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Prevalence, antibiotic spectrum and genetic relatedness in thermophilic *Campylobacter* species from poultry production environment of Punjab

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ABSTRACT

Campylobacter is responsible for human gastroenteritis worldwide. *C. jejuni* and *C. coli* are most frequently encountered in animals, birds and man, but mainly *C. jejuni* is particularly adapted to poultry. The disease in humans ranges from gastroenteritis to Guillain-Barre Syndrome. To know the status of *Campylobacter* species especially, *C. jejuni* in poultry environment in Punjab, 342 poultry fecal samples (228 from layers and 114 from broilers) were collected from 30 poultry farms of Punjab. Additionally, 27 fecal samples from poultry farm workers were also collected. Analysis of the samples revealed that 21 (6.14%) fecal samples (from poultry farm) and two (7.41%) from farm workers were positive for *Campylobacters* based on cultural and molecular detection. Out of 23 *Campylobacter* positive isolates, 19 (80.95%) were *C. jejuni* and 4 (19.04%) were *C. coli*. Antibiotic resistance in the isolates was low. The majority of the isolates were sensitive to macrolide and quinolone class of antibiotics which are important for the treatment of campylobacteriosis. Pulse Field Gel Electrophoresis (PFGE) showed high genetic diversity among *C. jejuni,* however, it was limited in *C. coli* isolates. Poultry production in Punjab poses a potential risk of campylobacteriosis. However, antibiotic resistance in the isolates was low.

Keywords: Antibiotic sensitivity test, *Campylobacter* species, *C. coli*, *C. jejuni*, Feces, PFGE, Poultry, *Sma*I

Campylobacteriosis is an important food-borne illness and the leading cause of human gastroenteritis worldwide. It is among the most common causes of diarrhea in travelers from developed nations. According to the Centre for Disease Control and Prevention, *Campylobacters* are the 4th major cause of food-borne illness, 3rd major cause of hospitalization and 5th main cause of human deaths due to food-borne infections annually in the USA alone (Scallen *et al.* 2011). Its incidence and antimicrobial resistance in the United States increased during 2004–2012 (Geissler *et al*. 2017).

Campylobacter species are widely distributed in nature. Several species of *Campylobacters* are encountered in animals, birds and man, but mainly *C. jejuni* and *C. coli* are important from a human health perspective. A majority of them are adapted to the gastro-intestinal tract of birds and animals. Amongst *Campylobacters*, mainly thermophilic *Campylobacter* species have been implicated in food-borne infections and *C. jejuni* is particularly adapted to poultry, which forms the largest reservoir. The contaminated water and foods of animal origin, especially improperly processed chicken have been implicated as vehicles for the transmission of campylobacteriosis. The disease in humans range from gastroenteritis to irritable bowel syndrome, reactive arthritis and Guillain-Barre Syndrome (Pope *et al.* 2007 and Marshall 2009). It is a self-limiting disease. However, antimicrobial therapy is warranted only for patients with severe disease or those at high risk for severe diseases, such as those with weakened immune system from medications or other illnesses. Indiscriminate and improper use of antibiotics is often considered an important driver for antimicrobial resistance (Wimalarathna *et al.* 2013). In recent years, there is an increasing trend of antimicrobial resistance in *Campylobacter* isolates worldwide, especially multidrug resistant (MDR) strains within the food chain (Perez-Boto *et al.* 2013). The concern exists that antimicrobial-resistant *Campylobacter* spp. from animals can be transmitted to humans through food.

Poultry rearing is actively done in the state of Punjab. The present study was undertaken to know the prevalence, antibiotic sensitivity and genetic variability of *Campylobacte*r spp. in the poultry production environment.

MATERIALS AND METHODS

Collection of samples: A total of 342 poultry fecal samples (228 from layers and 114 from broilers) were collected from 30 poultry farms located in Ludhiana, Sangrur, Hoshiarpur, Ropar districts and Chandigarh of Punjab from September 2015 to April 2016. In addition to that 27 fecal samples from poultry farm workers were also

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collected from these farms wherever workers volunteered. Samples were put in sterile sample collection tubes with cotton swab containing Cary-Blair transport medium.

Enrichment of samples: The samples were processed for isolation on the same day by mixing 1 ml of transport medium containing sample with 9 ml Preston broth supplemented with FBP (iron, bisulphate, pyruvate) and incubated under microaerophilic conditions (gas mix of 5% O_2, 10% CO_2, and 85% N_2) at 42°C for 48 hrs.

Selective plating: After incubation, a loop full of enriched inoculum from Preston broth was streaked onto modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) for primary isolation of thermophilic *Campylobacter* at 42°C for 48 hrs as mentioned earlier. Colonies showing typical greyish, flat, moistened mucoid dewdrop appearance were presumed to be *Campylobacter*. The presumptive *Campylobacter* colonies were subjected to Gram staining and catalase test. Colonies showing Gram-negative staining with characteristic spiral or curved slender rods morphologically and catalase-positive reaction were presumed to be *Campylobacter*. Presumptive *Campylobacter* colonies were subjected to oxidase test, micro-aerobic growth at 25°C, aerobic growth at 42°C, indoxyl acetate hydrolysis test and hippurate hydrolysis test. The isolates were differentiated as *C. jejuni* and *C. coli* based on hippurate hydrolysis test as *C. jejuni* is the only *Campylobacter* species that hydrolyses hippuric acid.

Molecular detection of Campylobacter: The isolation of genomic DNA from biochemically positive *Campylobacter* colonies was done by snap-chill method. Polymerase chain reaction (PCR) was carried out on extracted DNA targeting 16S *rRNA* gene which is highly conserved and genus-specific using published primers (Linton *et al.* 1997). The isolates which were positive for *Campylobacter* genus by PCR were further characterized into *C. jejuni* by targeting *hip*O gene and *C. coli* by targeting *asp*A genes (Linton *et al.* 1997). The amplification was carried out using a reaction volume of 25 µl containing 11 µl master mix (Promega, US), 1 µl forward primer (10 pmol/µl), 1 µl reverse primer (10 pmol/µl), 5 µl DNA template and 7µl sterilized nuclease-free water (NFW). Mastercycler Gradient Thermocycler (Applied biosystem) was used to perform the amplification reaction. The cycling conditions included an initial denaturation of DNA at 94°C for 2 min followed by 35 cycles each of 1 min denaturation at 95°C, annealing at 55°C for 1 min and extension at 72°C for 2 min, followed by a final extension of 5 min at 72°C and hold at 4°C.

Antibiotic sensitivity testing: Antibiotic sensitivity of confirmed *Campylobacter* isolates was performed by agar disc diffusion (Bauer *et al.* 1966). All the identified *Campylobacter* isolates were tested for their sensitivity to various clinically important antibiotics, viz. gentamicin (10 µg), erythromycin (15 µg), amikacin (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), tetracycline (30 µg) and nalidixic acid (30 µg) using antimicrobial discs (HiMedia Lab, Mumbai, India). The test was performed by applying bacterial inoculum (1.5×10^8 CFU/ml) having the same turbidity as that of 1.0 McFarland standard onto Mueller Hinton Agar (MHA) plate supplemented with 5% sheep blood using a sterile cotton swab. The swab was dipped in the test inoculum in a test tube and the excess inoculum was removed by squeezing the swab against the wall of the tube. MHA plate was then mopped with the swab while rotating the plate between each mop to make sure that the entire plate is covered. After application of the inoculum, the plate was allowed to dry for a few minutes. Four antibiotic discs were placed per plate. The plates were incubated in microaerophilic conditions at 42°C for 24 h. The zone of growth inhibition around each of the antibiotic discs was measured to the nearest millimeter. The zone diameter of each drug was interpreted as per CLSI Guidelines (Clinical and Laboratory Standards Institute) mentioned in the interpretation chart given by the antibiotic disc manufacturer. Standard strains *Campylobacter jejuni* ATCC strain 33560 and *Campylobacter coli* ATCC strain 33559 were used as positive control.

Genetic relatedness of Campylobacter *isolates:* Genetic relatedness of *Campylobacter* isolates was determined by Pulse field gel electrophoresis (PFGE) after digestion with restriction enzyme *Sma*I, using a CHEF MAPPER (Bio-Rad Laboratories, Hercules, CA, USA) following the Pulse Net protocol of Centre for Disease Control and Prevention, United States (CDC, USA). The complete protocol is given at https://www.cdc.gov/pulsenet/pdf/campylobacter-pfge-protocol-508c.pdf. The gel was visualized using Gel Documentation system (Syngene, USA). The gel image was then subjected to analysis using Bionumerics software (Applied Biosystems). Band molecular weights were determined by sample lanes to a standard molecular weight ladder (Lambda Ladder – Bio Rad). Banding patterns were converted into binary data based on the presence or absence of bands with each molecular weight and fingerprints were then compared using the Dice algorithm. The dendrogram was drawn using UPGMA (unweighted pair group method with averages) analysis of the PFGE patterns using the Dice algorithm with a 1% tolerance window.

RESULTS AND DISCUSSION

Prevalence of Campylobacter *spp.*: Out of 30 farms (20 layers and 10 broilers) examined, 16 farms were positive for *Campylobacter* giving a mean prevalence of 53.3% (16/30) based on the number of farms examined (Table 1). The prevalence within farms varied from 0% to 40%. Out of 342 fecal samples from 30 organized poultry farms, 21 fecal samples were positive for *Campylobacter* giving a mean fecal sample prevalence of 6.14% (21/342). Of these 21 *Campylobacter* positive isolates, 17 (80.95%) were *C. jejuni* and 4 (19.04%) were *C. coli*.

Out of 20 layer farms screened, 11 farms were positive for *Campylobacter* giving a prevalence of 55% (11/20) based on the layer farms examined. The prevalence within individual layer farms varied from 0% to 40%. Out of 228 fecal samples examined from 20 layer farms from different regions, 15 samples were positive for *Campylobacter*, thereby giving an overall prevalence of 6.57% (15/228) based on the number of fecal samples from layer farms (Table 1).

Out of 10 broiler farms studied, 5 farms were positive for *Campylobacter* giving a prevalence of 50% (5/10) based on the number of broiler farms examined. The prevalence within individual broiler farms ranged from 0% to 13.33%. Out of 114 fecal samples examined from 10 broiler farms from different regions, 6 fecal samples were positive for *Campylobacter*, thereby giving an overall prevalence of 5.2% (6/114) based on the number of fecal samples from broiler farms. Two Fecal samples from poultry farm workers of the two layer farms were also positive for *Campylobacter* (Table 1). The prevalence of *Campylobacter* reported in the present study was low compared to studies done elsewhere. The prevalence level varied in different studies reported from different areas. Rajagunalan *et al.* (2014) reported a prevalence of 15.89% for thermophilic *Campylobacters* in poultry fecal samples. Rizal *et al.* (2010) reported a prevalence of 17.14% in poultry feces. In another study, the prevalence ranged from 11.1% to 19.7% depending upon the poultry rearing system, with a higher prevalence in conventional rearing system (Novoa Rama *et al.* 2018). In a similar type of study, the conventional system of poultry rearing was found to be having a 73% prevalence of *Campylobacter* spp. as compared to organically raised (45%) birds (Bailey *et al.* 2019). There is a study that even reported a higher number of poultry farms positive for *Campylobacter* (82.5%, 33/40) (da Silva *et al.* 2016). In another study, García-Sánchez *et al.* (2020) reported 40% of the examined farms positive for *Campylobacter* and within farm prevalence range of 43.1–88.6%. Based on these studies we could say that type of poultry production system has an influence on *Campylobacter* prevalence in poultry farms with low prevalence reported from intensively reared farms. All the samples in the present study also came from intensively raised farms showing low prevalence. There was no difference noticed in the prevalence of *Campylobacter* even if the samples came from layers or broilers (Novoa Rama *et al.* 2018 and Bailey *et al.* 2019). Although, layer samples accounted for more *Campylobacter* isolates as more numbers of layer farms were sampled. Similar to our findings, *C. jejuni* was found to be the predominant species

Table 1. Prevalence of *Campylobacter* in samples from poultry and farm workers

District	No of poultry farms	Type of farms	No of faecal samples examined	No of positive samples (%)	Samples positive for *C. jejuni* (%)	Samples positive for *C. coli* (%)	No of stool sample from poultry farm workers	Stool samples positive (%)
Ludhiana	12	LF1	5	1 (20%)	1 (20%)	0	NA	0
		LF2	5	0	0	0	NA	0
		LF3	10	0	0	0	NA	0
		LF4	10	0	0	0	NA	0
		LF5	10	1 (10%)	0	1 (10%)	NA	0
		LF6	5	0	0	0	NA	0
		LF7	5	2 (40%)	1 (20)	1 (20%)	NA	0
		LF8	9	1 (11.11%)	1 (11.11%)	0	NA	0
		BF1	10	1 (10%)	1 (10%)	0	NA	0
		BF2	10	1 (10%)	1 (10%)	0	NA	0
		BF3	10	0	0	0	NA	0
		BF4	15	2 (13.3%)	2 (13.3%)	0	NA	0
Sangrur	7	LF9	10	0	0	0	NA	0
		LF10	10	1 (10%)	1 (10%)	0	NA	0
		LF11	15	1 (6.66%)	1 (6.66%)	0	NA	0
		LF12	10	0	0	0	NA	0
		LF13	15	0	0	0	NA	0
		LF14	15	3 (20%)	2 (13.3%)	1 (6.66)	NA	0
		LF15	15	1 (6.66%)	1 (6.66%)	0	NA	0
Hoshiarpur	6	BF5	10	0	0	0	NA	0
		BF6	15	1 (6.66)	1 (6.66)	0	NA	0
		BF7	10	0	0	0	NA	0
		BF8	15	0	0	0	NA	0
		BF9	12	1 (8.33%)	1 (8.33%)	0	NA	0
		BF10	12	0	0	0	NA	0
Chandigarh	2	LF16	20	1 (5%)	1 (5%)	0	5	1 (20%)
		LF17	20	2 (10%)	1 (5%)	1 (5%)	7	1 (14.2%)
Ropar	3	LF18	3	1 (33.3%)	1 (33.3%)	0	5	0
		LF19	3	0	0	0	5	0
		LF20	3	0	0	0	5	0
Total	30		342	21 (6.14%)	17 (80.95%)	4 (19.05%)	27	2 (7.4%)

as compared to *C. coli* (Novoa Rama *et al.* 2018 and Bailey *et al.* 2019). Poultry is an important source of pathogens to humans through contaminated meat, water and the environment. In our study, farmworkers of two farms were also positive for *Campylobacter*. In some similar studies elsewhere, occupational workers were found carrying *Campylobacter*. In one study, a total of l40 fecal samples from humans who were associated with poultry were positive (7.1%) for *Campylobacter* spp., of which 9 isolates were *C. jejuni* and one was *C. coli* (Chattopadhyay *et al.* 2001). In a study by Padungtod and Kaneene (2005), they found 9 out of 197 stool samples from farmworkers associated with poultry were positive for *Campylobacter* with a 4.6% prevalence.

Antibiotic resistance in Campylobacter *spp.*: The level of resistance in the isolates was towards the lower side. Out of 21 isolates from poultry farms and 2 isolates from farmworkers, 5 (21.7%) were susceptible to all the 7 antibiotics and 14 (60.9%) resistant to only one antibiotic tested (Table 2). Eighteen isolates were resistant to either one or more antibiotics. None of the isolates was multi drug resistant (MDR). *Campylobacter* isolates from layer farms showed resistance against amikacin (4/15, 26.6%) followed by tetracycline (4/15, 26.6%). Isolates from broiler farms showed resistance against amikacin (2/6, 33.3%) followed by gentamicin (1/16, 16.6%).

The presence of antibiotic resistance in food-borne pathogens is a concern. Such pathogens when transferred to humans are capable of creating serious health issues in a patient hospitalized due to severe clinical conditions. *Campylobacter* although is a self-limiting disease but in some cases, it leads to severe complications. Variable level of resistance of *Campylobacter* spp. has been reported to the different antibiotics in different studies. Most of the studies have recorded a high level of resistance to tetracycline in conventional as well as organically raised farms due to its therapeutic use post-viral infection to counter secondary bacterial infection or due to long term use of such antibiotics resulting in widespread dissemination even if they have not been used recently (Novoa Rama *et al*. 2018 and Bailey *et al.* 2019). However, the level of resistance in the present study is low. Tetracycline is one of the preferred drugs for the treatment of urinary tract infection, skin and respiratory infections (WHO 2014). Resistance of quinolone class of antibiotic was also low in the study as only 2 isolates each were found resistant to ciprofloxacin and nalidixic acid. In contrast, there are studies reporting high (100%) and low (6.1 and 7.4%, respectively) resistance to these groups of antibiotics (Sierra-Arguello *et al.* 2018 and Bailey *et al.* 2019). This group of antibiotics are the important drugs of choice for the treatment of campylobacteriosis in humans (Moore *et al.* 2005). Low resistance to erythromycin (macrolide) was also observed similar to the observation in another study,

Table 2. Antibiotic resistance spectrum of *Campylobacter* spp. from poultry and farm workers

Isolate	Species	Farm	Antibiotic						
			GEN	ERY	AMI	CIP	TET	C	NA
Isolate 07	*C. jejuni*	LF1	S	S	S	S	R	S	S
Isolate 19	*C. coli*	LF5	S	S	R	S	S	S	S
Isolate 26	*C. jejuni*	LF7	S	S	S	S	S	R	S
Isolate 35	*C. coli*	LF7	S	S	S	R	S	S	S
Isolate 42	*C. jejuni*	LF8	S	S	R	S	S	S	S
Isolate 65	*C. jejuni*	BF1	S	S	S	S	S	S	S
Isolate 70	*C. jejuni*	BF2	R	S	S	S	S	S	S
Isolate 85	*C. jejuni*	BF4	S	S	S	S	S	S	S
Isolate 92	*C. jejuni*	BF4	S	S	R	S	S	S	R
Isolate 113	*C. jejuni*	LF10	S	R	R	S	S	S	S
Isolate 158	*C. jejuni*	LF11	S	S	S	S	S	S	S
Isolate 176	*C. coli*	LF14	S	S	S	S	S	S	R
Isolate 208	*C. jejuni*	LF14	R	S	S	S	S	S	S
Isolate 214	*C. jejuni*	LF14	R	S	R	S	S	S	S
Isolate 221	*C. jejuni*	LF15	S	S	S	S	R	S	S
Isolate 235	*C. jejuni*	BF6	S	S	R	S	S	S	S
Isolate 240	*C. jejuni*	BF9	S	S	S	S	S	R	S
Isolate 254	*C. jejuni*	LF16	S	S	S	S	S	S	S
Isolate 265H	*C. jejuni*	LF16	S	S	S	S	S	S	S
Isolate 276H	*C. jejuni*	LF17	S	S	S	S	R	S	S
Isolate 284	*C. jejuni*	LF17	S	S	S	R	R	S	S
Isolate 301	*C. coli*	LF17	S	S	R	S	S	S	S
Isolate 320	*C. jejuni*	LF18	S	S	S	S	S	R	S
Total			3	1	6	2	4	3	2
(%)			(13.04%)	(4.34%)	(26.08%)	(8.69%)	(17.39%)	(13.04%)	(8.69%)

GEN, Gentamicin; ERY, erythromycin; AMI, amikacin; CIP, ciprofloxacin; TET, tetracycline; C, chloramphenicol; NA, nalidixic acid.

which is again an important drug for human campylobacteriosis (Abrahams *et al.* 2020). Human *Campylobacter* isolates in the study were sensitive to almost all antibiotics tested in the study, except one isolate reporting resistance to tetracycline.

Genetic relatedness in Campylobacter *isolates:* Twenty-three *Campylobacter* isolates generated 11 PFGE patterns (Fig. 1, PFGE patterns represented by a single strain). The similarity level of the *Campylobacter* isolates varied from 52% to 88%. We could identify two large clusters (PFGE pattern represented by multiple strains) at the similarity level above 50% (Fig. 1). The first cluster contained patterns 1 to 7 with 14 isolates, which shared a similarity level of 76%. The second cluster was identified with patterns 8 to 11 including 9 isolates with the similarity level of 72%. The *C. jejuni* isolates were grouped into 9 unique PFGE patterns. The 19 isolates of *C. jejuni* showed high genetic diversity. On the other hand, the *C. coli* isolates were grouped into two unique PFGE patterns, indicating limited diversity among the *C. coli* strains in poultry farms of Punjab.

In a study by Han *et al*. (2016) a total of 78 *C. jejuni* and 68 *C. coli* isolates, representing isolates of different origins, species and resistance patterns were selected for PFGE analysis. Thirty-three PFGE profiles were generated among 78 isolates of *C. jejuni* and 74 isolates exhibited high genetic diversity. In contrast to *C. jejuni*, the *C. coli* isolates were divided into 8 unique PFGE patterns and 7 clusters, indicating *C. coli* isolates were more closely related than the *C. jejuni* isolates. Similarly, in other studies also high genetic variability or diversity was reported in *Campylobacter* isolates (Novoa Rama *et al*. 2018 and Garcia-Sanchez *et al.* 2020)

Fecal samples from layer and broiler farms of Punjab were investigated for *Campylobacter* spp. Overall, 6.14% of the poultry fecal samples, 53.3% of the farms and 7.41%

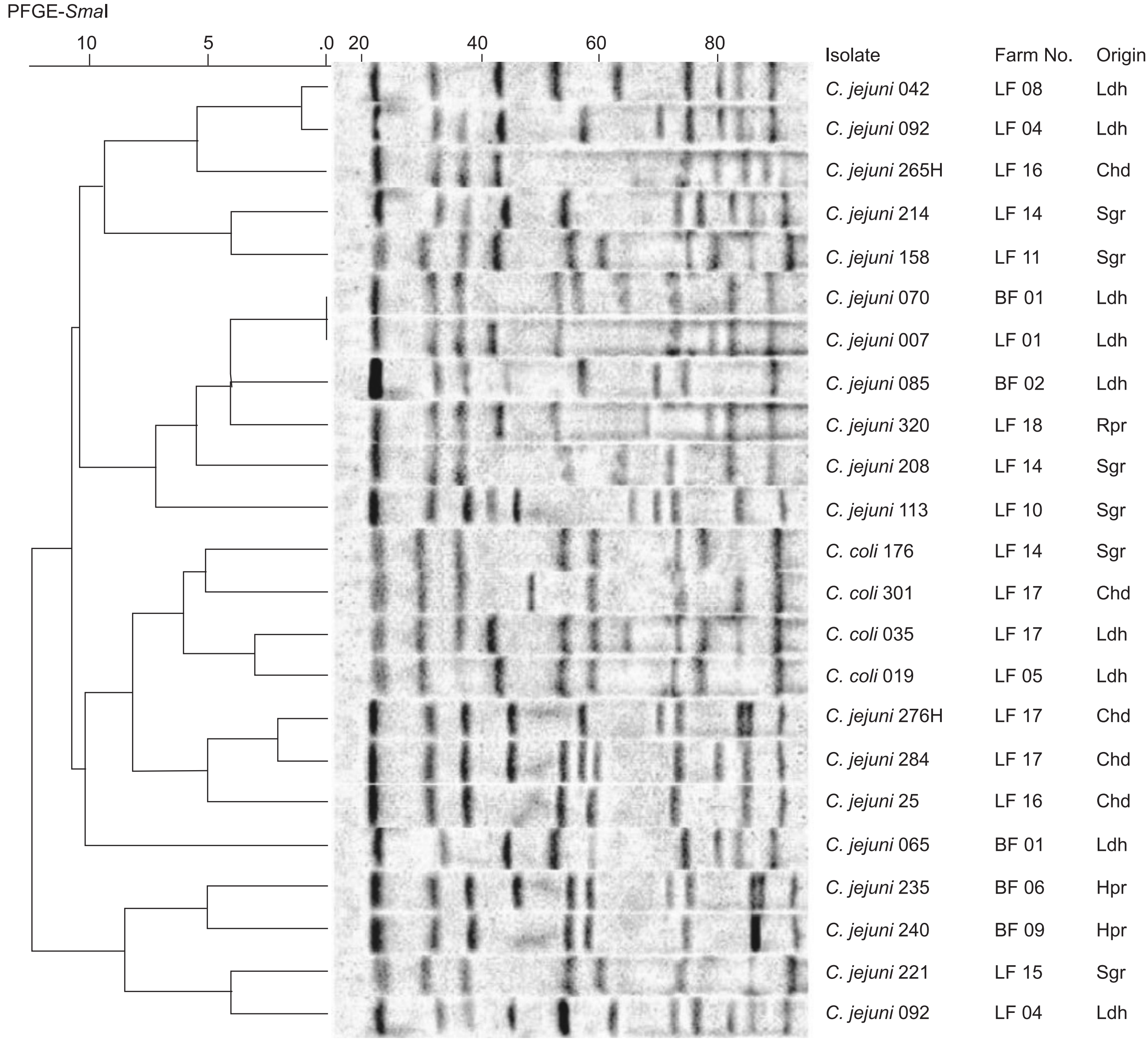

Fig. 1. Dendrogram of *Sma*I PFGE patterns of *Campylobacter* spp. from poultry and farm workers.

(2/27) farmworker samples were found positive for *Campylobacter* spp. Out of 23 isolates, 19 (80.95%) and 4(19.04%) were *C. jejuni* and *C. coli*, respectively. Antibiotic resistance in the isolates was low. The resistance to critical antibiotics used in human campylobacteriosis such as erythromycin, ciprofloxacin and nalidixic acid was also low. Genetic variability in *C. jejuni* isolates was high as compared to *C. coli* isolates and there was no correlation between the type of farm and location.

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