RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY (2011) 14:25-32
DOI: 10.2436/20.1501.01.132 ISSN: 1139-6709 **www.im.microbios.org**

Genes coding for virulence determinants of *Campylobacter jejuni* in human clinical and cattle isolates from Alberta, Canada, and their potential role in colonization of poultry

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Received 18 February 2011 · Accepted 30 March 2011

Summary. Forty nine *Campylobacter jejuni* isolates from cattle feces collected from Alberta feedlots and 50 clinical *C. jejuni* isolates from people in Alberta were tested for the presence of 14 genes encoding putative virulence factors by PCR. These included genes implicated in adherence and colonization (*flaC, cadF, docC, racR, jlpA, peb1,* and *dnaJ*), invasion (*virB11, ciaB, pldA,* and *iamA)* and protection against harsh conditions (*htrA, cbrA,* and *sodB*). The genes examined were widely distributed in both the cattle fecal isolates and the human isolates. Of the isolates tested, 67% contained all of the genes except *virB11*. The *cadF* gene was found in 100% of the isolates tested. The presence or absence of virulence-associated genes was not associated with the ability of the organism to colonize birds. All of the *C. jejuni* isolates used to challenge birds were able to colonize the animals regardless of virulence gene profile. While some diversity in the profile of the occurrence of virulence-associated genes in *C. jejuni* exists, the distribution of these putative virulence-associated genes isolates from feedlot cattle feces and humans in Alberta was similar. In addition it was not possible to predict the ability of the selected isolates to colonize young chicks based on the presence of these genes coding for virulence determinants. [**Int Microbiol** 2011; 14(1):25-32]

Keywords: *Campylobacter jejuni* · virulence genes · bacterial adherence · bacterial invasion · bacterial colonization

Introduction

Bacteria belonging to the genus *Campylobacter* are frequently isolated from people with gastrointestinal infections [3,22,40]. *Campylobacter jejuni* and *Campylobacter coli* are the two most common species isolated from human cases of campylobacteriosis [3,41], although *C. jejuni* is responsible for the majority of these infections [4]. The spectrum of disease observed in *C. jejuni*-infected individuals ranges from asymptomatic infection to severe enteritis, and may include fever, severe abdominal cramps and diarrhea with blood and mucus [2,10]. *Campylobacter jejuni* is also the infectious agent most frequently associated with the development of immunoreactive complications such as Guillain-Barré and Miller-Fisher syndromes [16], and recent evidence has revealed that *C. jejuni* infection may lead to the development of serological markers for celiac disease in people [43]. These complications enhance the significance of infection by this bacteria.

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Campylobacter jejuni can colonize a wide variety of birds and other warm-blooded animals including cattle, cats and dogs [36,37,40]. Poultry are considered the most common source of infection for people [22]. Recent studies have suggested that cattle may play a role in the epidemiology of campylobacteriosis [19,26,30]. Neilson et al. [29] reported identical typing patterns in isolates of *C. jejuni* from human and cattle. *Campylobacter jejuni* isolated from cattle are able to efficiently colonize poultry [45] suggesting that cattle could be a potential reservoir for poultry

Gastrointestinal colonization by *C. jejuni* in reservoir animals including cattle and poultry is complex and may be influenced by a variety of factors including flagella and secreted proteins [8,9,42]. *Campylobacter jejuni* has been found to colonize the mucus overlying cecal and intestinal epithelial cells in animals [9,31]. Potential virulence properties include flagella-mediated motility, adherence to intestinal mucosa, invasive and translocation capabilities, and production of toxins and secreted proteins [7,21,27,44]. Genes encoding these putative virulence determinants involved in human infection of *C. jejuni* have been identified [15,25,28]. However, only a few potential virulence factors have been investigated using defined mutants in chick colonization models [9,20], and epidemiological studies have shown a high degree of phenotypic and genotypic diversity in *C. jejuni* isolates [1,16,20].

The purpose of this study was to determine if 14 genes previously identified as coding for virulence determinants in *C. jejuni* were present in cattle feces and human clinical isolates from Alberta, Canada, and to determine the ability of these strains to colonize day-old chicks. The genes were selected based on (i) their ability for adherence and colonization of intestinal epithelial cells (*flaC* [38], *cadF* [27,45], *racR* [11], *docA* [20,23,28], *jlpA* [23], *peb1* [33] and *dnaJ* [45]); (ii) their ability to invade *in vitro* cell culture models (*virB11* [5], *ciaB* [24], *iamA* [14,28] and *pldA* [45]); and (iii) their ability to protect against harsh conditions *in vitro* growth conditions (*htrA* [12], *cbrA* [35] and *sodB* [34]).

Materials and methods

Human isolates. In 2005, eighty-eight human clinical *C. jejuni* isolates were sent to the Vaccine and Infectious Disease Organization (VIDO) from the Alberta Laboratory of Public Health, and represented people in three regional authorities in the province [19]. After stratification by feedlot, season and regional health authority of Alberta, 50 *C. jejuni* isolates were randomly selected and stored at –70°C in 25% glycerol for later virulence factor investigation and chick colonization experiments.

Bovine fecal *C. jejuni* isolates. In 2005 during a large epidemiological study in Alberta 1486 *C. jejuni* isolates from feedlot cattle feces in seven commercial feedlots were identified [19]. In brief, fresh, pen floor fecal pats were sampled using Starswab II (Starplex Scientific, Ontario, Canada) charcoal transport media swabs. Swabs were sent overnight to VIDO in Saskatoon, Saskatchewan, where each sample was streaked onto a Karmali selective agar plate, and the plates incubated at 43°C for 48–72 h under microaerophilic conditions (85% N_2, 10% CO_2 and 5% O_2). One colony considered positive for *Campylobacter* spp. from each plate (based on growth and morphology of the colony, and color of the cell mass) was selected and restreaked onto a Karmali agar plate and incubated at 42°C for a further 48 h under microaerophilic conditions.

Confirmation of bovine isolates as *C. jejuni*. Hippurate hydrolase testing was done according to the methods described by Gebhart et al. [18] with minor modifications. Briefly, a loopful of bacterial cells from the Karmali plates was emulsified in 100 µl of 1% aqueous sodium hippurate (Sigma, St. Louis, MO, USA) in a single well of a 96 well titer plate and incubated at 37°C for 2 h. After incubation, 50 µl of ninhydrin solution (3.5% ninhydrin in a 1:1 mixture of acetone and butanol) was slowly added to each well and re-incubated for 30 min at 37°C. A positive hippurate hydrolase test was based on the presence of any purple color in the wells, while clear to yellow wells indicated a negative test. Forty nine bovine isolates were randomly selected from all of the hippurate positive isolates after stratification by feedlot and season. These selected isolates had their identity as *C. jejuni* confirmed using multiplex PCR. Published PCR primers for *Campylobacter* spp. (23S rRNA), *C. jejuni* (*hipO*), and *C. coli* (*glyA*) were used, with initial denaturation at 95°C for 30 s, annealing at 59°C for 30 s, and two extension steps at 30 s and 7 min at 72°C. *Campylobacter jejuni* and *C. coli* controls were included in each PCR. Isolates identified as *C. jejuni* using both hippurate hydrolase testing and multiplex PCR were frozen at –70°C in 25% glycerol for virulence factor investigation and chick colonization studies.

***Campylobacter jejuni* reference strains and growth condition.** One hundred and two *C. jejuni* strains including 49 isolates from bovine feces, 50 human clinical isolates and three reference strains were tested in this study. Reference strains included two human clinical isolates NCTC 11168 [9,13,32] and 81–176 [20,21], and one animal isolate RM1221 [17]. All strains were grown on Mueller-Hinton agar (Oxoid) at 42°C under microaerophilic conditions (85% N_2, 10% CO_2 and 5% O_2) for 20 h.

DNA preparation. Chromosomal DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega). A loopful of fresh culture of *C. jejuni* was suspended in 600 µl of lysis buffer and mixed by gentle pipetting. The lysate was incubated at 80ºC for 5 min and allowed to cool to room temperature. Then 3 µl of RNAse solution was added and incubated at 37ºC for 1 h. After incubation, 200 µl of protein precipitation buffer was added and centrifuged at 13,000 ×*g* for 5 min. The supernatant was transferred to a tube containing 600 µl of isopropanol and centrifuged at 13,000 ×*g* for 5 min to sediment the DNA. Finally, the DNA pellet was washed with 70% ethanol and rehydrated in 100 µl of rehydration solution.

PCR primer design and amplification. The genes tested in this study, the primers used and annealing temperatures are listed in Table 1. All PCR amplifications were performed in a 50 µl mixture consisting of 5.0 µl 10X Taq buffer (GE Health Care), 0.5 µl Taq (GE Health Care), 4.0 µl dNTPs (dATP, dCTP, dTTP and dGTP each at 2.5 mM, GE Health Care), 1.0 µl extracted template DNA and 0.5 µl of a 20 pM solution of each primer. The reaction mixture was amplified using standardized cycling variables: initial denaturation at 95ºC for 1 min followed by 35 cycles of denaturation at 95ºC

for 30 s, variable annealing temperatures (Table 1) for 1 min, primer extension at 72ºC for 1 min and final extension at 72°C for 5 min. The results were visualized by electrophoresis on 2% agarose gels. Bands were stained with ethidium bromide and viewed using a Multi Image Light Cabinet (Alpha Innotech, USA).

Colonization of chickens. The ability of selected isolates to colonize one-day-old chicks was tested as previously described [13]. Briefly, 225 broiler chicks were obtained from a local hatchery in Saskatchewan on the day of hatch. Five chicks were euthanized and their cecal contents cultured for *Campylobacter* spp. The remaining birds were randomly assigned into groups

Table 1. Primers used for PCR analysis of human clinical and cattle feces isolates of *Campylobacter jejuni*

Primers	Sequence (5′→ 3′)	Annealing T (°C)	Product (bp)	Ref.
dnaJ-f	AAGGCTTTGGCTCATC	46	720	Dutta et al. [15]
dnaJ-r	CTTTTTGTTCATCGTT			
pldA-f	AAGCTTATGCGTTTTT	45	913	Dutta et al. [15]
pldA-r	TATAAGGCTTTCTCCA			
iaB-f	TTTCCAAATTTAGATGATGC	45	1165	Konkel et al. [24]
ciaB-r	GTTCTTTAAATTTTTCATAATGC			
jlpA-f	TAATACGACTCACTATAGGGGCCCATTAACATAGAAAACATGATA	50	1095	This study
jlpA-r	AATAGCTGTTTTGTTTTCAGCTTGC			
flaC-f	TAATACGACTCACTATAGGGATGATGATCTCTGATGCAACTATGA	50	712	This study
flaC-r	AAAGCAGCAGCATTTTCTTTTAGAT			
peb1-f	TAATACGACTCACTATAGGGGAAAATCTTTGTTAAAGTTGGCAGT	50	775	This study
peb1-r	TTTTCGCTAAAGCATCAATTTCATT			
docA-f	ATAAGGTGCGGTTTTGGC	50	725	Muller et al. [28]
docA-r	GTCTTTGCAGTAGATATG			
sodB-f	ATGATACCAATGCTTTTGGTGATTT	50	638	This study
sodB-r	TAATACGACTCACTATAGGGCATTTGCATAAAAGCTAACTGATCC			
cbrA-f	TAATACGACTCACTATAGGGTCAACTCTATCCTTGCCATTATCTT	50	1165	This study
cbrA-r	GTAGATATTGCTTTTGGTTTTGCTG			
htrA-r	TAATACGACTCACTATAGGGTAAGTTTAGCAAGTGCTTTATTTGC	50	1393	This study
htrA-f	AAAACCATTGCGATATACCCAAACT			
racR-f	GATGATCCTGACTTTG	45	584	Dutta et al. [15]
racR-r	TCTCCTATTTTTACCC			
iamA-f	GCACAAAATATATCATTACAA	52	518	Muller et al. [28]
iamA-r	TTCACGACTACTATGAGG			
virB11-f	TCTTGTGAGTTGCCTTACCCCTTTT	60	494	Kodinas et al. [25]
virB11-r	CCTGCGTGTCCTGTGTTATTTACCC			
cadF-f	TTGAAGGTAATTTAGATATG	45	400	Konkel e t al. [24]
cadF-r	CTAATACCTAAAGTTGAAAC			

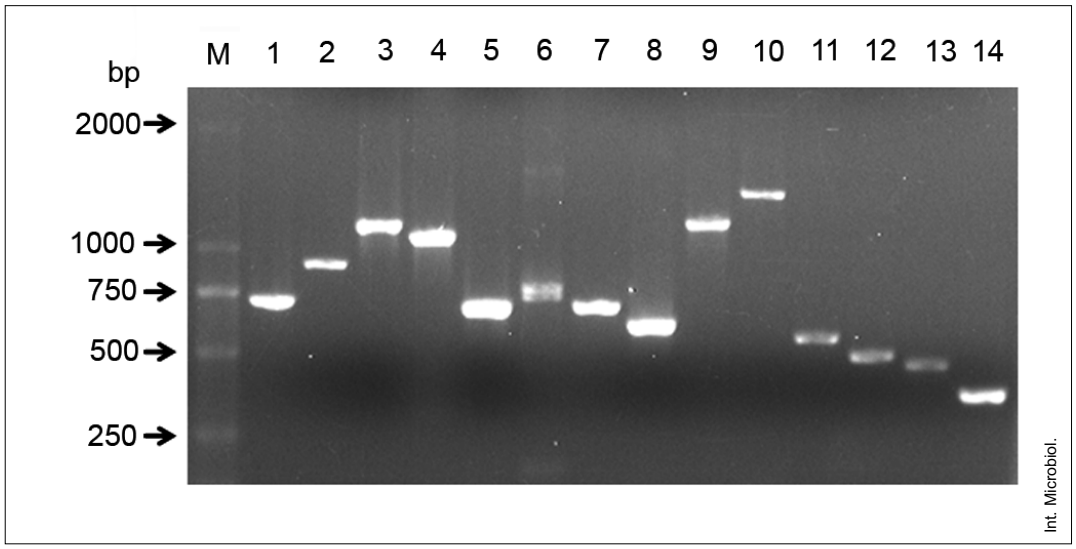

Fig. 1. Agarose gel electrophoresis of PCR products of *Campylobacter jejuni* genes coding for virulence determinants. Lanes: M, Gel Pilot Mid Range Ladder (250–2000 bps, Qiagen); 1, *dnaJ*; 2, *pldA*; 3, *ciaB*; 4, *jlpA*; 5, *flaC*; 6, *peb1*; 7, *docA*; 8, *sodB*; 9, *cbrA*; 10, *htrA*; 11, *racR*; 12, *iamA*; 13, *virB11*; 14, *cadF*.

of 20 birds and provided with feed and water *ad libitum*. Birds were cared for in accordance to guidelines of the Canadian Council for Animal Care.

Chicks were orally challenged with the test strains using 1.5×10^7 colony forming units (CFU) of *C. jejuni* in 0.5 ml of normal saline. Inocula for challenge experiments were produced by harvesting cells grown for 18 h under microaerophilic conditions at 37°C into cold 0.85% weight per volume (w/v) NaCl, diluting the cells to the indicated concentration in normal saline and maintaining the cells on ice until immediately before use. Viable cell counts were determined by plating serial dilutions onto Mueller-Hinton agar (Becton-Dickinson). Birds were maintained for seven days after challenge and then euthanized by cervical dislocation. Ceca were aseptically collected for qualitative as well as quantitative assessment of colonization. Intestinal colonization was established by culturing cecal contents (after appropriate dilution in 0.85% w/v NaCl) on Karmali agar (Bacto) under microaerophilic conditions at 42°C. On the basis of PCR results, 5 human clinical *C. jejuni* isolates and 5 cattle isolates were selected for inclusion in the chick colonization model. In addition, *C. jejuni* NCTC 1168 strain was chosen as a reference strain for the colonization model as it is well characterized and is known to be an efficient colonizer of the avian gastrointestinal tract [9,13].

Results

A total of 102 *C. jejuni* isolates including 49 human isolates, 50 cattle clinical isolates, and three reference strains were screened for the presence of 14 virulence-associated genes. The electrophoretic patterns of the amplified PCR products for these genes are shown in Fig. 1. With the exception of *virB11,* the genes examined were widely distributed in both the bovine fecal isolates and the human clinical isolates. While *virB11* was identified only sporadically (6% of human clinical isolates and 12% of cattle feces isolates), 67% of the isolates tested contained all of the other 13 putative virulence genes (Table 2). One of the 14 virulence-associated genes, *cadF,* was detected in all strains, and only 8% of isolates con-

Table 2. Presence of pathogenic genes in *Campylobacter jejuni* from clinical and carttle isolates

	Percentage of isolates testing positive													
Sources of *C. jejuni* (no. isolates)	*dnaJ*	*pldA*	*ciaB*	*jlpA*	*flaC*	*peb1*	*docA*	*sodB*	*cbrA*	*htrA*	*racR*	*iamA*	*virB11*	*cadF*
Human clinical isolates[a] (52)	94.23	94.23	92.31	96.15	88.46	88.46	94.23	94.23	92.31	92.31	94.23	92.31	5.77	100
Cattle feces isolates[b] (50)	90	90	90	90	94	94	88	98	90	86	92	94	12	100
Total (102)	92.16	92.16	91.18	93.14	91.18	91.18	91.18	96.08	91.18	89.22	93.14	93.14	8.82	100

[a]Clinical isolates included two reference strains: NCTC11168 and 81-176.
[b]Animal isolates included one reference strain RM1221.

Table 3. Prevalence of virulence genes in bovine and human isolates tested for the ability to colonize poultry

		Presence or absence of genes												
Sources	Strains	*dnaJ*	*pldA*	*ciaB*	*jlpA*	*flaC*	*peb1*	*docA*	*sodB*	*cbrA*	*htrA*	*racR*	*iamA*	*virB11*	*cadF*
Human	4174														
Human	4163													N[a]	
Human	4198								N						
Human	4196	N				N	N				N		N	N	
Human	4183	N	N	N	N	N	N	N	N	N	N	N	N	N	
Bovine	1645													N	
Bovine	2440	N		N			N			N	N			N	
Bovine	945		N	N	N	N		N	N	N	N	N	N	N	
Bovine	179	N	N					N		N	N	N	N	N	
Bovine	2650														
Human	NCTC11168[b]													N	

[a]N indicates the absence of the gene as determined by PCR.
[b]NCTC 11168 was used as a reference strain.

tained all of the 14 genes examined. Genes coding for various virulence determinants such as adherence *(dnaJ* and *jlpA*), invasion *(pldA* and *ciaB)* and protection against harsh condition (*htrA)* were identified less frequently in the cattle feces isolates than in the human clinical isolates. Only three virulence-associated genes (*flaC, peb1* and *sodB*) were detected in more of the cattle feces isolates than in the clinical human isolates (Table 2).

In the chick colonization model, all birds challenged were colonized by both human clinical and bovine *C. jejuni* isolates (Table 3), and the colonized birds appeared healthy with no signs of disease. Nine of the 10 *C. jejuni* isolates studied colonized all twenty chicks (100%), and bacterial loads from the test isolates were of levels similar to those of the reference NCTC 11168 strain. Only bovine isolate (179) colonized 80% birds at a median level lower than the *C. jejuni* NCTC 11168 strain (data not shown). This bovine isolate lacked most of the virulence-associated genes including adherence and colonization genes *racR*, *docA* and *dnaJ,* invasion genes *iamA, virB11* and *pldA,* and stress genes *htrA* and *cbrA.* However, other genes involved in adherence and secretory protein production (*cadF, peb1, flaC, jlpA* and *ciaB*) were identified (Table 3). Two isolates (human 4183 and bovine 945) lacked several virulence-associated genes but were still able to colonize chicks to the same extent as the control *C. jejuni* strain NCTC 11168. In addition, seven *C. jejuni* isolates lacking the *virB11* gene and three isolates lacking the *flaC* gene were also able to colonize chicks effectively.

Discussion

Control and prevention of campylobacteriosis in humans require knowledge of virulence factors, infection mechanisms and risk factors [31]. While poultry is considered to be the primary source of *Campylobacter* for people [20,45], it is also important to establish the potential role of other reservoir hosts in the epidemiology of campylobacteriosis. A large epidemiological study in Alberta revealed that *Campylobacter* including *C. jejuni* are commonly found in the feces of feedlot cattle [19]. In addition, genetic similarity between human clinical *C. jejuni* isolates collected within the same geographical areas and during the same time frame as the feedlot cattle survey was observed using DNA microarray [19]. The purpose of our study was to determine if *C. jejuni* genes previously established to be involved in the colonization of poultry and people could be identified in the feedlot cattle and human clinical *C. jejuni* isolates from Alberta. As

a means of evaluating the clinical attributes of these strains, selected isolates were chosen to colonize 1-day-old chicks.

This study described the patterns of virulence-associated genes in 102 strains; 51 animal isolates and 51 human clinical isolates. The results suggest that diversity in the profile of virulence-associated genes from *C. jejuni* exists; however the distribution of these genes was relatively similar in both human clinical and bovine isolates. This is in contrast to other published studies, in which proportionally higher numbers of virulence-associated genes were identified in human clinical isolates [15,39]. This research also contrasted to previously published data in which the prevalence of various genes coding for virulence determinants (*pldD, ciaB, dnaJ and racR*) in their isolates reached 100% [15,39].

Our results identified one virulence-associated gene, *cadF*, in 100% of strains tested as has been observed by Bang et al. [6], Datta et al. [15] and Talukder et al. [39]. This gene has been reported to encode a fibronectin-binding outer-membrane protein [27], and is suspected to be involved in bacterial adhesion to the extracellular matrix of the gastrointestinal epithelium [27]. As such, this gene may be required for intestinal colonization. Note that the gene with the lowest prevalence among the *C. jejuni* isolates tested was the plasmid-associated gene *virB11*. Only 6% of the human *C. jejuni* isolates contained this gene, compared to 12% of the cattle isolates. *VirB11* mutant strains have been shown to have reduced virulence with regard to adhesion and invasion in vitro and to cause milder clinical manifestations in infected animals [5]. Our data support recent reports that suggest that this gene is more often absent in human isolates [6,15,39], and therefore may not be as important a virulence factor in human cases of infection.

Similar proportions of some virulence-associated genes were observed when cattle feces and human clinical *C. jejuni* isolates were compared, including genes putatively responsible for: adherence and colonization of intestinal epithelial cells (periplasmic cytochrome C peroxidase *docA*, flagellar filamentous protein, *flaC*); the up regulation of the two-component system response regulator protein (*racR*); the surface lipoprotein (*jlpA*); the outer membrane receptor protein (*peb1*); and the heat shock protein (*dnaJ*). In addition, the detection rates for *ciaB* (gene for a secreted protein), *iamA* and *pldA* (invasion-associated genes), and *htrA, cbrA* and *sodB* (protection genes against harsh conditions) were also relatively similar in cattle and human isolates. As people often become clinically ill from *Campylobacter* infections while cattle may carry *Campylobacter* in their intestinal tract without evidence of clinical disease, the similar proportions of these genes in the strains from the two sources does little to identify useful potential gene targets for future research related to strain virulence.

Previous gene manipulation studies have revealed that some of the genes examined in this study (*sodB, racR, ciaB, flaC, virB11* and *cadF*) may be involved specifically in *C. jejuni*-host interactions [5,23,45]. It has been reported that manipulation of flagellar genes (*sodB, racR* and *cadF)* may reduce the colonization ability of *C. jejuni* strains [20,45]. However, our PCR results suggested that the presence of these virulence-associated genes did not influence the colonization ability of *C. jejuni* strains in poultry. Only one isolate tested in this study was a poor colonizer in our chick colonization model. In addition, isolates from both human and bovine sources that lacked several virulence associated genes (*flaC*, *virB11, sodB* and *racR*) were as effective colonizers as the control strain. Our screening revealed that the absence of the *virB11* gene did not reduce the colonization ability of several *C. jejuni* isolates, while three of 10 *C. jejuni* isolates that lacked the flagellar gene *flaC* colonized well.

The expression of virulence is multifactorial, complex and dynamic, and future studies using molecular techniques with higher resolution and discriminatory power may be beneficial in exploring *C. jejuni* virulence-associated genes. In this study, we used PCR (based on known genetic sequences) to detect the presence or absence of virulence-associated genes in the cattle and human *C. jejuni* isolates; however this technique does not evaluate gene function. PCR does not give information about expression levels, nor does it describe deletions or insertions in the gene sequence; all of which may contribute to virulence. In addition, we chose to explore only a subset of putative *C. jejuni* virulence-associated genes, those involved in infection, and as virulence determinants research continues it might be found that other genes or combinations of genes should be targeted. However, the results of this study add insight into the distribution of our chosen genes in cattle and human strains in Alberta, and describe the ability of these strains to colonize poultry.

In conclusion, our results suggest that while diversity in the profile of virulence-associated genes from *C. jejuni* exists, the detection rates of many of these genes examined were similar in cattle feces and human clinical *C. jejuni* isolates from Alberta. In addition, the presence or absence of the 14 virulence-associated genes investigated was not predictive of the ability of selected isolates to colonize chicks. The results suggest that research should continue into targets for genes that may help to explain virulence differences between human and animal *C. jejuni* strains. Transmission of campy-

lobacter in Alberta is probably a complex web involving poultry, livestock, wild animals and birds, people and the environment. This study also suggests that strains of *C. jejuni* isolated from feedlot cattle may be effective colonizers of poultry; and that cattle may play a role in the epidemiology of *Campylobacter* in Alberta.

Acknowledgements. Financial support for this study has been provided by the Canadian Poultry Research, Avian Gut Microbiology program, the Council Agriculture Council of Saskatchewan through the Advancing Canadian Agriculture and Agri-Food Saskatchewan (ACAAFS) program. Funding for the ACAAFS program is provided by Agriculture and Agri-Food Canada. We also gratefully acknowledge funding and support from the Natural Sciences and Engineering Research Council (NSERC; A.P. holds an NSERC senior industrial research chair in food and water safety vaccines), B.C. Cattlemen's Association, Vétoquinol, and Western College of Veterinary Medicine interprovincial graduate student fellowship. Published with the permission of the Director of VIDO as journal series #566.

Competing interests. None declared.

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