In vitro Selection of Enteric Microflora for Potential Use as a Competitive Exclusion Culture Against Campylobacter in Poultry†

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Abstract: The administration of nonpathogenic microflora in neonatal poultry has been employed to reduce or eliminate the colonization of enteric pathogens. This concept, also called Competitive Exclusion (CE), although effective against Salmonella, has not consistently worked against Campylobacter. Most CE cultures are developed by randomly collecting enteric bacteria without any preselection criteria for bacteria. It may be possible to enhance the efficacy of a CE against Campylobacter by preselecting enteric microflora with the ability to inhibit Campylobacter, in vitro. With this goal, an assay was developed to test individual isolates with the ability to reduce or eliminate Campylobacter growth, in vitro. Individual isolates (n = 137) were collected from ceca of both juvenile and adult poultry and tested for efficacy against Campylobacter. Isolates were serially diluted (10⁰ or 10⁵ CFU/well) and added to 96 well polystyrene plates containing 1 x 10⁴ CFU C. jejuni or C. coli/well. Plates were incubated at 42°C in a microaerophilic environment for 24-48 h. Following incubation, a 1 µl loop from each well was streaked onto Campy-Cefex agar plate and incubated at 42°C in a microaerophilic environment for 24-48 h. Twenty-three isolates were identified with the ability to inhibit C. jejuni or C. coli growth in vitro. This research demonstrates that it is possible to pre-screen enteric isolates for Campylobacter inhibition for use as competitive exclusion cultures.

Key words: Campylobacter, enteric microflora, competitive exclusion, foodborne pathogens

INTRODUCTION

Campylobacter and Salmonella are the most commonly reported pathogens causing food borne infections in the United States (CDC, 2011). An estimated 2.1 to 2.4 million cases are reported annually (Altekruse et al., 1999; CDC, 2010). High proportions of retail chicken and turkey product are contaminated with Campylobacter and Salmonella (Norkrans and Svedhem, 1982; Genigeorgis et al., 1986, Stern and Line, 1992; Zhao et al., 2001; Fratamico, 2003) and epidemiological evidence has implicated raw poultry products as a significant source of human infection (Blaser, 1997; Friedman et al., 2004).

One approach to reducing pathogen contamination in poultry is to administer Competitive Exclusion (CE) cultures as a preharvest preventative measure. Competitive exclusion can broadly be defined as the oral administration of non-pathogenic intestinal bacteria which are able to establish and eventually colonize the digestive tract and maintain or increase the natural flora to prevent or reduce colonization of pathogenic organisms (Fuller, 1989; Vanbelle et al., 1990; Griggs and Jacob, 2005). First described by Nurmi and Rantala (1973), CE has been an effective method of control for salmonellosis in commercial poultry flocks. Since this initial report, numerous researchers have described the beneficial effects of CE against Salmonella (Snoeyenbos et al., 1978; Rigby and Pettit, 1980; Impey et al., 1982, 1984; Stavric et al., 1987; Schneitz et al., 1990; Cox et al., 1991; Schneitz and Nuotio, 1992; Bailey, 1993; Bielke et al., 2003; Nisbet et al., 1993, 1994, 1996; Corrier et al., 1994, 1995a,b; Hume et al., 1996a,b; Methner et al., 1997; Mead, 2000; Stern et al., 2001; Schneitz, 2005; Wolfenden et al., 2007; Zhang et al., 2007a; Mountzouris et al., 2009; Higgins et al., 2010).

Although selection of bacteria for CE cultures against Salmonella are effective in poultry, efforts to develop consistent CE cultures against Campylobacter, have produced limited or inconsistent success (Shanker et al., 1990; Stern et al., 2001; Mead, 2002). In an effort to overcome these problems, pre-screening enteric bacteria for the ability to inhibit Campylobacter, may enhance the efficacy of competitive exclusion products. Therefore, the purpose of this study was to develop an in vitro screening method capable of identifying bacterial candidates against Campylobacter.

MATERIALS AND METHODS

Collection of cecal isolates: Cecal samples from 6 to 74 wk of age chickens (n = 46) were collected aseptically...
at the University of Arkansas poultry farm. The cecal contents were squeezed into sterile tubes and diluted with Butterfield’s Phosphate Diluent (BD - Difco, Becton Dickinson, MD) in three ten-fold dilutions. One hundred microliters of each solution was spread on Blood Agar Plates (BAP - Difco, Becton Dickinson, MD) and the plates were aerobically incubated for 24 h at 37°C. Isolated colonies were picked and streaked onto Tryptic Soy Agar (TSA; EMD, NJ) to assure purity and incubated aerobically at 37°C for 24 h. Single, isolated colonies were individually grown in 5 mL of Tryptic Soy Broth (TSB - Difco, Becton Dickinson, MD) for 8 h or until turbid. Stocks of those bacteria were prepared by centrifuging TSB at 3000 rpm for 10 min, pouring off supernatant and resuspending in 2 mL TSB with 20% filter-sterilized glycerol, the suspensions were dispensed into 1 mL aliquots and stored at -80°C.

**Screening for in vitro efficacy against Campylobacter:**

American Type Culture Collection (ATCC) 33291 Campylobacter jejuni and a wild type poultry Campylobacter coli strain were used in separate experiments. An aliquot of frozen Campylobacter culture was taken up by sterile loop and inoculated into 5 ml Campylobacter Enrichment Medium (CEM) without antibiotics and incubated for 24-48 h at 42°C in a micro-aerophilic environment (5% O₂, 10% CO₂ and 85% N₂) and pipetted into 96 well plates. For the bacterial challenge, a total of 137 cecal isolates were tested for their ability to inhibit Campylobacter, in vitro. A frozen culture of each isolate was thawed, picked up with a sterile plastic loop (10 µL Bac-Loops®) and inoculated into 10 mL CEM without antibiotics and allowed to grow for 12 h at 37°C. Isolates exhibiting turbidity were diluted with CEM and diluted to a final concentration of 10⁵ or 10⁶ CFU/mL and dispensed in 96 well NUNC™ brand flat bottomed polystyrene plates. The treatments included: 1) Campylobacter alone (positive control), 2) cecal bacterial isolates alone (negative control) or 3) co-incubation with Campylobacter and individual cecal isolates. The final concentration in each well was 10⁵ CFU/mL Campylobacter and either 10⁴ or 10⁵ CFU/mL of each enteric isolate. The 96 well plates were incubated in micro-aerophilic condition at 42°C for 24-48 h. Following incubation, the contents of the individual challenge or control were streaked onto Campy-Cefox plates with 1 µl Bac-Loops® for comparison. The plates were incubated in a micro-aerophilic environment (5% O₂, 10% CO₂ and 85% N₂) at 42°C and were read after 24-48 h. This allowed a subjective assessment of Campylobacter survival following co-incubation with the cecal bacteria. The Campylobacter positive control plates were defined as 0% inhibition. Campylobacter inhibition following cecal bacterial challenges were placed into 5 categories. Those being, 0%, 10-24%, 25-49%, 50-74% or 75-100% inhibition of Campylobacter growth.

**RESULTS**

**Development of in vitro screening procedure for cecal microflora efficacy against Campylobacter:** One hundred and thirty-seven isolates were collected and tested for their ability to inhibit the in vitro growth of Campylobacter. Of the 137 isolates, 14 were highly efficacious in reducing growth of both C. jejuni or C. coli at concentrations of 10⁵ and 10⁶ CFU/mL per bacterial isolate in vitro (Table 1). Furthermore, for most of these isolates, efficacy was improved at the 10⁶ CFU/mL versus 10⁵ CFU/mL concentration. An additional nine isolates demonstrated a consistent inhibition in Campylobacter growth (both C. jejuni and C. coli) at the 10⁶ CFU/mL per bacterial isolate treatment. For these nine isolates, reduced efficacy was observed, defined as a reduction in 25-50% Campylobacter growth, at the 10⁵ CFU/mL concentration (Table 1). The remaining 114 isolates had either inconsistent or no inhibition of Campylobacter growth rates when tested in our in vitro system.

Table 1: Percent inhibition of Campylobacter jejuni or Campylobacter coli when co-incubated with individual poultry enteric isolates, in vitro*  

<table>
<thead>
<tr>
<th>Number of bacterial isolates</th>
<th>Enteric isolate (10⁵ CFU/mL)</th>
<th>Enteric isolate (10⁶ CFU/mL)</th>
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<tbody>
<tr>
<td></td>
<td>C. jejuni</td>
<td>C. coli</td>
</tr>
<tr>
<td>3</td>
<td>75-100%</td>
<td>75-100%</td>
</tr>
<tr>
<td>5</td>
<td>50-74%</td>
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<tr>
<td>2</td>
<td>75-100%</td>
<td>50-74%</td>
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<tr>
<td>4</td>
<td>50-74%</td>
<td>50-74%</td>
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<td>3</td>
<td>Less than 25-49%</td>
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<td>2</td>
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<td>4</td>
<td>Less than 25-49%</td>
<td>50-74%</td>
</tr>
<tr>
<td>114</td>
<td>No or inconsistent efficacy</td>
<td>No or inconsistent efficacy</td>
</tr>
</tbody>
</table>

*Percent Campylobacter inhibition was determined following co-incubation with cecal bacteria in vitro for 24-48 h at 42°C. Campylobacter inhibition (%) was estimated by comparing the growth of Campylobacter co-incubated with individual isolates versus Campylobacter alone (0% inhibition). Campylobacter inhibition was placed into 5 categories: 0%, 10-24%, 25-49%, 50-74% or 75-100%
DISCUSSION

Previous efforts to develop Competitive Exclusion (CE) cultures against *Campylobacter* colonization in poultry have had limited success (Mead, 2002). These poor results may be due to the lack of pre-selection criteria used for collection of bacteria for development of CE cultures. Most CE cultures are obtained by randomly collecting cecal micro flora from healthy poultry (Nurmi and Rantala, 1973; Stavric et al., 1987; Nisbet et al., 1993; Corrier et al., 1995a,b). This strategy has successfully been used for developing effective CE cultures against *Salmonella* (Bailey et al., 1988; Aho et al., 1989; Qin et al., 1995; Stern et al., 2001; Heres et al., 2003), but not against *Campylobacter* (Soerjadi-Liem et al., 1984; Stern et al., 2001; Mead, 2002; Patterson and Burkholder, 2003).

Using a novel approach, we have successfully developed a technique to pre-select for cecal microbes with the potential to reduce *Campylobacter* growth in vitro. This technique is selective, with varying degrees of efficacy against *Campylobacter*. Of the 137 isolates tested, 23 bacterial isolates demonstrated the ability to consistently reduce *Campylobacter* growth and 14 of these isolates were highly efficacious in our system. Several investigators have attempted to improve CE cultures by mimicking properties of efficacious bacteria, defining cultures or quantitating the effect within the gastrointestinal tract. Schoeni and Doyle (1992) isolated cecum-colonizing bacteria that produced anti-*Campylobacter* metabolites from *C. jejuni*-free hens and demonstrated that these isolates could provide some protection against *C. jejuni* in chicks. These investigators focused on mechanisms of *Campylobacter* colonization in birds, specifically the chemotraction of *Campylobacter* to mucin and its ability to use mucin as a substrate. They isolated potential CE bacteria that could grow on mucin as a sole substrate and that occupied the same niche in the cecum as *Campylobacter*. Zhang and co-workers (2007b) have also developed a unique method for collecting isolates with *in vitro* efficacy against *Campylobacter*. This group identified chickens that did not become colonized with *Campylobacter* and isolated bacteria from the enteric tracts of these birds. Using this strategy they have also demonstrated *in vivo* efficacy of defined bacterial isolates against *Salmonella* (Zhang et al., 2007a).

The mechanisms by which the enteric isolates from this study inhibited *Campylobacter* in our system are probably due to either out-competing *Campylobacter* for nutrients and/or producing products (i.e. bacteriocins) that inhibit the pathogen’s ability to grow. Although competition for nutrients has been proposed for microorganisms (Grover, 2009; Grover et al., 2011; Saito and Miki, 2010), to our knowledge, this type of interaction has not been demonstrated in the poultry gut. For bacteriocins, however, previous research has shown efficacy against *Campylobacter* in poultry. Bacteriocins are defined as biologically active proteinaceous compounds produced by certain strains of bacteria which are bactericidal to other closely related bacteria (Tagg et al., 1976). It has been demonstrated that bacteriocins can significantly reduce *Campylobacter* colonization in broiler chickens and turkeys (Stern et al., 2005, 2006; Cole et al., 2006). It has been suggested that bacteriocin treatment may be an effective and feasible strategy to reduce *Campylobacter* in poultry (Lin, 2009; Svetoch and Stern, 2010). In conclusion, we have developed an *in vitro* method capable of identifying bacterial CE isolates with the potential to reduce *Campylobacter* in poultry.

REFERENCES


Zhang, G., M.A. Li and M.P. Doyle, 2007b. Potential competitive exclusion bacteria from poultry inhibitory to *Campylobacter jejuni* and *Salmonella*. J. Food Protect., 70: 867-873.