levels of antimicrobial residues. These concentrations, which are far too low to be of any potential concern for the product after weeks of metabolism, may occasionally interfere with competitive exclusion culture establishment when chicks are treated at the hatchery. The presently described bioassay is very simple to use, is quite inexpensive, and does not require any substantial equipment investment at the hatchery. Importantly, this assay may be performed on a sample of yolk sacs obtained from 18-day embryos at the time of transfer from incubators to hatching cabinets at the hatchery. With results obtained substantially in advance of hatch, implementation of this assay may predict some hatches that are not candidates for competitive exclusion culture. This information could save substantial money on potentially wasted competitive exclusion culture costs and may improve our understanding of the value of competitive exclusion for protection of young chicks from *Salmonella* and other enteric pathogens. Further evaluations, under commercial conditions, will help to determine how useful this assay will be and if there is indeed a direct correlation of competitive exclusion efficacy and an absence of detectable antimicrobial residues.

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