Phenotyping and Genotyping of *Campylobacter coli* in Pigs from Farm to Fork

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Abstract

Introduction: *Campylobacter* are estimated to cause 2.4 million illnesses annually in the United States, and most of these illnesses are food-related. Pigs can be sub-clinically infected with these pathogens, and fecal contamination of meat during processing is a food safety risk. The goal of this study is to determine the clonal relatedness of selected *Campylobacter coli* isolates from an individually-identified cohort of pigs collected from five sample types on the farm to processing continuum. Methods: Samples were collected at five stages throughout the processing. Feces were collected on the farm and in lairage. A hide swab was collected before scalding and the entire carcass was swabbed immediately before chilling. For each individually identified carcass, a meat sample (ribs) was also collected. *Campylobacter* was cultured and speciated. Antibiotic susceptibility testing using the microbroth dilution system (Sensititer) was performed on the selected isolates. Multi-locus Sequence Typing (MLST) is currently underway to ascertain clonal relatedness. Results: There were 11 instances where *Campylobacter coli* was isolated from the farm, lairage, hide, carcass and rib of the exact same animal. The most common antibiotic resistance patterns for the sample types were: farm, pansusceptible [n=3], ArCaErTe [n=2], and Te [n=2]; lairage ArCaErTe [n=5]; hide, Te [n=7]; carcass, Te [n=7]; meat ArCaEr [n=3], and ArCaErNiTe [n=3]. Lairage and farm appeared to be the most common source of *Campylobacter* contamination of meat.

Introduction

Approximately 1.4–2.3 million persons become infected with *Campylobacter* in the United States each year, and most of these illnesses are food-related (Samuel et al 2004). Pigs can be sub-clinically infected with *Campylobacter* and fecal contamination of meat during processing is a food safety risk. There have not been any reports of sequencing *Campylobacter* isolated from the same animal from different samples along the farm to fork continuum. This is especially important in defining the role of peri-harvest (on farm, in lairage) factors for contamination of the final meat product and to help assess the level of food safety risk. MLST is a very discriminatory DNA fingerprinting method. By addressing the issue of the clonality of *Campylobacter* in longitudinally collected samples from the same animals, it will allow greater understanding of the sources of contamination and will help to address the most effective measures to reduce contamination.

Material and Methods

Samples. Five different samples were taken from each individually identified animal (fecal sample from farm, fecal sample collected during lairage, hide swab, carcass swab, and final meat product). Approximately 40g of feces were collected from each of the pigs within 48 hours of being shipped to lairage. Another fecal sample (same weight) was collected rectally post mortem (lairage fecal sample). In lairage, the most visually contaminated section of the hide (~625cm²) was sampled using a sponge (1.5” x 3”, Biotrace) pre-moistened with buffered peptone water (BPW). Three sections were sampled, each with a separate sterile sponge. The entire hot carcass of each animal was sampled post washing (cold water) and pre chilling. Three sponges were used to sample each half of the carcass. A final meat product was
obtained from the same animals a week after they were harvested. Approximately 6 pounds of ribs was collected from each of the pigs. All samples were transported back to the lab on ice and processed immediately, except for the fecal sample from farm, which were stored at 4°C for 48h in order to process with the other samples.

**Campylobacter Culture.** The fecal samples from farm and fecal samples from lairage were processed by weighing 1g of feces into 9ml of BPW. One-hundred microliters of the mixture was plated in duplicate on Campy-Cefex plates and incubated under microaerophilic conditions for 48h at 42°C. The hide swab samples were processed by mixing the sponge with 30ml of Bolton broth. The samples were incubated under microaerophilic conditions for 48h at 42°C. An aliquot of 100µl from each enriched sample was plated onto Campy-Cefex plates and incubated under microaerophilic conditions for 48h at 42°C. The 3 sponges from the carcass swab were pooled and mixed with 90ml of Bolton broth. The samples then followed the protocol for the hide swabs from this point on. Approximately one pound of ribs was mixed with 500ml of Bolton broth. The ribs were incubated in the Bolton broth for 48h at 42°C under microaerophilic conditions. One hundred µl of the mixture was plated onto Campy-Cefex as for previous samples. Suspect Campylobacter colonies from all positive pigs from each sample type were saved at -80°C for further identification.

**Antibiotic Susceptibility.** Antimicrobial susceptibility of isolates was performed using an approved standard broth microdilution method according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines (NCCLS, 1999). The experiment used commercially prepared Campy plates (Sensititre, TREK™ Diagnostic Systems Inc., Westlake OH). Cutoff values are established based upon NCCLS standards and the cutoffs used by the United States National Antibiotic Monitoring System (NARMS). The following antibiotics were tested: (Ar – Azithromycin, Ca – Clindamycin, CIP – Ciprofloxacin, Er – Erythromycin, Ff – Florencicol, Gm – Gentamycin, NI – naladixic acid, Te – Tetracycline). Minimum inhibitory concentrations (MIC) will be determined in accordance with the manufacturer’s instructions. Briefly, colonies will be selected from a primary agar plate medium and emulsified into 4 ml of sterile MH broth. The turbidity of this emulsion will be adjusted to a 0.5 McFarland Standard. From the adjusted suspension, 10 µl will be transferred to a tube of Mueller-Hinton broth with laked horse blood. Fifty µl of the inoculated broth will be added to each well of a Sensititre plate containing several concentrations of each antimicrobial. Plates are incubated at 42°C for 18-24 hours under microaerophilic conditions. After incubation, minimum inhibitory level was determined using the SensiTouc (TREK™ Diagnostic Systems Inc).

**Multi Locus Sequence Typing.** PCR was performed on the purified DNA (Tissue Kit, Qiagen) of the isolates for all the following seven housekeeping genes described at MLST.net: aspA (aspatase), gltA (glutamine synthetase), glnA (citrate synthase), glyA (serine hydroxy methyl transferase), pgm (phospho glucomutase), tkt (transketolase), uncA (ATP synthase alpha subunit). Ready-to-Go PCR beads (Amersham Pharmacia Biotech AB) was used for all PCR amplifications with the addition of 0.75 µl primer (10 pmol/µl) and 1 µl of template DNA (25 to 100 pg) in a final volume of 25 µl per reaction. The reaction conditions were: initial denaturation at 95°C for 5 min, followed by 30 cycles of the following, 95°C for 1 min, primer annealing at 55°C for 1 min 30 sec, and extension at 72°C for 1 min. Thermal cycling was conducted with a MJ PTC 200 thermal cycler. The PCR products were purified by use of the Qiagig multiwell PCR purification kit (Qiagen), and the concentration estimated using a ND-1000 NanoDrop UV-Vis spectrophotometer (NanoDrop Technologies). Sequencing reactions were conducted in a volume of 20 µl containing 1 µl purified PCR product, 2 µl primer (10 pmol/ µl), 1.5 µl sequencing buffer (Beckman Coulter, Fullerton, CA, USA), 2 µl DTCS Quick Start Master Mix (Beckman Coulter, Fullerton, CA, USA), and 13.5 µl molecular grade water. Thermal cycling conditions for sequencing reactions were set up according to the manufacturer’s instructions (Beckman Coulter). Unincorporated dye terminators were removed by the DyeEx 96 Kit (Qiagen), and the sequenced products were separated and detected with a CEQ 8000 Genetic Analysis System (Beckman Coulter). Alleles and sequence types (STs) were assigned by submitting the DNA sequence to the Campylobacter MLST database.
(http://campylobacter.mlst.net). The isolates were analyzed as follows: isolates with six or more shared alleles at each locus will be considered members of the same clonal complex. The degree of clonality was determined using the index of association and phylogenetic analysis as shown previously (Jolley et al. 2001 and Kumar et al. 2004). A minimum spanning tree was created using BioNumerics software version 4.0 (Applied Maths, Kortrijk, Belgium). The ClustalW software (available at http://www.ebi.ac.uk/clustalw) was used to perform the sequence alignments.

Results

There were 11 instances where Campylobacter coli were found from the farm, lairage, hide, carcass and rib of the same animal. The most common antibiotic resistance patterns for the sample types were: farm, pan-susceptible [n=3], ArCaErTe [n=2], and Te [n=2]; lairage ArCaErTe [n=5]; hide, Te [n=7]; carcass, Te [n=7]; meat ArCaEr [n=3], and ArCaErNITe [n=3] (Table 1). After re-isolating the Campylobacter from -80°C cryopreservation some of the isolates did not speciate as C. coli using primers specific for the ceuE gene, which encodes for a putative virulence determinant, or C. jejuni using primers specific for the hipQ gene, which encodes for the hippurase enzyme (Gebreyes et al., 2005). However, Campylobacter specific 16S rDNA primers gave a positive result for all of the 11 non-speciated isolates (Oyarzabal et al., 2006), implying the occurrence of Campylobacter spp other than C. coli or C. jejuni. Multi-locus sequence typing is currently underway.

<table>
<thead>
<tr>
<th>Pig</th>
<th>Farm</th>
<th>Lairage</th>
<th>Hide</th>
<th>Carcass</th>
<th>Meat</th>
</tr>
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<tbody>
<tr>
<td>2</td>
<td>ArCaErGmNITe</td>
<td>Te</td>
<td>Te</td>
<td>Te</td>
<td>ArCaErNl*</td>
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<tr>
<td>3</td>
<td>Te</td>
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<td>Te</td>
<td>Te</td>
<td>ArCaGmNITe*</td>
</tr>
<tr>
<td>4</td>
<td>ArCaErFFTe</td>
<td>ArCaErTe</td>
<td>Te</td>
<td>Te</td>
<td>ArCaEr*</td>
</tr>
<tr>
<td>6</td>
<td>Pan Susceptible</td>
<td>ArCaErTe</td>
<td>Ca</td>
<td>Te</td>
<td>ArCaErNITe*</td>
</tr>
<tr>
<td>7</td>
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<td>Pan Susceptible</td>
<td>ArCaErNITe*</td>
</tr>
<tr>
<td>10</td>
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<td>Te</td>
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</tr>
<tr>
<td>26</td>
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<td>Te</td>
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</tr>
<tr>
<td>31</td>
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<td>Te</td>
<td>Pan Susceptible</td>
<td>ArCaErTe</td>
</tr>
<tr>
<td>43</td>
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<td>ArCaErTe</td>
<td>Te</td>
<td>Te</td>
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</tr>
<tr>
<td>76</td>
<td>Te</td>
<td>ArCaErTe</td>
<td>Te</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>85</td>
<td>Pan Susceptible</td>
<td>No growth</td>
<td>No growth</td>
<td>CaEr*</td>
<td>Te</td>
</tr>
</tbody>
</table>

Table 1 Results from micro broth antibiotic dilution to the following antibiotics: (Ar – Azithromycin, Ca – Clindamycin, CIP – Ciprofloxacin, Er – Erythromycin, FF – Florfenicol, Gm – Gentamicin, Nl – Naladixic acid, Ti – Telithromycin, and Te – Tetracycline).

* Unknown Campylobacter sp. (not C. coli or C. jejuni)

Discussion

The majority of the Campylobacter isolates, particularly from farm and lairage, appear to be phenotypically similar to isolates from rib meat. Culture methods may have had a role in selecting for certain isolates with distinct resistance patterns. Since we have found that a majority of the meat isolates were not C. coli it is apparent that these isolates are not clonal to the isolates found upstream in the processing. This could indicate that the meat is becoming contaminated at the meat processing stage (from the environment, knives, meat handlers) or that the isolates are able to survive better in this type of environment compared to the other stages of samples.

Conclusion

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Since the Multi Locus Sequence Typing has not been completed at the time this paper was written we are
not able to ascertain the clonality of the isolates. It is clear, however, that the meat samples that did not
speciate as C. coli are not clonal to the majority of the isolates from the same pig collected upstream of the
processing. The picture will be clearer once the MLST has been completed. Care still needs to be taken
when harvesting and processing meat to try and prevent the contamination of foodborne pathogens.

References

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