Identification of Possible Virulence Marker from *Campylobacter jejuni* Isolates

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A novel protein translocation system, the type-6 secretion system (T6SS), may play a role in virulence of *Campylobacter jejuni*. We investigated 181 *C. jejuni* isolates from humans, chickens, and environmental sources in Vietnam, Thailand, Pakistan, and the United Kingdom for T6SS. The marker was most prevalent in human and chicken isolates from Vietnam.

Campylobacter species are the principal bacterial cause of human foodborne enterocolitis worldwide (*1*). Despite the global significance of *C. jejuni* as a leading cause of diarrheal disease (*2*), the mechanisms of pathogenesis of *C. jejuni* are not well understood. Research on *Campylobacter* epidemiology has largely been conducted in high-income countries and therefore may not be representative of global patterns.

Recently, a novel class of protein translocation system was identified in gram-negative bacteria. This system, named the type-6 secretion system (T6SS), has been found to play roles in pathogen–pathogen and host–pathogen interactions and has a major effect on virulence in a range of pathogens, including *Vibrio cholerae* (*3*–*6*) (reviewed in *7*,*8*). A functional T6SS was recently identified in *C. jejuni* (*9*,*10*) and found to have several roles in virulence, influencing cell adhesion, cytotoxicity toward erythrocytes, and colonization of mice (*9*,*10*). However, it is unknown whether T6SS changes the effects of these pathogens in human infection.

In this study, we aimed to determine whether presence of T6SS in *C. jejuni* is potentially a marker associated with more severe human disease. Moreover, because human infection with *C. jejuni* is often associated with contact with poultry, we investigated whether poultry harbor *C. jejuni* that possess T6SS.

The Study

To partially address bias toward study of *C. jejuni* strains from high-income countries and the under-representation of strains from Asia in previous studies, we previously sequenced the genomes of 12 clinical isolates of *C. jejuni* from Asia: 4 from Thailand, 3 from Pakistan, and 5 from Vietnam (J. Harrison, unpub. data; Figure 1). We noted that 8 (67%) of these isolates possessed a cluster of genes homologous to the recently described T6SS (Figure 1). This finding was in contrast to findings regarding previously sequenced *C. jejuni* genomes; only 10 (14%) of 71 previously sequenced *C. jejuni* strains possessed an apparently intact T6SS gene cluster (Figure 1; full listing of genomes is in online Technical Appendix Table 1, wwwnc.cdc.gov/EID/article/20/6/13-0635-Techapp1.pdf). Several other strains from our study and previously sequenced strains contained ≥1 T6SS genes but not a complete T6SS cluster. Figure 1 shows the presence and absence of each T6SS gene in each available genome sequence (J. Harrison, unpub. data) and the previously sequenced strains. A nonrandom distribution of T6SS can be seen across the phylogenetic diversity of *C. jejuni*; T6SS is limited to certain clades, and degeneration of the T6SS gene cluster apparently occurs in parallel within several of those clades (Figure 1).

Our genome sequencing analysis indicated that strains possessing a complete T6SS cluster could be distinguished by the presence of the *hcp* gene (Figure 1) (*9*,*10*). Therefore, we used *hcp* as a proxy for determining the presence of a functional T6SS in 181 *C. jejuni* isolates from chickens, humans, and environmental sources (collections of the Oxford University Clinical Research Unit and the University of Exeter; online Technical Appendix Table 2). We designed and used a multiplex PCR (online Technical Appendix Table 3) to screen for the presence of *hcp* in these isolates; the conserved *C. jejuni* housekeeping gene, *gltA*, was used as a positive control.

Of the 181 isolates, 28 originated from chickens in the United Kingdom and 21 from chickens in Vietnam. The *hcp* gene was found significantly more often in isolates

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DOI: http://dx.doi.org/10.3201/eid2006.130635

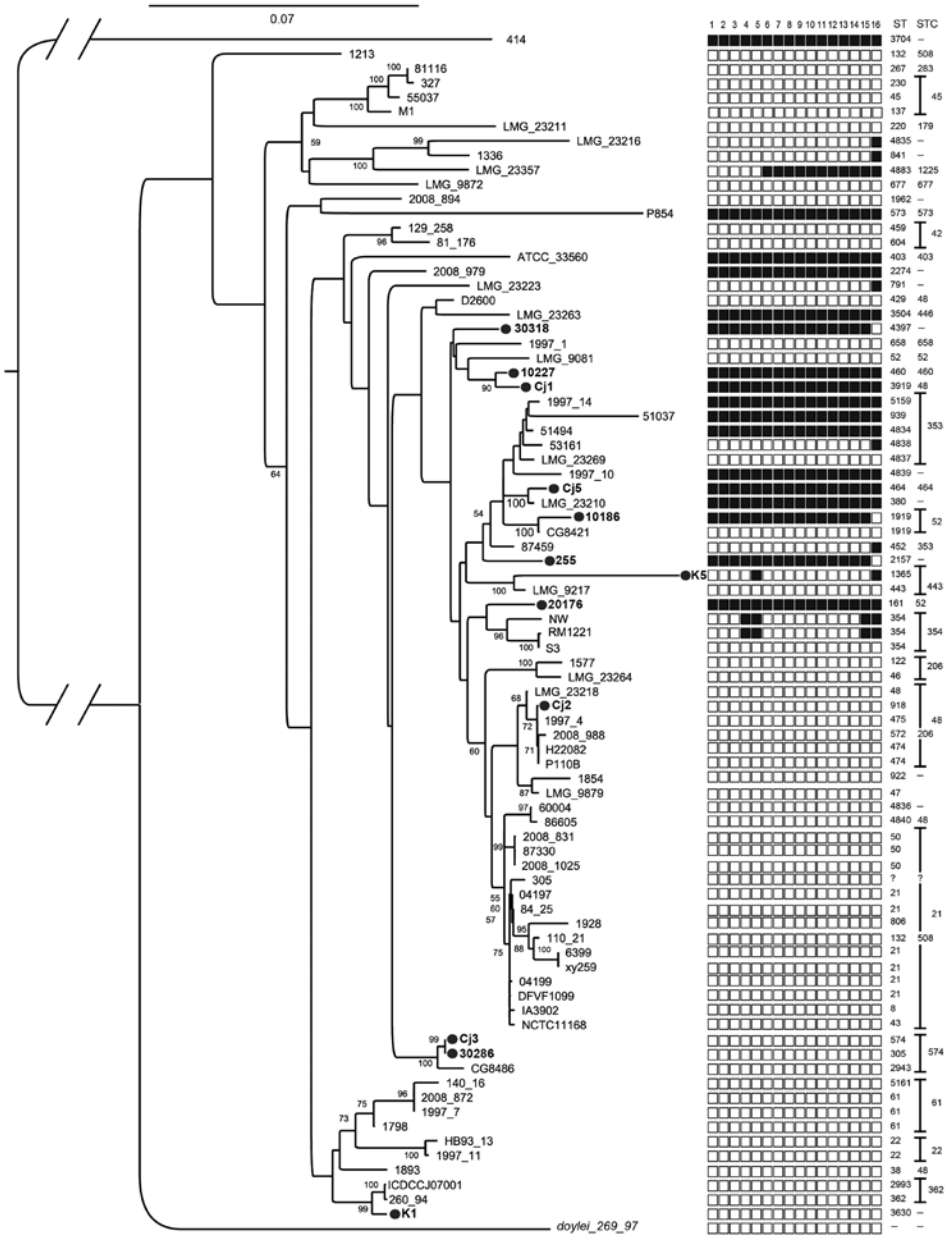

Figure 1. Distribution of the type-six secretion system (T6SS) marker across the phylogenetic diversity of *Campylobacter jejuni* strains, as determined by multilocus sequence analysis. We generated a maximum-likelihood tree from concatenated nucleotide alignments of 31 housekeeping genes; nucleotide sequences were aligned by using MUSCLE (www.drive5.com/muscle) and masked by using GBLOCKS (http://molevol.cmima.csic.es/castresana/Gblocks.html). Maximum-likelihood analysis was done by using the GTR model in PhyML (http://code.google.com/p/phyml/). Numbers on nodes denote bootstrap values (1,000 bootstrap replicates); values <50 are not shown. Black circles indicate strains whose genomes were sequenced in this study (GenBank accession nos. AUUQ00000000, AUUP00000000, AUUO00000000, AUUN00000000, AUUM00000000, AUUL00000000, AUUK00000000, AUUJ00000000, AUUI00000000, ARWS00000000, AUUH00000000, AUUG00000000). We inferred the presence/absence of each of the T6SS genes on the basis of TBLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch) searches against the predicted proteins sequences from *C. jejuni* strain 414 (National Center for Biotechnology Information reference sequence no. NZ_CM000855). Presence or absence of each gene is indicated by a black or white square, respectively, for each strain: column 1, *hcp*; column 2, *icmF*_1; column 3, *icmF*_2; column 4, *vasK*; column 5, *FHA*; column 6, *vasF*; column 7, *vasE*; column 8, *vasD*; column 9, *impA*; column 10, *impD*; column 11, *impC*; columns 12 and 13, conserved hypotheticals; column 14, *vasA*; column 15, *vasB*; column 16, *vgrg*. The sequence type (ST) and ST complex (STC) columns represent global multilocus sequence types as described by the Oxford multilocus sequence typing scheme (http://pubmlst.org). ?, unknown ST; –, isolate could not be allocated to a specific ST or STC. Scale bar indicates nucleotide substitutions per site. Further details of the isolates are provided in online Technical Appendix Table 2 (wwwnc.cdc.gov/EID/article/20/6/13-0635-Techapp1.pdf).

from Vietnam (15 [71.4%] isolates) than in those from the United Kingdom (1 [3.5%] isolate) ($p<0.01$ by 2-sample *Z*-test; online Technical Appendix Figure 1). An additional 38 of the isolates were from humans in the United Kingdom and 33 from humans in Vietnam; again, the *hcp* gene was significantly more prevalent in isolates from Vietnam (20 [60.6%] isolates) than those from the United Kingdom (1 [2.6%] isolate) ($p<0.01$ by 2-sample *Z*-test; online Technical Appendix Figure 2).

We also found that patients infected with *hcp*-positive *C. jejuni* experienced bloody diarrhea more commonly than those infected with *hcp*-negative *C. jejuni*. For the 36 isolates for which detailed clinical data on patients were available, 6 (31.6%) of 19 patients in Vietnam who were infected with *hcp*-positive *C. jejuni* had bloody diarrhea, compared with 1 (5.9%) of 17 patients infected with *hcp*-negative *C. jejuni* ($p<0.05$ by 2-sample *Z*-test) (Figure 2). These results suggest a potential correlation between T6SS and bloody diarrhea, a serious clinical manifestation of the infection that results in higher rates of hospitalization and greater need for treatment with antimicrobial drugs (*11*). Moreover, *Campylobacter*-related septicemia developed in the 1 patient in the United Kingdom who was infected with a T6SS-positive strain (*11*). These data suggest that infection with the *C. jejuni* T6SS genotypic strains is associated with more severe disease. However, for sample bias to be ruled out, a comprehensive study is required in which the prevalence of T6SS is measured in *C. jejuni* samples from patients with mild and severe forms of infection.

We found a number of *C. jejuni* strains from humans and poultry that possessed the T6SS cluster, although some strains showed a slightly modified gene order (online Technical Appendix Table 1 and Figure 3). However, most (61 [85.9%] of 71) of the previously sequenced *C. jejuni* isolates lacked a complete T6SS gene cluster (Figure 1); this finding might explain why T6SS was not discovered in *C. jeuni* sooner. Conversely, our PCR-based study frequently identified the *hcp* marker in isolates from Thailand, Pakistan, and Vietnam (Table). We cannot be certain that all of the isolates with the *hcp* marker possessed a complete and functional T6SS gene cluster, but the *hcp* gene is consistently associated with the presence of a complete T6SS cluster in all available sequenced *C. jejuni* genomes (Figure 1). This correlation lends confidence to the use of *hcp* as a proxy.

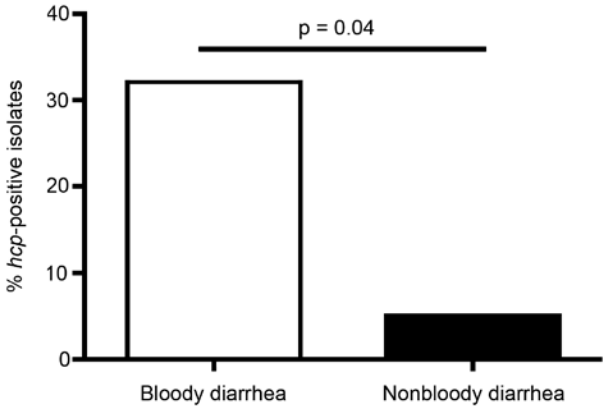

Figure 2. Percentage of *hcp*-positive *Campylobacter jejuni* strains isolated from patients in Vietnam who had bloody diarrhea and nonbloody diarrhea. Patients who were hospitalized because of *C. jejuni* infection were scored for the presence of bloody diarrhea or nonbloody diarrhea, and presence of the *hcp* type-six secretion system (T6SS) marker in strains isolated from the patients was determined. Of patients with bloody diarrhea, 32% were infected with *hcp*-positive strains; of patients with nonbloody diarrhea, 5% were infected with *hcp*-positive strains.

Poultry are a well-documented reservoir of human *Campylobacter* infection (*12*). We found that *Campylobacter* strains harboring the *hcp* marker were significantly associated with chickens in Asia. Large numbers of poultry are imported into North America and Europe from low-income countries, including Thailand (*13*). This process could introduce T6SS-positive *Campylobacter* genotypes into the food chains of the importing countries, posing a potential emerging threat to public health.

Conclusions

Our results suggest that the T6SS may be more prevalent in *C. jejuni* in Vietnam, Pakistan, and Thailand than in the United Kingdom. Furthermore, our results suggest that *hcp* may be a marker associated with severe human disease caused by *C. jejuni* infection in Vietnam, although there is no evidence that the association is causal. Chickens imported from these countries could be a source of *hcp*-positive strains and may have the potential to cause severe human infection.

Table. Overview of *Campylobacter jejuni* strains containing type-six secretion system genetic marker *hcp*, by country and isolate source

	No. *hcp*-positive strains/total no. strains (%)				
Isolate source	United Kingdom	Vietnam	Pakistan	Thailand	Total
Human	1/38 (2.6)	20/33 (60.6)	2/13 (15.4)	1/3 (33.3)	24/87 (27.6)
Chicken	1/28 (3.9)	15/21 (71.4)	1/2 (50)	0	17/51 (33.3)
Other	5/26 (19.2)	1/14 (7.1)	1/3 (33.3)	0	7/43 (16.3)
Total	7/92 (7.6)	36/68 (54.4)	4/18 22.2)	1/3 (33.3)	48/181 (26.5)

Acknowledgments

We thank Konrad Paszkiewicz and Karen Moore for assistance with whole-genome sequencing.

The work was partly supported by the UK Biotechnology and Biological Sciences Research Council, award BB/1024631/1 to R.T., D.S., and O.C.; by a Wellcome Trust Institutional Strategic Support Award (WT097835MF); and by a studentship awarded to J.H.

Mr Harrison is a PhD student at the University of Exeter under the supervision of D.S. His research focuses on using bioinformatic methods to investigate the comparative genomics of emerging diseases and plant-associated microbes.

References

1. Adak GK, Meakins SM, Yip H, Lopman BA, O'Brien SJ. Disease risks from foods, England and Wales, 1996–2000. Emerg Infect Dis. 2005;11:365–72. http://dx.doi.org/10.3201/eid1103.040191
2. Allos BM. *Campylobacter jejuni* infections: update on emerging issues and trends. Clin Infect Dis. 2001;32:1201–6. http://dx.doi.org/10.1086/319760
3. Das S, Chakrabortty A, Banerjee R, Roychoudhury S, Chaudhuri K. Comparison of global transcription responses allows identification of *Vibrio cholerae* genes differentially expressed following infection. FEMS Microbiol Lett. 2000;190:87–91. http://dx.doi.org/10.1111/j.1574-6968.2000.tb09267.x
4. Ishikawa T, Sabharwal D, Bröms J, Milton DL, Sjöstedt A, Uhlin BE, et al. Pathoadaptive conditional regulation of the type VI secretion system in *Vibrio cholerae* O1 strains. Infect Immun. 2012;80:575–84. http://dx.doi.org/10.1128/IAI.05510-11
5. Parsons DA, Heffron F. sciS, an icmF homolog in *Salmonella enterica* serovar *Typhimurium*, limits intracellular replication and decreases virulence. Infect Immun. 2005;73:4338–45. http://dx.doi.org/10.1128/IAI.73.7.4338-4345.2005
6. Pukatzki S, Ma AT, Sturtevant D, Krastins B, Sarracino D, Nelson WC, et al. Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. Proc Natl Acad Sci U S A. 2006;103:1528–33. http://dx.doi.org/10.1073/pnas.0510322103
7. Cascales E. The type VI secretion toolkit. EMBO Rep. 2008;9:735–41. http://dx.doi.org/10.1038/embor.2008.131
8. Mulder DT, Cooper CA, Coombes BK. Type VI secretion system-associated gene clusters contribute to pathogenesis of *Salmonella enterica* serovar *Typhimurium.* Infect Immun. 2012;80:1996–2007. http://dx.doi.org/10.1128/IAI.06205-11
9. Lertpiriyapong K, Gamazon ER, Feng Y, Park DS, Pang J, Botka G, et al. *Campylobacter jejuni* type VI secretion system: roles in adaptation to deoxycholic acid, host cell adherence, invasion, and in vivo colonization. PLoS ONE. 2012;7:e42842. http://dx.doi.org/10.1371/journal.pone.0042842
10. Bleumink-Pluym NMC, van Alphen LB, Bouwman LI, Wösten MMSM, van Putten JPM. Identification of a functional type VI secretion system in *Campylobacter jejuni* conferring capsule polysaccharide sensitive cytotoxicity. PLoS Pathog. 2013;9:e1003393. http://dx.doi.org/10.1371/journal.ppat.1003393
11. Kuşkonmaz B, Yurdakök K, Yalçin SS, Ozmert E. Comparison of acute bloody and watery diarrhea: a case control study. Turk J Pediatr. 2009;51:133–40.
12. Harris NV, Weiss NS, Nolan CM. The role of poultry and meats in the etiology of *Campylobacter jejuni/coli* enteritis. Am J Public Health. 1986;76:407–11. http://dx.doi.org/10.2105/AJPH.76.4.407
13. Food and Agriculture Organization of the United Nations. Agribusiness handbook: poultry meat and eggs. 2010 [cited 2013 Apr 1]. http://www.fao.org/fileadmin/user_upload/tci/docs/1_AH9-Poultry%20Meat%20&%20Eggs.pdf

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