

POULTRY/EGGS

COMPETITIVE EXCLUSION BACTERIA AGAINST *SALMONELLA* COLONIZATION IN CHICKENS (G. Zhang, L. Ma, and M. P. Doyle)

Poultry and poultry products are important sources of human foodborne salmonellosis. Newly hatched chickens are highly susceptible to infection and colonization by *Salmonella*. Pretreatment of chicks with microflora obtained from the GI tract of adult healthy chickens can protect chicks from *Salmonella* infection; however, undefined cultures from chicken GI tract may transfer bacterial pathogens or bacteria that confer antibiotic resistance into new flocks. The objective of this project is to develop a defined competitive exclusion product that can reduce or eliminate *Salmonella* colonization in poultry.

The first step was to isolate from healthy chickens competitive exclusion bacterial candidates having antimicrobial activity to *Salmonella* in vitro. Using selective and non-selective media, 143 isolates with inhibitory activity to six *Salmonella* strains of poultry origin (2 *Salmonella* Enteritidis, 1 *Salmonella* Typhimurium, 1 *Salmonella* Heidelberg, 1 *Salmonella* Kentucky, and 1 *Salmonella* Senftenberg) were obtained from 9 donor chickens.

Seventeen trials have been carried out to test the effectiveness of many of the competitive exclusion bacterial candidates in reducing *Salmonella* colonization of chicks. The general design of these trials was similar for all, although there were slight variations between different trials. Each trial included 18 treatments, with 10 to 25 chicks per treatment. Undiluted overnight CE bacteria (approximately 10^7 - 10^8 cfu/chick) were fed to chicks at day-of-hatch and the following day. Chicks were challenged with different strains of *Salmonella* (*S.* Typhimurium, *S.* Kentucky, and *S.* Enteritidis) (ca. 10^4 cfu/chick) when they were 3 days old. They were necropsied at ca. 10 days of age. Cecal contents from each chick were enumerated on BGA plates; at the same time, they were enriched in RV broth and streaked onto BGA plates. Results were obtained for (a) the percentage of *Salmonella*-positive birds out of the total number tested in each treatment and (b) the level of *Salmonella* carriage (expressed as the geometric mean of the counts per gram of cecal content for all chicks tested in the group). CE cultures grown in MRS broth before feeding to chicks yielded inconsistent results. Modifying MRS broth to provide nutritional conditions more similar to that of the intestinal tract of chicks were *Salmonella*-positive. The effectiveness of many of the CE bacteria against *Salmonella* in chickens varied; however, six CE isolates showed consistent effectiveness against *Salmonella* colonization in chickens in repeated trials. They reduced *Salmonella* carriage in cecal content by more than 2 log₁₀ cfu/g on average for three trials. The percentage of chickens *Salmonella*-positive after treatment with these CE bacteria was ca. 30%, whereas ca. 80% of control (no CE bacteria treatment) were *Salmonella*-positive. Currently, we are testing different combinations of these six CE bacteria to identify the best combinations. Cultural conditions for growing the best performing CE bacteria will be optimized, and these CE bacteria will be identified and further characterized for phenotypic and genotypic properties.

CAMPYLOBACTER SPP. ENUMERATION IN BROILER FECES AND PROCESSED CARCASSES (N. J. Stern and M. C. Robach)

Enumeration of *Campylobacter* spp. on Campy-Cefex agar from 50 carcasses, before and after chilling, was conducted in both 1995 and 2001. One day prior to processing, feces were also collected from each of the broilers for enumeration. A significant reduction in the levels of the organism on freshly processed broiler carcasses was observed from 1995 ($10^{4.11}$ cfu/carcass) to 2001 ($10^{3.05}$ cfu/carcass). Levels of *Campylobacter* spp. found in production and processing were not strongly correlative and suggested the existence of complex parameters involving production factors and variables associated with flock transport and the processing of the broilers.

RELATIONSHIP OF *CAMPYLOBACTER* SPP. IN ICELANDIC POULTRY OPERATIONS AND INCIDENCE OF HUMAN *CAMPYLOBACTERIOSIS*

(N. J. Stern, K. L. Hiatt, G. A. Alfredsson, K. G. Kristinsson, J. Reiersen, H. Hardardottir, H. Briem, E. Gunnarsson, F. Georgsson, R. Lowman, E. Berndtson, A. M. Lammerding, G. M. Paoli, and M. T. Musgrove)

Domestic cases of human campylobacteriosis in Iceland during 1999 reached peak levels of 116 cases per 100,000 population. Over the same period, 62% of broiler carcass rinses were contaminated with *Campylobacter* spp. The incidence of campylobacteriosis in humans decreased to 33 cases per 100,000 population in 2000, and only 15% of the broiler flocks tested *Campylobacter* spp. positive. Several factors account for the large reduction in poultry-borne campylobacteriosis and include public education, enhanced on-farm biological security measures, and carcass freezing. Additional information is being sought to understand the decline in campylobacteriosis such that a risk model for *Campylobacter* spp. transmission may be generated for this well-defined system.

GENOTYPE ANALYSES OF *CAMPYLOBACTER* ISOLATED FROM THE GASTROINTESTINAL TRACTS AND THE REPRODUCTIVE TRACTS OF BROILER BREEDER ROOSTERS

(K. L. Hiett, G. R. Siragusa, N. A. Cos, R. J. Buhr, M. T. Musgrove, N. J. Stern, and J. L. Wilson)

The pathways involved in *Campylobacter* contamination of poultry flocks, horizontal transmission and/or vertical transmission, remain unclear. In this study, *Campylobacter* isolated from feces, cloacal swabs, ceca, semen, and vas deferens of 12 breeder roosters were genotyped by both flagellin A short variable region (*flaA* SVR) DNA sequence analysis and repetitive element (rep)-polymerase chain reaction (PCR). *Campylobacter* was isolated from multiple sites in 9 of 12 roosters. *Campylobacter* isolated from five of the nine roosters demonstrated closely related M SVR DNA sequences as well as rep-PCR patterns. These isolates were collected from both the gastrointestinal and the reproductive tracts or from the gastrointestinal tract alone. Isolates from two of the remaining four roosters originated from both the gastrointestinal tract and the reproductive tracts and were distinct by both typing methods. Distinct isolates from the remaining two roosters originated from only the reproductive tract. No relationships between the genotypes and the sample type could be determined. Additional studies will need to be conducted to determine if the presence of *Campylobacter* within the rooster leads to contamination of the broiler offspring via the fertilized egg.

INTESTINAL CARRIAGE OF *CAMPYLOBACTER* AND *SALMONELLA* IN TURKEYS IN RESPONSE TO SUB-THERAPEUTIC LEVELS OF ANTIMICROBIALS IN FEED **(N. A. Cox, S. E. Craven, M. T. Musgrove, M. E. Berrang, and N. J. Stern)**

Since the 1950s, antimicrobials have been added to poultry feed at sub-therapeutic levels to minimize illness and promote growth. Despite the benefits to the agricultural industry and domestic animals, there are fierce debates worldwide on whether or not this practice carries a consequence in terms of human health. Turkeys and broilers provided these additives have increased weight gain, muscle yield, and feed conversion, in part due to decreases in diseases such as coccidiosis and necrotic enteritis. Benefits achieved by adding these compounds to animal feeds are attributed in part to a shift in the gut microflora. However, studies have been published in which it was determined that competitive exclusion cultures, administered to birds to control colonization by human pathogens such as *Salmonella*, can be negatively affected by antimicrobials commonly used in poultry rations. Other published studies have reported an increase in *Salmonella* levels when experimentally challenged birds were fed diets containing low levels of antimicrobials. This study demonstrated that although naturally occurring populations of *Campylobacter* were virtually unaffected by antimicrobial feed additives, *Salmonella* populations were significantly decreased when commercial turkeys were fed rations containing flavomycin, virginiamycin, or monensin.

APPLICATION OF NESTED PCR TO DETECTION OF *SALMONELLA* IN POULTRY ENVIRONMENT **(J. J. Maurer)**

Isolation of *Salmonella* from poultry environmental and processing plant samples requires sampling large numbers of areas within the poultry house or plant. Subsequently, this results in a large volume of work for a microbiology laboratory especially when the protocol requires inclusion of a delayed secondary enrichment for isolation of *Salmonella*. This study examined the use of PCR to identify those secondary enrichments containing *Salmonella*. The unique *Salmonella* virulence gene *invA* was chosen as the target for development of a nested-PCR because of its uniform distribution among *Salmonella* serotypes. The use of nested-PCR primers increased sensitivity of detection 100-fold, resulting in detection of as few as 4 cells. There was a statistically significant, strong positive correlation between PCR and culture results as determined by chi-squared test ($p < 0.001$) and

kappa test (0.915; excellent agreement). Using PCR as a screen of primary enrichments for presumptive *Salmonella* contamination, we improved our efficiency at isolating *Salmonella* upon secondary enrichment by 20 % and no false negatives were observed. This method will not only validate the use of secondary enrichment procedures, but also reduce costs and manpower for surveillance of *Salmonella*.

DETECTION OF *SALMONELLA* AND *CAMPYLOBACTER* IN POULTRY BY PCR-ELISA (J. J. Maurer)

Contamination of retail poultry by *Campylobacter spp.* and *Salmonella enterica* is a significant source of human diarrheal disease. Isolation and identification of these microorganisms requires a series of biochemical and serological tests. In this study, *Campylobacter ceuE* and *Salmonella invA* genes were used to design probes in PCR-ELISA, as an alternative to conventional bacteriological methodology, for the rapid detection of *Campylobacter jejuni*, *Campylobacter coli*, and *Salmonella enterica* from poultry samples. With PCR-ELISA (40 cycles), the detection limit for *Salmonella* and *Campylobacter* was 2×10^2 cfu/ml and 4×10^1 cfu/ml, respectively. ELISA increased the sensitivity of the conventional PCR method by 100- to 1,000-fold. DNA was extracted from carcass rinses and tetrathionate enrichments and used in PCR-ELISA for the detection of *Campylobacter* and *S. enterica*, respectively. With PCR-ELISA, *Salmonella* was detected in 20 of 120 (17%) chicken carcass rinses examined, without the inclusion of an enrichment step. Significant correlation was observed between PCR-ELISA and cultural methods (kappa = 0.83; chi-squared test: $p < 0.001$) with only one false negative (1.67%) and four false positives (6.67%) when PCR-ELISA was used to screen sixty tetrathionate enrichment cultures for *Salmonella*. With PCR-ELISA, we observed positive correlation between the ELISA absorbance (Optical Density_{405nm}) and *Campylobacter* cell number in carcass rinse, as determined using standard culture methods. Overall, PCR-ELISA is a rapid and cost-effective approach for the detection and enumeration of *Salmonella* and *Campylobacter* on poultry.

A RESTRICTION FRAGMENT LENGTH POLYMORPHISM BASED POLYMERASE CHAIN REACTION AS AN ALTERNATIVE TO SEROTYPING FOR IDENTIFYING *SALMONELLA* SEROTYPES (J. J. Maurer)

The phase 1 (*fliC*) and phase 2 (*fljB*) *Salmonella* flagella genes were analyzed by RFLP-PCR to aid in the identification of different *Salmonella* serotypes. Twenty-four phase 1 flagellin and eight phase 2 flagellin genes could be differentiated among each other using restriction endonucleases *Sau3A* and *HhaI* in RFLP-PCR analysis. These flagellin genes comprise the major antigenic formulas for fifty-two serotypes of *Salmonella spp.*, which include the common serotypes found in poultry and other important food animal species. With the knowledge of the O antigen composition determined from conventional O serotyping, ninety percent of the *Salmonella* serotypes could be identified using this double restriction enzyme RFLP analysis of *fliC* and *fljB* genes. This RFLP-PCR flagellar typing scheme was successfully applied to the identification of serotype for 112 *Salmonella* isolates obtained from poultry environments. There was a significant correlation between RFLP PCR and conventional serotyping (chi-square $p < 0.001$). Overall, PCR-RFLP proved to be a fast, accurate and economic alternative approach to serotyping *Salmonella spp.* such that it is now part of diagnostic services offered to the poultry industry for identifying *Salmonella* serotypes.

RAPID DETECTION OF POULTRY *SALMONELLA ENTERICA* SEROTYPES ENTERITIDIS, HADAR, HEIDELBERG, AND TYPHIMURIUM BY MULTIPLEX PCR TO COMMON O AND H ANTIGEN GENES AND ALLELES (J. J. Maurer)

There are over 2,000 different *Salmonella* serotypes but few are commonly associated with human disease. Currently, serotyping relies on a time consuming process that requires implementing a battery of antisera for typing O and H antigens of *Salmonella enterica*. A rapid multiplex PCR-based molecular serotyping scheme was developed with an ability to identify *S. enterica* serotypes Enteritidis, Hadar, Heidelberg, and Typhimurium isolated from the poultry environment. The nucleotide sequence of the O-antigen biosynthesis *rfb* operons for *Salmonella* O serogroups D1, B, C1, C2, and E1 were analyzed to identify sequences suitable for designing multiplex primer sets. By analyzing the highly variable nucleotide sequences in the central region of the phase-1 and phase-2 *Salmonella* flagella genes *fliC* and *fljB*, multiplex PCR's were designed to identify the *fliC* genes associated with the phase 1 antigen types i, g, m, r, z₁₀ and the *fljB* genes belonging to two major phase 2 antigen

complexes, 1,2, 1,5, 1,6, 1,7 and e,n,x, e,n,z₁₅. These antigens constitute the antigenic formulas for common clinical and poultry *S. enterica* serotypes. The multiplex PCR conditions were optimized and discrimination was achieved, identifying O or H antigen gene(s) and alleles based upon the unique sizes of the amplicons from multiplex PCR. The serotyping, multiplex PCR was comparable to the standard serological typing methods for correctly identifying *S. enterica* serotype ($\kappa = 1.00$; chi-squared test: $p < 0.001$). Coupled with primary enrichment using tetrathionate brilliant green broth, multiplex PCR correctly identified poultry samples contaminated with *S. enterica* Enteritidis, Kentucky, and Typhimurium. These multiplex PCR assays for detecting specific O and H antigen gene alleles can become a rapid and cost-effective alternative approach to serotyping for the identification of common poultry *Salmonella* serotypes. In fact, this multiplex PCR has now become part of diagnostic services offered to poultry industry for identifying *Salmonella* serotypes.

**DIRECT MICROSCOPIC OBSERVATION AND VIABILITY DETERMINATION
OF *CAMPYLOBACTER JEJUNI* ON CHICKEN SKIN
(W. Chantarapanont, M. Berrang, and J. F. Frank)**

The objective of this study was to develop a method to determine survival of *Campylobacter jejuni* at specific sites on chicken skin and to use this method to observe survival of *C. jejuni* at various locations on the skin during storage. This method employs confocal laser scanning laser microscopy (CSLM) to visualize *Campylobacter jejuni* transformed with a green fluorescent protein plasmid (GFP-*Campylobacter*) and stained with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). The green fluorescence of *C. jejuni* cells and the red fluorescent CTC-formazan in viable *Campylobacter* cells were clearly visible on chicken skin. GFP-*Campylobacter* remaining on the chicken skin surface after rinsing were mostly located in crevices, entrapped inside feather follicles with water, and entrapped in the surface water layer. Most viable cells were entrapped with water in the skin crevices and feather follicles. These sites provide a suitable microenvironment for GFP-*Campylobacter* to survive. The population of *C. jejuni* on chicken skin decreased by 1 log₁₀ unit during storage at 25°C for 24 h. *Campylobacter jejuni* located in sites 20-30 μm beneath the chicken skin surface maintained viability during incubation at 25°C. *C. jejuni* on chicken skin stored at 4°C maintained constant numbers during 72 h of incubation with no significant changes in population of feather follicles or crevices. Live and dead cells were initially retained with water on the skin and penetrated into skin follicles and channels during storage. Microscopic observations of GFP-producing cells allowed identification of survival niches for *C. jejuni* present on chicken skin.