Title: Genotyping of *Campylobacter coli* in Pigs from Farm to **Fork - NPB # 08-263**

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Industry Summary

Campylobacter is a bacterial organism that cause 2.4 million foodborne illnesses annually in the United States. Pigs can be infected with these pathogens without symptoms, and fecal contamination of meat during processing could be of high food safety risk. The goal of this study is to determine the persistence of *Campylobacter coli* in the farm to processing continuum. Samples were collected at five sample types. 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 (rib) was also collected. *Campylobacter* was cultured and speciated. Antibiotic susceptibility was tested. DNA fingerprinting was done to ascertain clonal relatedness. 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], and Te [n=2]; lairage ArCaErTe [n=3]; hide, Te [n=7]; carcass, Te [n=7]; meat Te [n=2]. *Campylobacter* isolates from meat appear to be phenotypically similar and they are within the same fingerprint group as isolates collected from farm, lairage, hide and carcass. The high relatedness of isolates from farm, lairage, hide and carcass indicate that origin of carcass contamination could well be from on-farm or lairage/ processing sources. There were six new strains identified.

Scientific Abstract

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 the same pigs collected from five sample types on the farm to processing continuum. Samples were collected at five sample types. Feces were collected on the farm and in lairage. A hide swab was collected before scalding and the

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entire carcass was swabbed immediately before chilling. For each individually identified carcass, a meat sample (rib) 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 is currently underway to ascertain clonal relatedness. 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], and Te [n=2]; lairage ArCaErTe [n=3]; hide, Te [n=7]; carcass, Te [n=7]; meat Te [n=2]. *Campylobacter* isolates from meat appear to be phenotypically similar and they are within the same clonal complex as isolates collected from farm, lairage, hide and carcass. The clonal groups of isolates have isolates including farm, lairage, hide and carcass indicating that during the process the carcass can be contaminated by any/all of the previous means including on-farm sources. There were 6 new Sequence Types identified from unique allele reassortments. The results show that clonal isolates of *Campylobacter coli* are recovered from the final meat product that were also isolated from feces, hide and the carcass of the same animal showing the high persistence.

Introduction

Approximately 2.4 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.

Objectives

Aim 1. To determine clonality of isolates from on-farm sources to those collected during harvest and postharvest stages using Multi Locus Sequence Typing (MLST).

Aim 2. To determine persistence of Campylobacter coli strains from the same pigs at each of the five sample types.

Materials 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 (~625cm^2) 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 – Florfenicol, Gm – Gentamycin, Nl – Naladixic acid, Tt – Telithromycin, 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 SensiTouch (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), *glnA* (glutamine synthetase), *gltA* (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 Qiaquick 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 ethanol precipitation, 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;Kumar et al., 2004). A minimum spanning tree was created using Bionumerics software version 4.0 (Applied Maths, Kortrijik, Belgium). The ClustalW software (available at http://www.ebi.ac.uk/clustalw) was used to perform the sequence alignments.

Results

Aim 1. To determine clonality of isolates from on-farm sources to those collected during harvest and postharvest stages using Multi Locus Sequence Typing (MLST).

After analyzing the results of the MLST it is shown that all of the isolates from meat are within the same clonal complex as isolates collected from the other sample types (Figure 2). ST-1579 was within the same clonal complex as isolates recovered from the farm, lairage, hide, carcass and meat. ST-1107 and ST-1056 are also within the same clonal complex and include isolates from farm, lairage, hide, carcass and meat. These results indicate that isolates recovered from on-farm sources, during harvest and postharvest are all within the same clonal complexes and are clonal. Therefore, it ascertains that meat contaminated with *Campylobacter coli* strains may have partially originated from the farm.

Aim 2. To determine persistence of *Campylobacter coli* strains from the same pigs at each of the five sample types.

In two pigs the sequence type that was isolated from the meat was also isolated from the same pig (Table 2). In the case of pig number 26, ST-1056 was isolated from the meat and from the lairage but not on-farm. In pig number 43 St-1056 was isolated from the meat and from the lairage and hide. These results indicate that certain *Campylobacter coli* strains can persist in the same pigs from farm to slaughter. Additionally, unique strains that persist in the lairage environment could also result in meat contamination.

Discussion

Campylobacter isolates from meat appear to be phenotypically similar and they are within the same clonal complex as isolates collected from farm, lairage, hide and carcass

The clonal groups of isolates have isolates including farm, lairage, hide and carcass indicating that during the process the carcass can be contaminated by any/all of the previous means

There were 6 new Sequence Types identified from unique allele re-assortments. This is an important finding to add to the global database of sequence types.

The variation of culture methods between fecal samples (farm and lairage) and the hide, carcass and meat samples may have a role in selecting for certain isolates.

The results indicate that clonal isolates of *Campylobacter coli* are being recovered from the final meat product that were also isolated from feces, hide and the carcass of the same animal. Further study and realization of novel ideas to reduce contamination of the final meat product are still warranted.

Pig	Farm	Lairage	Hide	Carcass	Meat
2	ArCaErGmNlTe	Te	Te	Te	No growth
3	Te	Pan Susceptible	Te	Te	ArCaErNlTe
4	ArCaErFfTe	No growth	Te	Te	No growth
6	Pan Susceptible	ArCaErTe	Ca	Te	No growth
7	Pan Susceptible	No growth	Pan Susceptible	Pan Susceptible	No growth
10	ErNlTe	No growth	Pan Susceptible	Te	ArGmNlTe
26	No growth	ArCaErNlTe	Te	Te	Pan Susceptible
31	ArCaErTe	ArCaErFfNlTe	Te	Pan Susceptible	ArCaErTe
43	ClPNlTe	ArCaErTe	Te	Te	Te
76	Te	ArCaErTe	Te	No growth	No growth
85	Pan Susceptible	No growth	No growth	No growth	Te

Table 1. Antibiotic Resistance Patterns of the *Campylobacter coli* isolates (Ar – Azithromycin, Ca – Clindamycin, CIP – Ciprofloxacin, Er – Erythromycin, Ff – Florfenicol, Gm – Gentamycin, Nl – Naladixic acid, Tt – Telithromycin, Te – Tetracycline)

Pig	Farm	Lairage	Hide	Carcass	Meat	Date of Harvest
2	ST-1436	St-1056	ST-1556	**ST-4086**	No growth	Oct. 12, 2006
3	**ST-4086**	ST-1056	ST-854	**ST-4086**	ST-1107	Oct. 12, 2006
4	ST-854	No growth	ST-854	ST-1056	No growth	Oct. 12, 2006
6	ST-854	ST-854	ST-854	**ST-4085**	No growth	Oct. 12, 2006
7	ST-854	No growth	ST-854	ST-854	No growth	Oct. 12, 2006
10	ST-854	No growth	ST-828	**ST-4085**	ST-1056	Oct. 12, 2006
26	No growth	ST-1056	**ST-4087**	ST-854	ST-1056	Feb. 1, 2007
31	ST-1107	**ST-4083**	**ST-4088**	ST-854	ST-1579	Feb. 1, 2007
43	ST-854	**ST-4084**	ST-1056	ST-1056	ST-1056	Feb. 8, 2007
76	**ST-4085**	**ST-4084**	**ST-4086**	No growth	No growth	Feb. 22, 2007
85	ST-854	No growth	No growth	No growth	ST-1056	March 1, 2007

Table 2. Sequence types of the isolates in this study (sequence types in bold were new types as a result of unique allele re-assortments).

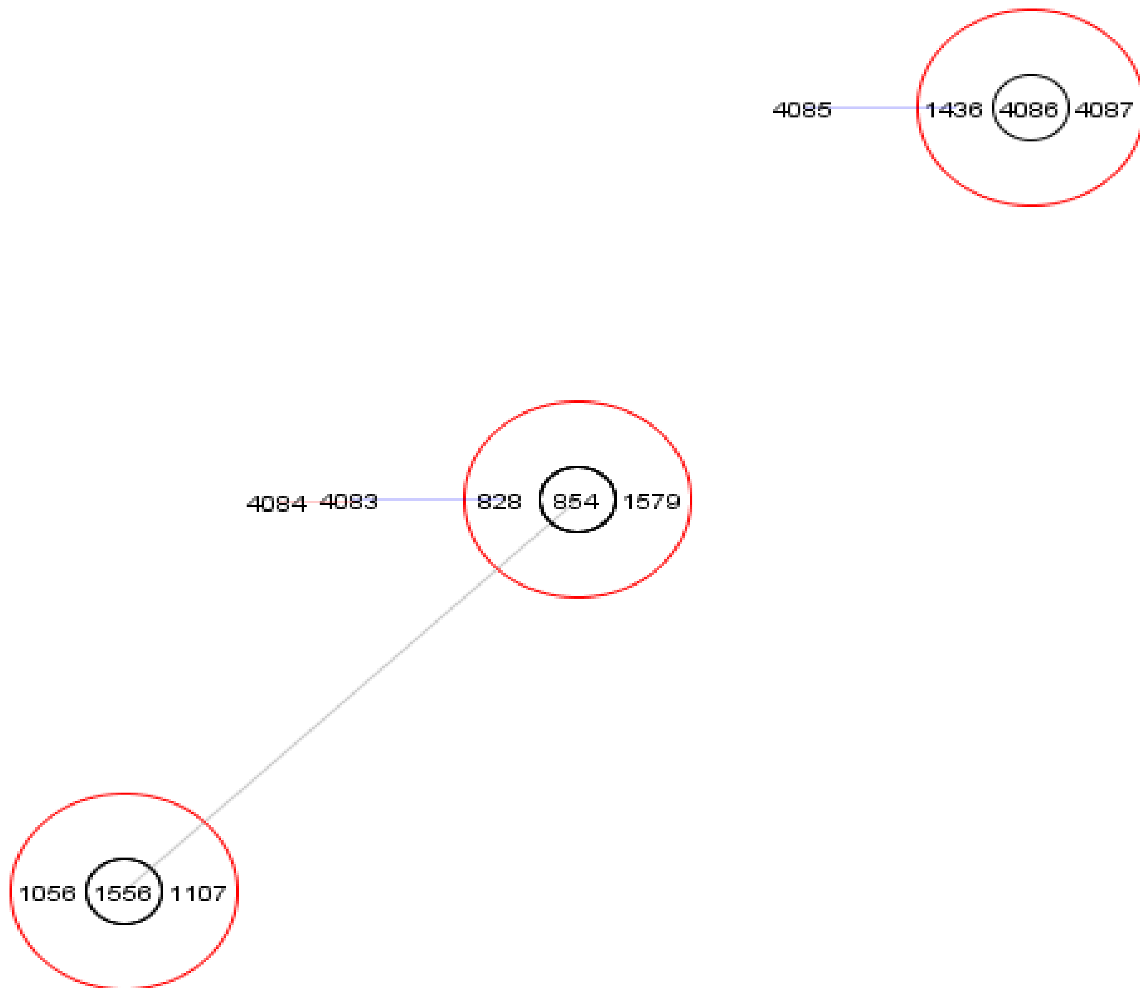
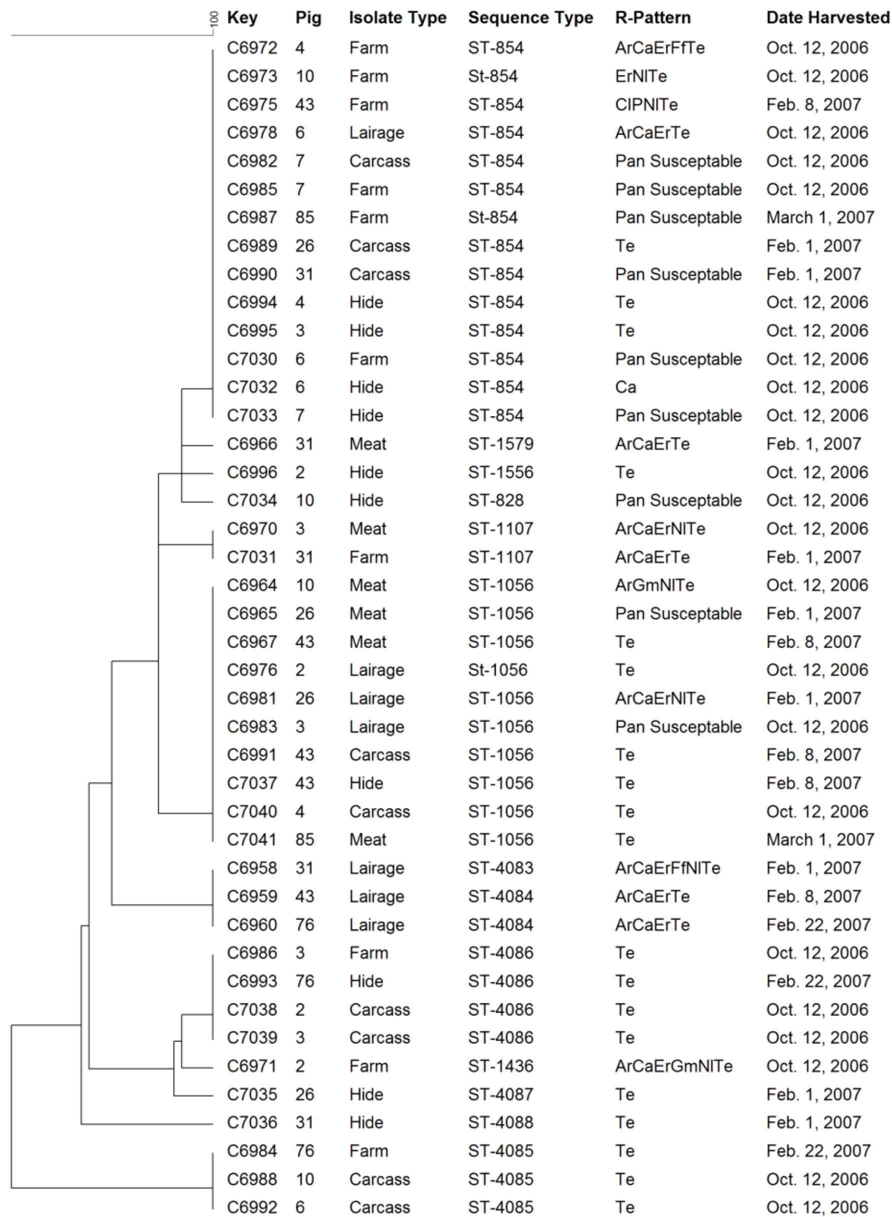


Figure 1. Based Upon Related Sequence Types Analysis (BURST) of Sequence Types found in this study



Key	Pig	Isolate Type	Sequence Type	R-Pattern	Date Harvested
C6972	4	Farm	ST-854	ArCaErFfTe	Oct. 12, 2006
C6973	10	Farm	St-854	ErNlTe	Oct. 12, 2006
C6975	43	Farm	ST-854	ClPNlTe	Feb. 8, 2007
C6978	6	Lairage	ST-854	ArCaErTe	Oct. 12, 2006
C6982	7	Carcass	ST-854	Pan Susceptable	Oct. 12, 2006
C6985	7	Farm	ST-854	Pan Susceptable	Oct. 12, 2006
C6987	85	Farm	St-854	Pan Susceptable	March 1, 2007
C6989	26	Carcass	ST-854	Te	Feb. 1, 2007
C6990	31	Carcass	ST-854	Pan Susceptable	Feb. 1, 2007
C6994	4	Hide	ST-854	Te	Oct. 12, 2006
C6995	3	Hide	ST-854	Te	Oct. 12, 2006
C7030	6	Farm	ST-854	Pan Susceptable	Oct. 12, 2006
C7032	6	Hide	ST-854	Ca	Oct. 12, 2006
C7033	7	Hide	ST-854	Pan Susceptable	Oct. 12, 2006
C6966	31	Meat	ST-1579	ArCaErTe	Feb. 1, 2007
C6996	2	Hide	ST-1556	Te	Oct. 12, 2006
C7034	10	Hide	ST-828	Pan Susceptable	Oct. 12, 2006
C6970	3	Meat	ST-1107	ArCaErNlTe	Oct. 12, 2006
C7031	31	Farm	ST-1107	ArCaErTe	Feb. 1, 2007
C6964	10	Meat	ST-1056	ArGmNlTe	Oct. 12, 2006
C6965	26	Meat	ST-1056	Pan Susceptable	Feb. 1, 2007
C6967	43	Meat	ST-1056	Te	Feb. 8, 2007
C6976	2	Lairage	St-1056	Te	Oct. 12, 2006
C6981	26	Lairage	ST-1056	ArCaErNlTe	Feb. 1, 2007
C6983	3	Lairage	ST-1056	Pan Susceptable	Oct. 12, 2006
C6991	43	Carcass	ST-1056	Te	Feb. 8, 2007
C7037	43	Hide	ST-1056	Te	Feb. 8, 2007
C7040	4	Carcass	ST-1056	Te	Oct. 12, 2006
C7041	85	Meat	ST-1056	Te	March 1, 2007
C6958	31	Lairage	ST-4083	ArCaErFfNlTe	Feb. 1, 2007
C6959	43	Lairage	ST-4084	ArCaErTe	Feb. 8, 2007
C6960	76	Lairage	ST-4084	ArCaErTe	Feb. 22, 2007
C6986	3	Farm	ST-4086	Te	Oct. 12, 2006
C6993	76	Hide	ST-4086	Te	Feb. 22, 2007
C7038	2	Carcass	ST-4086	Te	Oct. 12, 2006
C7039	3	Carcass	ST-4086	Te	Oct. 12, 2006
C6971	2	Farm	ST-1436	ArCaErGmNlTe	Oct. 12, 2006
C7035	26	Hide	ST-4087	Te	Feb. 1, 2007
C7036	31	Hide	ST-4088	Te	Feb. 1, 2007
C6984	76	Farm	ST-4085	Te	Feb. 22, 2007
C6988	10	Carcass	ST-4085	Te	Oct. 12, 2006
C6992	6	Carcass	ST-4085	Te	Oct. 12, 2006

Figure 2. Dendrogram built by pairwise comparison of the consensus sequences

References

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