


Helicobacter pullorum Isolated from Fresh Chicken Meat: Antibiotic Resistance and Genomic Traits of an Emerging Foodborne Pathogen

Vítor Borges,^a Andrea Santos,^b Cristina Belo Correia,^c Margarida Saraiva,^c Armelle Ménard,^{d,e} Luís Vieira,^f Daniel A. Sampaio,^f Miguel Pinheiro,^g João Paulo Gomes,^a  Mónica Oleastro^b

Bioinformatics Unit, Department of Infectious Diseases, National Institute of Health, Lisbon, Portugal^a; National Reference Laboratory for Gastrointestinal Infections, Department of Infectious Diseases, National Institute of Health, Lisbon, Portugal^b; Department of Nutrition, National Institute of Health, Porto, Portugal^c; Laboratoire de Bactériologie, Université de Bordeaux, Bordeaux, France^d; INSERM U853, Bordeaux, France^e; Innovation and Technology Unit, Department of Human Genetics, National Institute of Health, Lisbon, Portugal^f; School of Medicine, University of St Andrews, St Andrews, United Kingdom^g

Meat and meat products are important sources of human intestinal infections. We report the isolation of *Helicobacter pullorum* strains from chicken meat. Bacteria were isolated from 4 of the 17 analyzed fresh chicken meat samples, using a membrane filter method. MIC determination revealed that the four strains showed acquired resistance to ciprofloxacin; one was also resistant to erythromycin, and another one was resistant to tetracycline. Whole-genome sequencing of the four strains and comparative genomics revealed important genetic traits within the *H. pullorum* species, such as 18 highly polymorphic genes (including a putative new cytotoxin gene), plasmids, prophages, and a complete type VI secretion system (T6SS). The T6SS was found in three out of the four isolates, suggesting that it may play a role in *H. pullorum* pathogenicity and diversity. This study suggests that the emerging pathogen *H. pullorum* can be transmitted to humans by chicken meat consumption/contact and constitutes an important contribution toward a better knowledge of the genetic diversity within the *H. pullorum* species. In addition, some genetic traits found in the four strains provide relevant clues to how this species may promote adaptation and virulence.

Infectious diarrhea is a major cause of morbidity and mortality throughout the world, particularly in children (1). Besides the most commonly associated pathogens, such as *Salmonella enterica*, *Campylobacter* spp., and Shiga toxin-producing *Escherichia coli*, infectious intestinal pathogens also include *Helicobacter* species. Among these, *Helicobacter pullorum* has been detected in poultry as well as in human samples (2, 3).

H. pullorum is a Gram-negative, gently curved rod, with an unsheathed monopolar flagellum (4). This enterohepatic *Helicobacter* species was initially isolated from the liver, duodenum, and cecum of asymptomatic poultry, but it has also been associated with enteritis and vibronic hepatitis in broiler chickens and laying hens (4). In poultry, *H. pullorum* was found to colonize the cecum at high concentrations, as well as to be present on poultry carcasses, possibly due to contamination during slaughtering. Therefore, the potential role of this bacterium as an emerging foodborne human pathogen needs to be considered (3, 5–7). In humans, *H. pullorum* is considered an emergent agent implicated in several digestive pathologies, such as gastroenteritis (2) and chronic inflammatory conditions of the intestine (8, 9) and liver (for a review, see reference 10 and references cited therein). However, the lack of available detection tools and the difficulties in cultivating this fastidious organism result in an underdetection of the pathogen and underdiagnosis of the associated infection. Despite its zoonotic potential, there is no proof to date that one can acquire *H. pullorum* infection by the handling and consumption of raw or undercooked broiler chicken meat.

In the present work, we report the isolation of *H. pullorum* from chicken meat and present antimicrobial susceptibility data and genomic features of four isolates, supporting the view that the foodborne bacterium *H. pullorum* exhibits traits that may play a role in adaptation and virulence.

MATERIALS AND METHODS

Food samples, isolation, and identification. Meat samples were collected from 15 different producers, in the context of the Portuguese Inspection Plan for Surveillance and Monitoring of Foodstuffs, comprising seven regions, distributed as follows: North (one producer, one region), Centre (six producers, two regions), Lisbon Metropolitan Area (six producers, two regions), and South (two producers, two regions). The samples were processed according to the ISO 10272-1:2006 protocol for plating, which specifies the use of an alternative plating medium (11). For each meat sample, 25 g of fresh meat was homogenized in a stomacher, and 225 ml of Bolton broth (Bolton base medium CM0983, selective supplement SR0183E, and lysed horse blood SR0048C; Oxoid, Hampshire, United Kingdom) was added, for selective pre-enrichment. The samples were incubated at $37 \pm 1^\circ\text{C}$ for 4 to 6 h, followed by 44 ± 4 h at $41.5 \pm 1^\circ\text{C}$, under microaerophilic conditions with hydrogen (Anoxomat; MART Microbiology BV, Drachten, The Netherlands). Then, 100 μl of the mixture was applied onto a 0.65- μm cellulose filter membrane (Advantec MFS, CA, USA) and placed directly on Columbia agar with 5% sheep blood (bio-Mérieux, Marcy l'Etoile, France). This plate was incubated aerobically at room temperature for 15 min; the membrane was then removed, and the plate was incubated under a hydrogen-enriched microaerophilic atmosphere for 44 ± 4 h at $41.5 \pm 1^\circ\text{C}$.

Five colonies from each colony type were chosen from each plate, tested by Gram stain and oxidase test, and then subcultured on blood agar

Received 6 August 2015 Accepted 14 September 2015

Accepted manuscript posted online 18 September 2015

Citation Borges V, Santos A, Correia CB, Saraiva M, Ménard A, Vieira L, Sampaio DA, Pinheiro M, Gomes JP, Oleastro M. 2015. *Helicobacter pullorum* isolated from fresh chicken meat: antibiotic resistance and genomic traits of an emerging foodborne pathogen. *Appl Environ Microbiol* 81:8155–8163. doi:10.1128/AEM.02394-15.

Editor: C. M. Dozois

Address correspondence to Mónica Oleastro, monica.oleastro@insa.min-saude.pt.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

plates if they were Gram negative, oxidase positive, and spiral shaped. Colonies that were not characteristic of *Campylobacter* spp. were identified by 16S rRNA gene amplification followed by Sanger sequencing, as previously described (12), using the universal forward 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse 1492R (5'-TACGGYTACCTGTTACGACTT-3') primers (13).

Antibiotic susceptibility testing. MICs of three antimicrobial agents, namely, ciprofloxacin, erythromycin, and tetracycline, were determined using Etest strips. Briefly, bacterial suspensions (adjusted to an opacity equivalent to an 0.5 McFarland opacity standard) were inoculated on Mueller-Hinton agar supplemented with 5% defibrinated horse blood (Oxoid, Basingstoke, Hants, United Kingdom) and 20 mg/liter β -NAD (Sigma-Aldrich, St. Louis, MO) and incubated under a microaerobic hydrogen-enriched atmosphere, at $41 \pm 1^\circ\text{C}$ for 48 h. Besides the isolates collected in this study, six additional *H. pullorum* clinical strains, presumably susceptible to ciprofloxacin, were included. These strains had been isolated from stool samples of patients suffering from gastroenteritis and are designated as follows: 2007-1794, 2008-547, 2010-1828, 2012-216H, 2014-133H, and 2014-1734 (the isolation year is used as the first number in the strain identifications).

Whole-genome sequencing (WGS). Total DNA was extracted using the QIAmp DNA minikit (Qiagen) according to the manufacturer's instructions. The yield and quality of the purified DNA were assessed with a Qubit assay (Quant-iT double-stranded DNA [dsDNA] assay kit, broad range; Life Technologies, MA, USA) and agarose gel (0.7%) electrophoresis. High-quality DNA samples were then selected to prepare sequencing libraries using the Nextera XT DNA sample preparation kit. Library samples were subjected to cluster generation and paired-end sequencing (2 by 250 bp) on a MiSeq sequencer (Illumina Inc., San Diego, CA, USA), according to the manufacturer's instructions. The number of passing filter reads obtained per sample ranged from 1.0 to 1.9 million. The FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and FASTX (http://hannonlab.cshl.edu/fastx_toolkit/) software tools were applied to evaluate and improve the quality of the raw sequence data, respectively. Subsequently, high-quality reads were assembled *de novo* using Velvet (version 1.2.10) (14) (several assemblies using different k-mer sizes were run), where the best assembly was assumed as the one with the best cumulative rank for N_{50} , the number of contigs/scaffolds, and the length of the largest contig/scaffold. The obtained mean depth of coverage per sample ranged from 135- to 195-fold. The final contigs/scaffolds were visually inspected (using Tablet 1.14.04.10) (15) and properly corrected prior to annotation using the NCBI Prokaryotic Genomes Annotation Pipeline 2.3.

Comparative genomic analyses. With the exception of the four genomes sequenced in the present study, only the draft genome sequence of the human MIT 98-5489 *H. pullorum* isolate (16) is currently available (GenBank accession no. [ABQU00000000.1](https://www.ncbi.nlm.nih.gov/nuclseq/ABQU00000000.1)). Therefore, in order to achieve a wide picture of the genetic diversity within the *H. pullorum* species, this genome was included in all comparative genomic analyses. First, the 5 draft sequences were aligned using the progressive algorithm of MAUVE software (version 2.3.1) (17), and a core alignment was extracted by keeping and concatenating regions where genomes aligned over at least 500 bp. This core genome alignment, encompassing about 70 to 83% of each genome sequence, was subsequently applied to identify genomic regions with high single nucleotide polymorphism (SNP) density using DnaSP v5 software (18), with a window and step sizes of 1,000 bp. MEGA5 software (19) (<http://www.megasoftware.net>) was also used to estimate overall genetic distances among the *H. pullorum* genomes. Individual alignments of highly polymorphic genes and genes with SNPs likely to be associated with antibiotic resistance were further analyzed using MEGA5 to confirm annotation, homology, and polymorphism. Identification of positional homologous coding sequences (CDSs) was facilitated by using MAUVE, assuming a minimum of 60% nucleotide identity and a minimum of 70% length coverage. DnaSP and SWAAP (version 1.0.3) software analyses were further applied to evaluate the variability within rele-

vant CDSs. The major differences in the accessory genome, particularly in regions showing strain specificity, were also studied. The identification of putative plasmids was performed by checking contigs (minimum size, 1,000 bp) with high depth of coverage and evidence of circularity. The plasmid copy numbers were estimated by comparing the mean plasmid sequence coverage to that of the remaining contigs/scaffolds. Large insertions and deletions (indels) were identified following both the inspection of the positional homolog in MAUVE and *nucmer* analyses using MUMmer 3.23 (minimum homology of 75% and a stretch window of 100 bp) (<http://www.tigr.org/software/mummer>) (20). For confirmation purposes, reads from the *H. pullorum* isolates collected in this study were additionally mapped using Bowtie2 (version 2.1.0 [<http://bowtie-bio.sourceforge.net/bowtie2/index.shtml>]) (21) against all genomic regions that displayed strain specificity or were absent in particular strains. BLASTn (<http://blast.be-md.ncbi.nlm.nih.gov/Blast.cgi>) and PHAST (<http://phast.wishartlab.com/>) analyses (22) were additionally applied over these regions to check homology and to identify, annotate, and graphically display prophage sequences, respectively.

Nucleotide sequence accession numbers. Newly determined sequence data were deposited in GenBank under accession numbers [JNOB00000000](https://www.ncbi.nlm.nih.gov/nuclseq/JNOB00000000), [JNOC00000000](https://www.ncbi.nlm.nih.gov/nuclseq/JNOC00000000), [JNUR00000000](https://www.ncbi.nlm.nih.gov/nuclseq/JNUR00000000), and [JNOA00000000](https://www.ncbi.nlm.nih.gov/nuclseq/JNOA00000000).

RESULTS

***H. pullorum* isolation, identification, and antibiotic susceptibility.** We isolated *H. pullorum* from four fresh raw chicken meat samples out of 17 analyzed. These four samples originated from three different producers, two located in the Centre region and one in the South of Portugal. Three strains were isolated from refrigerated chicken carcass meat, and the fourth was isolated from seasoned-meat preparations intended to be cooked before consumption (Table 1). The small gray-white colonies grown were different from *Campylobacter* species. Subculturing and Gram staining revealed small curved Gram-negative bacilli identified as *H. pullorum* by 16S rRNA gene sequencing. WGS was carried out for further analysis of comparative genomics. The draft genome sequence of the *H. pullorum* MIT 98-5489 strain (16) isolated in Canada from the stools of a patient suffering from gastroenteritis (corresponding to strain H436 in reference 23) was included.

The MIC values of the *H. pullorum* isolates collected in this study are shown in Table 1. For all four isolates, the MIC of ciprofloxacin was >32 mg/liter, and for three of those, the functional ACA \rightarrow ATA substitution previously reported was detected in *gyrA* (24). This mutation was also detected in the human MIT 98-5489 strain (16). Missense mutations in *gyrA* reported to confer resistance to ciprofloxacin in other enterohepatic *Helicobacter* species were not detected in any of the four strains (16). The six additional *H. pullorum* strains isolated from patients exhibited a low range of MICs of ciprofloxacin (0.023 to 0.047 mg/liter), and none of these strains harbored the ACA \rightarrow ATA transition in *gyrA*. In addition, no distinctive mutations were found in *gyrB* when comparing the group with low MIC values of ciprofloxacin (GenBank accession numbers of *gyrB*: KR014216, KR014217, KR014218, KR014219, KR014220, and KR014221) with the group of the four strains isolated in this study presenting with high MIC values. Neither the topoisomerases IV encoded by *parC* and *parE* nor the plasmid-mediated quinolone resistance gene, *qnr*, was found in the four genomes of the meat isolates and in the human strain MIT 98-5489.

For erythromycin, only strain 229336/12 had a high MIC (>256 mg/liter), and the transition A2075G in the 23S rRNA gene associated with macrolide resistance was detected in this strain

(25). Concerning tetracycline, only strain 229254/12 presented a MIC of >256 mg/liter, and it presented a single transversion, A926T, in the 16S rRNA gene. Both these mutations were detected in the strain MIT 98-5489 but in none of the meat isolates presenting low MICs of erythromycin and tetracycline (Table 1) (16).

Core genome analysis. Draft *H. pullorum* genomes were extrapolated/estimated to vary from 1.7 Mb to 2.1 Mb, with a G+C content of ~34% (Table 1). By comparing the mean depth of coverage obtained for the rRNA genes with the one calculated for single-copy genes (e.g., *gyrA*), we confirmed that *H. pullorum* harbors three copies of the ribosomal operon (26).

The core genomes were highly similar (>98%), with a mean number of 22,031.5 (standard error [SE], ±91.9) nucleotide differences, although marked by highly polymorphic regions (named A to R in Fig. 1 and Table 2). Eighteen polymorphic genes were found to belong to different functional categories, like metabolic enzymes, outer membrane proteins (OMP), and restriction modification systems.

A more detailed SNP analysis of the identified polymorphic OMP-encoding gene (label C, Fig. 1 and Table 2), varying in length from 1,407 to 1,488 bp, showed a particular pattern of variability, with 7 localized variable regions interspersed with highly conserved sequences throughout the gene (Fig. 2A), similarly to the *Campylobacter* major protein (*cmp*) gene encoding the major outer membrane protein (MOMP) of *Campylobacter jejuni* (27). The high density of nonsynonymous substitutions observed in the variable regions of this OMP-encoding gene (Fig. 2B) suggests that they are likely to correspond to surface-exposed regions of the OMP.

Other genes were highly polymorphic (Fig. 1 and Table 2), such as a gene encoding a protein belonging to the pyridoxamine 5'-phosphate oxidase-related family (label D), as well as a gene coding for a putative cytotoxin (label I) which was highly variable in length (from 2,649 to 2,817 bp) among the four *H. pullorum* isolates and the strain MIT 98-5489. This cytotoxin presented the highest variability at both nucleotide and protein levels (*p*-distances of 0.208 and 0.280, respectively), which was mostly concentrated within the 5' end and the middle region of the gene. *In silico* analysis of functional domains showed that the conserved 3' region shows features of an autotransporter β-domain (residues 592 to 882, according to the numbering of the HPMG_01076 gene of strain MIT 98-5489), with a putative signal peptide including a cleavage site (data not shown). The cytotoxin may belong to a gene family since a total of four putative paralog genes were found in the genome of all four *H. pullorum* strains studied, as well as in strain MIT 98-5489.

BLAST searches against the recently described genomes of other *Helicobacter* species (16) revealed the presence of protein homologs to both the *Campylobacter*-like MOMP and the cytotoxin for the species *Helicobacter canadensis* (strain NCTC 13241; MIT 98-5491) (blastp; query cover, 100%; identity, >76%; E value, 0, for both proteins; GenBank accession numbers WP_006656911.1 and WP_006656715.1, respectively) and "*Helicobacter winghamensis*" (strain ATCC BAA-430) (blastp; query cover, >98%; identity, >42%; E value, <1e-168, for both proteins; GenBank accession numbers WP_006801784.1 and WP_006802875, respectively).

Accessory genome analysis. The analysis of the accessory genome of *H. pullorum* showed several genomic regions which were highly variable in length and with G+C contents deviating from

TABLE 1 Origin, genome characteristics, and antimicrobial susceptibility of the four *Helicobacter pullorum* strains isolated from chicken meat and comparison with the human clinical isolate H436 (MIT 98-5489)

Isolate name	Origin	Accession no.	Genomic data			Antibiotic resistance-associated SNP ^a			Antimicrobial susceptibility (MIC, mg/liter)		
			Genome size (Mb)	GC content (%)	No. of putative plasmids	Fluoroquinolone: <i>GyrA</i> , Thr84Ile mutation	Macrolide: 23S rRNA gene, ^c AAA2055-2057GAA mutation	Tetracycline: 16S rRNA gene, ^e transversion AGA925-927TGA	Ciprofloxacin	Erythromycin	Tetracycline
229313/12 ^d	Refrigerated chicken carcass meat	JNOB000000000	1.69	34.56	0	Yes	No	No	>32	0.25	0.25
229334/12	Seasoned-meat preparations	JNOC000000000	2.13	33.92	0	Yes	No	No	>32	0.125	0.5
229336/12	Refrigerated chicken carcass meat	JNUR000000000	1.81	34.20	1	No	Yes	No	>32	>256	0.38
229254/12	Refrigerated chicken carcass meat	JNOA000000000	1.81	34.23	2	Yes	No	Yes	>32	0.25	>256
MIT 98-5489 ^a	Human, stool, gastroenteritis	ABQU000000000.1	1.95	34.15	0	Yes	Yes	Yes	NA ^e	NA	NA

^a *H. pullorum* strain MIT 98-5489 was isolated in Canada from the stools of a patient suffering from gastroenteritis and sequenced by Shen et al. (16).

^b Numbering according to the annotated genomes.

^c All three operons of the 23S and 16S rRNA genes harbored the SNPs.

^d A complete 36.4-kb prophage is integrated in the bacterial genome of this strain.

^e NA, not available.

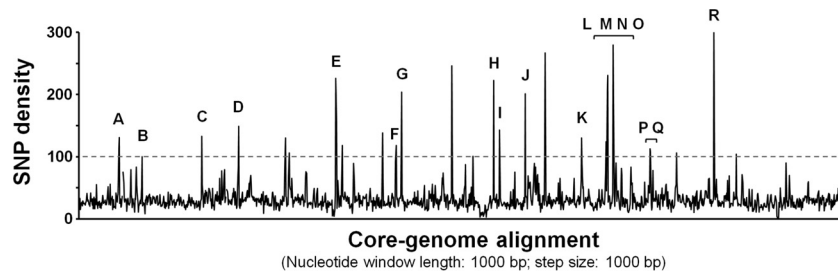


FIG 1 Analysis of polymorphism in the *Helicobacter pullorum* core genome. The graph shows the SNP density throughout a core genome alignment encompassing about 70 to 83% of each draft genome sequence under evaluation. An SNP density threshold of 100/1,000 was defined to highlight the most polymorphic regions among the *H. pullorum* strains studied. Genes found to be highly polymorphic are labeled with letters and listed in Table 2. Nonlabeled peaks refer to artifacts associated with the random junction of noncontiguous blocks (see Materials and Methods for details).

the genome average (minimum, 29.42%; maximum, 35.26%) (Table 3). These include (i) two putative cryptic plasmids present in strain 229254/12 and one putative conjugative plasmid present in strain 229336/12, (ii) several strain-specific regions with contiguous CDSs coding for hypothetical proteins, (iii) a complete 36.4-kb prophage integrated in the bacterial genome of strain 229313/12, and (iv) a putative type VI secretion system (T6SS), present in all strains except for 229313/12.

The prophage sequence in strain 229313/12 revealed a G+C content of 35.54% and 47 predicted CDSs, representing 33,266 bp. The sequence showed homology with phage sequences found in several bacterial genera, including a predicted integrase, a transposase, and tail proteins. The last are intercalated with sequences coding for putative proteins of bacterial origin of related species, such as *C. jejuni*, *Arcobacter butzleri*, and *Helicobacter hepaticus*, and other DNA-associated open reading frames (ORFs) whose functions could not be predicted. The strain 229313/12 also

showed another exclusive sequence of 11 kbp, most likely corresponding to a remnant prophage, with homology to *C. jejuni* prophages.

The T6SS genetic cluster was well conserved in three out of the four chicken meat isolates and strain MIT 98-5489, comprising 13 ORFs that encode T6SS predicted proteins, including the T6SS hallmark genes: hemolysin-coregulated protein (*hcp*) and valine glycine repeat G (*vgrG*) genes (Fig. 3 and Table 4). The *H. pullorum* T6SS genetic cluster was quite similar in organization to the *C. jejuni* T6SS, except that in the latter, the 13 T6SS genes are contiguous, whereas in *H. pullorum*, the T6SS genes are organized in three blocks (two of which are inverted, related to the *C. jejuni* gene organization) which are intercalated with genes encoding mostly proteins with no assigned function. The central block contains most of the T6SS genes. Among the extreme blocks, one is comprised of the genes *fha*, *vasK*, and *hcp* whereas the other (the noninverted block) corresponds to the *vgrG* gene (Fig. 3). It is

TABLE 2 Highly polymorphic genes in *Helicobacter pullorum* strains isolated from chicken meat

Label	Locus tag ^a	Gene product	<i>p</i> -distance	
			nt ^d	aa ^e
A	HPU229254_06545	Dihydroxy acid dehydratase (<i>ilv</i>)	0.081	0.050
B	HPU229254_07315	Putative outer membrane protein	0.052	0.052
C	HPU229254_07240 ^b	Outer membrane protein	0.110	0.133
D	HPU229254_09065	Antibiotic resistance protein (pyridoxamine 5'-phosphate oxidase related)	0.112	0.108
E	HPU229254_01025	TonB-dependent receptor	0.103	0.092
F	HPU229254_01370	Outer membrane phospholipase A family protein/porin	0.099	0.102
G	HPU229254_01245	Putative maltose <i>O</i> -acetyltransferase	0.088	0.076
H	HPU229254_03825	1-Deoxy-D-xylulose 5-phosphate reductoisomerase (<i>dxr</i>)	0.107	0.097
I	HPU229254_03755 ^c	Cytotoxin	0.208	0.280
J	HPU229254_03030	TonB-dependent receptor	0.156	0.164
K	HPU229254_04335	Modification methylase	0.078	0.062
L	HPU229254_02485	Arginase	0.068	0.057
M	HPU229254_03115	Transporter/outer membrane efflux protein	0.179	0.186
N	HPU229254_03120	Transporter/putative efflux system membrane fusion protein	0.096	0.093
O	HPU229254_04745	Asparagine synthase	0.031	0.023
P	HPU229254_05270	Homoserine <i>O</i> -succinyltransferase (MetA)	0.057	0.034
Q	HPU229254_05315	Hypothetical protein	0.163	0.183
R	HPU229254_06390	Type II restriction modification enzyme/restriction endonuclease	0.132	0.176

^a The locus designations are based on a draft genome annotation of the 229254/12 strain (GenBank accession no. [JNOA00000000](https://doi.org/10.1093/nar/nqaa000)).

^b The corresponding gene names for the strains 229313/12, 229334/12, 229336/12, and MIT 98-5489 are HPU229313_06420, HPU229334_02340, HPU229336_08255, and HPMG_00190, respectively.

^c The corresponding gene names for the strains 229313/12, 229334/12, 229336/12, and MIT 98-5489 are HPU229313_03315, HPU229334_07720, HPU229336_02900, and HPMG_01076, respectively.

^d nt, nucleotide.

^e aa, amino acid.

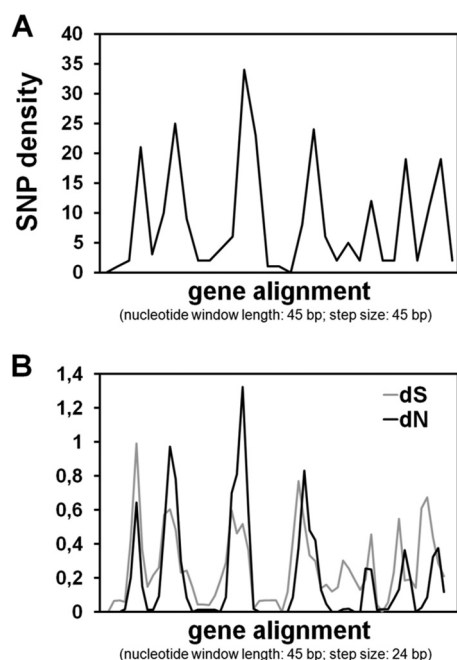


FIG 2 Sequence variation within the gene encoding the outer membrane protein HPU229254_07240. (A) SNP density (calculated through DnaSP) based on the nucleotide alignment of the sequences of the five *H. pullorum* strains under study. (B) Analysis of dN (number of nonsynonymous substitutions per nonsynonymous site) and dS (number of synonymous substitutions per synonymous site) over the same alignment (using SWAAP). Step size was adjusted according to software specificities and to improve visualization.

important to note that all 13 T6SS-associated proteins display a high level of similarity between the two species (Table 4).

DISCUSSION

The present study reports the isolation of *H. pullorum* from fresh raw chicken meat. Although *H. pullorum* is present in poultry, representing a risk to human health, and has been isolated in cases of human intestinal and liver disease, it has never been identified

or cultured on poultry meat before. The finding of *H. pullorum* in four out of 17 chicken meat samples for human consumption suggests that this organism may pose a risk of zoonotic foodborne transmission, as is known to occur with *Campylobacter* sp., which is presently recognized as a major human enteropathogen worldwide. In support of this, a few studies using PCR reported high (although variable) frequencies of *H. pullorum*, ranging from 33.6% (28) to 100% (6, 26) in poultry cecal samples and 37% in chicken meat products (7). Presently, the difficulty in culturing this fastidious microorganism and the use of inappropriate culture media and conditions, in clinical, veterinary, and food laboratories, have hampered the correct establishment of *H. pullorum* as a foodborne pathogen. In this study, we demonstrate the usefulness of a membrane filter method for isolating *H. pullorum* from meat samples, as was previously described for poultry cecal samples (6). The application of this strategy may also be highly valuable for the accurate and sensitive detection/isolation of *Campylobacter* and *Helicobacter* species in those laboratory settings.

The four *H. pullorum* isolates collected in this study were tested for antibiotic susceptibility to the antibiotics commonly used to treat *Campylobacter* and *Helicobacter* infections. According to previous data on *in vitro* susceptibility of *H. pullorum* to antimicrobial agents, we may assume that all strains were resistant to fluoroquinolones; one isolate was also resistant to erythromycin, which is the first-choice antibiotic against *Campylobacterales* infections; and another was resistant to tetracycline (6, 24, 29). The MIC values of the resistant isolates were all high, suggesting a high level of resistance to those three antibiotics. Although to date there are no studies on antibiotic susceptibility comprising a large number of *H. pullorum* isolates, high resistance to these antibiotics has been described in poultry isolates of one study from Italy including nine isolates (24) and of one study from Egypt including 20 isolates (30). In contrast, lower MIC values were described for 15 isolates in another study from Italy (6) and in a study from Belgium comprising 23 strains from poultry and two human isolates (29). Resistance to ciprofloxacin, erythromycin, and tetracycline corresponded to SNPs previously reported in *gyrA*, 23S rRNA genes, and 16S rRNA genes, respectively (16, 24). For the cipro-

TABLE 3 Most relevant genetic differences in accessory genome of *Helicobacter pullorum*

Isolate	Contig	Contig position (bp)	Predicted length (bp)	% G+C	Observation
229254/12	55		5,149	30.73	Putative plasmid (probably more than 10 copies per chromosome)
229254/12	88		4,149	33.21	Putative plasmid (probably more than 10 copies per chromosome)
229313/12	7	94358–130742	36,385	35.26	PHAST result: intact prophage
229313/12	29	30609–41976	11,368	29.42	Remnant prophage? BLAST results: <i>Campylobacter jejuni</i> putative prophages; no significant PHAST result
229313/12	48		9,097	30.89	Predicted annotation includes several hypothetical proteins
229334/12	58	1–7656	7,656	32.84	Predicted annotation includes several hypothetical proteins
229334/12	102		7,140	33.33	Predicted annotation includes several hypothetical proteins
229334/12	103		13,867	29.96	Predicted annotation includes several hypothetical proteins
229334/12	107		15,702	31.75	Predicted annotation includes several hypothetical proteins
229334/12	111		9,151	31.56	Predicted annotation includes several hypothetical proteins
229334/12	114		8,140	32.94	Predicted annotation includes several hypothetical proteins
229336/12	46		24,483	29.48	Putative conjugative plasmid (probably fewer than two copies per chromosome); BLAST results: <i>Campylobacter jejuni</i> putative plasmids and integrative conjugative elements
All but 229313/12	1 ^a		25,000–27,000	35.12	Type VI secretion system locus (Fig. 3)

^a The T6SS genetic cluster is located in the contig "1" for all strains, except for strain 229334/12, where it is fragmented in more than one contig (contigs 1, 2, and 3).

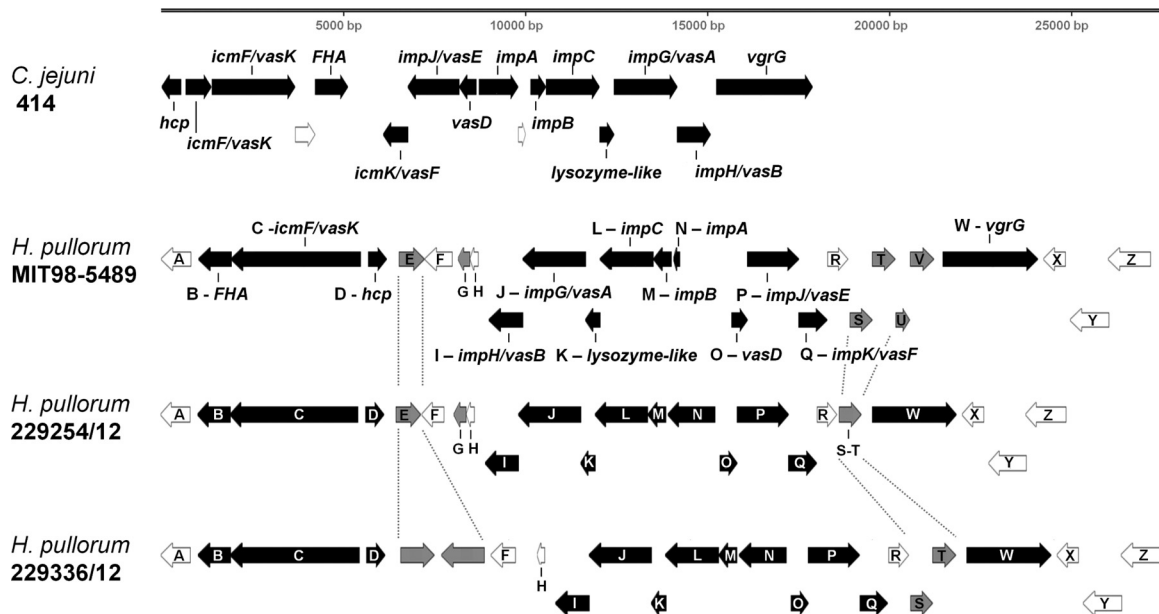


FIG 3 Genetic organization of the T6SS cluster in *Helicobacter pullorum*. The T6SS gene order and content are displayed for *Helicobacter pullorum* strains and also for *Campylobacter jejuni* strain 414 (top row), for comparative purposes. Genes are plotted as arrows (size proportional to gene length) whose direction indicates the chromosomal strand. Genes encoding proteins related to the T6SS are in black. Genes located in regions displaying variability in the genetic content among *Helicobacter pullorum* strains (highlighted by gray dashed lines) are in gray. The gene organization of the T6SS for strain 229334/12 is not shown because assembly issues yielded the T6SS genes in more than one contig. However, the draft genome of this strain carries all of the genes related to the T6SS.

floxacin-resistant strain for which no mutations in *gyrA* and *gyrB* could be assigned, we cannot exclude the putative involvement of efflux pumps in such resistance phenotypes, in line with what has been demonstrated for *Campylobacter* (31).

The present study also allowed the release of four *H. pullorum* genomes in addition to the single genome available to date (16), contributing to a better knowledge of the genome make-up of this emerging foodborne pathogen. Among the most polymorphic genes found in the *H. pullorum* core genome (Table 2), including some genes encoding proteins with unknown function, we highlight a gene belonging to the pyridoxamine 5'-phosphate oxidase-related family. Although members of this family (e.g., Nim proteins) have been associated with resistance to 5-nitroimidazole, the molecular basis of this resistance involves several bacterial players, so any correlation between the multiple polymorphisms found and susceptibility to nitroimidazoles would be speculative (32). Another interesting example stands for a length-variable OMP-encoding gene, whose polymorphism pattern, relying on 7 variable domains intercalated with conserved regions, resembles that of the *Campylobacter* major protein (*cmp*) gene of *C. jejuni*. This gene encodes a major OMP that displays features of Gram-negative bacterial porins and has important antigenic properties, making it a potential target for vaccine development (27). In addition, polymorphisms in *cmp* seem to reflect phylogenetic relations between *C. jejuni* strains, suggesting that a sequence variation of *cmp* might be exploited for molecular typing or phylogenetic analysis of *C. jejuni*. The identification of a *cmp* ortholog in the *H. pullorum* genome suggests that this gene may also code for a porin and may be involved in antigenic variability, constituting a promising research target.

The most polymorphic gene identified in the *H. pullorum* genomes codes for a putative cytotoxin, displaying high genetic vari-

ability within the 5' and middle regions of the gene. This gene pattern shares similarities with the *vacA* gene, encoding the vacuolating cytotoxin of the gastric pathogen *Helicobacter pylori*, for which the signal, intermediate, and middle genotypes are strong markers of severe gastric disease (33). The well-conserved 3' region of the *H. pullorum* cytotoxin shows features of an autotransporter β -domain, which is characteristic of secreted proteins (34). Accordingly, the cytotoxin also showed a putative signal peptide with a cleavage site, corroborating the hypothesis that it is a secreted protein. This scenario again parallels the vacuolating cytotoxin of *H. pylori*, whose gene structure, protein secretion, and proteolytic processing are characteristic of autotransporter proteins secreted by Gram-negative bacteria (35). Finally, three additional paralogs of the cytotoxin were found in the four genomes of chicken meat *H. pullorum* isolates as well as in the human strain MIT 98-5489, once more mirroring the scenario of the *vacA* gene. Altogether, these data strongly suggest that *H. pullorum* is likely to possess an additional cytotoxin besides the cytolethal distending toxin, which was present in the four meat isolates and is believed to be ubiquitous in *H. pullorum* (36). The genetic heterogeneity and role of this additional cytotoxin in the virulence of *H. pullorum* deserve to be further explored. Homologs of both these genetic traits, the *Campylobacter*-like MOMP and the cytotoxin, were also present in the genomes of two strains from other enterohepatic *Helicobacter* species, namely, *H. canadensis* and *H. winghamensis*. Similar to *H. pullorum*, both these species are considered emerging human pathogens with diverse animal reservoirs and also lack a flagellar sheath and the gamma-glutamyl transpeptidase (4, 16, 37, 38). Therefore, the contributions of the *Campylobacter*-like MOMP and the putative cytotoxin to the host adaptation and to the virulence of these pathogens warrant further investigation. In contrast, neither *H. canadensis* nor *H. wingha-*

TABLE 4 Type VI secretion system in *Helicobacter pullorum* strains isolated from chicken meat^a

Label	Locus tag for strain:			Predicted protein/putative domains	BLASTp against <i>C. jejuni</i> 414 ^b		
	MIT 98-5489	229254/12	229336/12		Cover (%)	Identity (%)	E value
A	HPMG_00002	HPU229254_00020	HPU229336_00010	Flagellar hook-associated protein (FlgK)			
B	HPMG_00003	HPU229254_00030	HPU229336_00020	Predicted T6SS component/FHA domain	98	52	3e-93
C	HPMG_00004	HPU229254_00035	HPU229336_00025	T6S protein IcmF/VasK	64	64	0
D	HPMG_00005	HPU229254_00040	HPU229336_00030	T6S effector Hcp1 family/T6SS component Hcp (secreted cytotoxin)	100	82	8e-111
E	HPMG_00006	HPU229254_00045	— ^c	Acetyltransferase (GNAT) family			
F	HPMG_00007	HPU229254_00050	HPU229336_00045	Helicase/NYN domain			
G	HPMG_00008	HPU229254_00055		Helicase			
H	HPMG_00009	HPU229254_00060	HPU229336_00055	Hypothetical protein			
I	HPMG_00010	HPU229254_00070	HPU229336_00060	T6S protein ImpH/VasB	100	61	6e-132
J	HPMG_00011	HPU229254_00075	HPU229336_00065	T6S protein ImpG/VasA	100	63	0
K	HPMG_00012	HPU229254_00080	HPU229336_00070	T6SS lysozyme-like protein	98	61	1e-23
L	HPMG_00013	HPU229254_00085	HPU229336_00075	T6S protein ImpC	100	83	0
M	HPMG_00014	HPU229254_00090	HPU229336_00080	T6S protein ImpB	83	77	7e-72
N	HPMG_00015 ^d	HPU229254_00095	HPU229336_00085	T6S ImpA-related N-terminal protein	74	41	5e-72
O	HPMG_00016	HPU229254_00100	HPU229336_00090	T6S lipoprotein VasD/SciN	80	69	2e-58
P	HPMG_00017	HPU229254_00105	HPU229336_00095	T6S protein ImpJ/VasE	99	67	0
Q	HPMG_00018	HPU229254_00110	HPU229336_00100	T6S protein ImpK/VasF; OmpA/MotB domain	81	62	6e-84
R	HPMG_00019	HPU229254_00115	HPU229336_00105	Hypothetical protein/restriction endonuclease-like superfamily			
S	HPMG_00020	— ^e	HPU229336_00110	Hypothetical protein/phosphatidylinositol-4-phosphate 5-kinase			
T	HPMG_00021	— ^e	HPU229336_00115	Hypothetical protein			
U	HPMG_00022			Hypothetical protein			
V	HPMG_00023			Hypothetical protein			
W	HPMG_00024	HPU229254_00125	HPU229336_00120	T6SS VgrG protein/phage late control gene D protein	92	47	0
X	HPMG_00025	HPU229254_00130	HPU229336_00125	Hypothetical protein			
Y	HPMG_00026	HPU229254_00135	HPU229336_00130	Hypothetical protein/predicted ATP-dependent endonuclease of the OLD family			
Z	HPMG_00027	HPU229254_00140	HPU229336_00135	Hypothetical protein			

^a T6SS genes are highlighted in bold.

^b BLASTp result (nonredundant protein sequence, blastp) of the T6SS proteins from *Helicobacter pullorum* against the genome of *Campylobacter jejuni* subsp. *jejuni* 414 (GenBank accession number [NZ_CM000855](#)) described in reference 41.

^c The 229336/12 strain has two putative ORFs (HPU229336_00035 and HPU229336_00040) in this region.

^d This region has annotation issues in the genome of the strain MIT 98-5489.

^e The 229254/12 strain has a putative gene fusion (HPU229254_00120) encompassing the ORFs HPMG_00020 and HPMG_00021.

menis displays the virulence-associated T6SS (16), which was found to be carried by three of the four *H. pullorum* isolates in this study, and also in sheathed-flagellum enterohepatic *Helicobacter* species, i.e., *Helicobacter cinaedi* and *Helicobacter bilis*. The T6SS is used by Gram-negative bacteria to deliver effector proteins into both eukaryotic and prokaryotic neighboring cells to mediate virulence and competition, respectively (39). The presently characterized T6SS consists of 13 noncontiguous genes, including the hallmark *hcp* and *vgrG* genes, and closely resembles the T6SS genes of *C. jejuni* at both gene organization and content levels (Fig. 3) (40). The existence of a putative functional T6SS in *H. pullorum* was recently proposed by Sirianni et al., who correlated the mechanism of action of this cluster with the transcellular invasion of Caco-2 intestinal epithelial cells by this pathogen (41). In *C. jejuni*, the prevalence of the T6SS varies greatly among isolates and appears to depend on the geographic origin of the strain, being more prevalent among strains from Asia (from 60.6% to 71.5%) than those from Western countries (<10%) (40, 42). In addition, the T6SS is associated with more severe forms of *C. jejuni* infection, such as bloody diarrhea and bacteremia, supporting the contribution of the system to the virulence of this pathogen (40, 42). Therefore, the presence of a complete and most likely functional T6SS in three out of the four *H. pullorum* isolates suggests that this system is common among this species, constituting an important virulence mechanism.

Globally, the comparative genomic analysis has shown some extent of plasticity in the *H. pullorum* genome (Table 3), and it is likely that other genomic features found in the accessory genome also contribute to adaptation and virulence, as in the case of a prophage. It is well known that phages integrated into the genomes of their hosts influence both bacterial behavior and virulence (43). In *C. jejuni*, prophages promote genomic rearrangements and resistance to DNA transformation (44), as well as in *H. pylori*, for which the prevalence of prophages has been underestimated until recently (45). Considering that one isolate revealed a complete prophage and also sequences from another putative remnant prophage, we hypothesize that this type of genetic element may play a role in *H. pullorum* adaptation and pathogenicity. Also, since phages are important drivers of microbial evolution, this finding should be important for further understanding and prediction of genetic diversity in *H. pullorum*.

In conclusion, our data indicate that the emerging pathogen *H. pullorum* can be transmitted to humans by chicken meat consumption/contact, suggesting the need to expand already-existent surveillance strategies at food level. In addition, this study has greatly enhanced the knowledge of genetic diversity within the *H. pullorum* species, with some highlighted genetic traits, such as OMPs, cytotoxins, prophages, and the T6SS, providing clues to how this species promotes adaptation and virulence.

ACKNOWLEDGMENT

This work was funded by the Portuguese National Institute of Health grant for Research and Development.

REFERENCES

- Aboutaleb N, Kuijper E, van Dissela J. 2014. Emerging infectious colitis. *Curr Opin Gastroenterol* 30:106–115. <http://dx.doi.org/10.1097/MOG.000000000000030>.
- Steinbrueckner B, Haertel G, Pelz K, Weiner S, Rump JA, Deissler W, Bereswill S, Kist M. 1997. Isolation of *Helicobacter pullorum* from patients with enteritis. *Scand J Infect Dis* 29:315–318. <http://dx.doi.org/10.3109/00365549709019053>.
- Ceelen L, Decostere A, Verschraegen G, Ducatelle R, Haesebrouck F. 2005. Prevalence of *Helicobacter pullorum* among patients with gastrointestinal disease and clinically healthy persons. *J Clin Microbiol* 43:2984–2986. <http://dx.doi.org/10.1128/JCM.43.6.2984-2986.2005>.
- Stanley J, Linton D, Burnens A, Dewhirst FE, On SL, Porter A, Owen RJ, Costas M. 1994. *Helicobacter pullorum* sp. nov.—genotype and phenotype of a new species isolated from poultry and from human patients with gastroenteritis. *Microbiology* 140:3441–3449. <http://dx.doi.org/10.1099/13500872-140-12-3441>.
- Atabay H, Corry J, On S. 1998. Identification of unusual *Campylobacter*-like isolates from poultry products as *Helicobacter pullorum*. *J Appl Microbiol* 84:1017–1024. <http://dx.doi.org/10.1046/j.1365-2672.1998.00438.x>.
- Zanoni R, Rossi M, Giacomucci D, Sanguinetti V, Manfreda G. 2007. Occurrence and antibiotic susceptibility of *Helicobacter pullorum* from broiler chickens and commercial laying hens in Italy. *Int J Food Microbiol* 116:168–173. <http://dx.doi.org/10.1016/j.ijfoodmicro.2006.12.007>.
- González A, Piqueres P, Moreno Y, Cañigral I, Owen RJ, Hernández J, Ferrús MA. 2008. A novel real-time PCR assay for the detection of *Helicobacter pullorum*-like organisms in chicken products. *Int Microbiol* 11:203–208.
- Laharie D, Asencio C, Asselineau J, Bulois P, Bourreille A, Moreau J, Bonjean P, Lamarque D, Pariente A, Soulé J-C, Charachon A, Coffin B, Perez P, Mégraud F, Zerbib F. 2009. Association between enterohepatic *Helicobacter* species and Crohn's disease: a prospective cross-sectional study. *Aliment Pharmacol Ther* 30:283–293. <http://dx.doi.org/10.1111/j.1365-2036.2009.04034.x>.
- Bohr U, Glasbrenner B, Primus A, Zagoura A, Wex T, Malfertheiner P. 2004. Identification of enterohepatic *Helicobacter* species in patients suffering from inflammatory bowel disease. *J Clin Microbiol* 42:2766–2768. <http://dx.doi.org/10.1128/JCM.42.6.2766-2768.2004>.
- Pellicano R, Ménard A, Rizzetto M, Mégraud F. 2008. Helicobacters and liver diseases. Association or causation? *Lancet Infect Dis* 8:254–260. [http://dx.doi.org/10.1016/S1473-3099\(08\)70066-5](http://dx.doi.org/10.1016/S1473-3099(08)70066-5).
- International Organization for Standardization. 2006. ISO 10272-1:2006. Microbiology of food and animal feeding stuffs. Horizontal method for detection and enumeration of *Campylobacter* spp. Part 1: detection method. International Organization for Standardization, Geneva, Switzerland.
- Ferreira S, Júlio C, Queiroz J, Domingues F, Oleastro M. 2014. Molecular diagnosis of *Arcobacter* and *Campylobacter* in diarrhoeal samples among Portuguese patients. *Diagn Microbiol Infect Dis* 78:220–225. <http://dx.doi.org/10.1016/j.diagmicrobio.2013.11.021>.
- Suzuki M, Giovannoni S. 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl Environ Microbiol* 62:625–630.
- Zerbino D, Birney E. 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res* 18:821–829. <http://dx.doi.org/10.1101/gr.074492.107>.
- Milne I, Stephen G, Bayer M, Cock PJ, Pritchard L, Cardle L, Shaw PD, Marshall D. 2013. Using Tablet for visual exploration of second generation sequencing data. *Brief Bioinform* 14:193–202. <http://dx.doi.org/10.1093/bib/bbs012>.
- Shen Z, Sheh A, Young S, Abouelliel A, Ward DV, Earl AM, Fox JG. 2014. Draft genome sequences of six enterohepatic *Helicobacter* species isolated from humans and one from rhesus macaques. *Genome Announc* 2(5):00857–14. <http://dx.doi.org/10.1128/genomeA.00857-14>.
- Darling A, Mau B, Perna N. 2010. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. *PLoS One* 5:e11147. <http://dx.doi.org/10.1371/journal.pone.0011147>.
- Librado P, Rozas J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25:1451–1452. <http://dx.doi.org/10.1093/bioinformatics/btp187>.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739. <http://dx.doi.org/10.1093/molbev/msr121>.
- Kurtz S, Phillippy A, Delcher A, Smoot M, Shumway M, Antonescu C, Salzberg SL. 2004. Versatile and open software for comparing large genomes. *Genome Biol* 5:R12. <http://dx.doi.org/10.1186/gb-2004-5-2-r12>.
- Langmead B, Salzberg S. 2012. Fast gapped-read alignment with Bowtie 2. *Nat Methods* 9:357–359. <http://dx.doi.org/10.1038/nmeth.1923>.
- Zhou Y, Liang Y, Lynch K, Dennis J, Wishart D. 2011. PHAST: a fast phage search tool. *Nucleic Acids Res* 39:W347–W352. <http://dx.doi.org/10.1093/nar/gkr485>.
- Gibson J, Ferrus M, Woodward D, Xerry J, Owen R. 1999. Genetic diversity in *Helicobacter pullorum* from human and poultry sources identified by an amplified fragment length polymorphism technique and pulsed-field gel electrophoresis. *J Appl Microbiol* 87:602–610. <http://dx.doi.org/10.1046/j.1365-2672.1999.00858.x>.
- Pasquali F, Rossi M, Manfreda G, Zanoni R. 2007. Complete nucleotide sequence of the *gyrA* gene of *Helicobacter pullorum* and identification of a point mutation leading to ciprofloxacin resistance in poultry isolates. *Int J Antimicrob Agents* 30:222–228. <http://dx.doi.org/10.1016/j.ijantimicag.2007.04.017>.
- Kuijper E, Stevens S, Imamura T, De Wever B, Claes E. 2003. Genotypic identification of erythromycin-resistant campylobacter isolates as *Helicobacter* species and analysis of resistance mechanism. *J Clin Microbiol* 41:3732–3736. <http://dx.doi.org/10.1128/JCM.41.8.3732-3736.2003>.
- Manfreda G, Parisi A, Lucchi A, Zanoni R, De Cesare A. 2011. Prevalence of *Helicobacter pullorum* in conventional, organic, and free-range broilers and typing of isolates. *Appl Environ Microbiol* 77:479–484. <http://dx.doi.org/10.1128/AEM.01712-10>.
- Zhang Q, Meitzler J, Huang S, Morishita T. 2000. Sequence polymorphism, predicted secondary structures, and surface-exposed conformational epitopes of *Campylobacter* major outer membrane protein. *Infect Immun* 68:5679–5689. <http://dx.doi.org/10.1128/IAI.68.10.5679-5689.2000>.
- Ceelen L, Decostere A, Van den Bulck K, On SLW, Baele M, Ducatelle R, Haesebrouck F. 2006. *Helicobacter pullorum* in chickens, Belgium. *Emerg Infect Dis* 12:263–267. <http://dx.doi.org/10.3201/eid1202.050847>.
- Ceelen L, Decostere A, Devriese L, Ducatelle R, Haesebrouck F. 2005. *In vitro* susceptibility of *Helicobacter pullorum* strains to different antimicrobial agents. *Microb Drug Resist* 11:122–126. <http://dx.doi.org/10.1089/mdr.2005.11.122>.
- Mohamed M, Ibrahim R, Shahata M, El-Refai E. 2010. *Helicobacter pullorum* among poultry in Assiut-Egypt: genetic characterization, virulence and MIC. *Int J Poult Sci* 9:521–526. <http://dx.doi.org/10.3923/ijps.2010.521.526>.
- Ge B, McDermott P, White D, Meng J. 2005. Role of efflux pumps and topoisomerase mutations in fluoroquinolone resistance in *Campylobacter jejuni* and *Campylobacter coli*. *Antimicrob Agents Chemother* 49:3347–3354. <http://dx.doi.org/10.1128/AAC.49.8.3347-3354.2005>.
- Löfmark S, Edlund C, Nord CE. 2010. Metronidazole is still the drug of choice for treatment of anaerobic infections. *Clin Infect Dis* 50(Suppl 1):S16–S23. <http://dx.doi.org/10.1086/647939>.
- Memon A, Hussein N, Miendje Deyi V, Burette A, Atherton J. 2014. Vacuolating cytotoxin genotypes are strong markers of gastric cancer and duodenal ulcer-associated *Helicobacter pylori* strains: a matched case-control study. *J Clin Microbiol* 52:2984–2989. <http://dx.doi.org/10.1128/JCM.00551-14>.
- Grijpstra J, Arenas J, Rutten L, Tommassen J. 2013. Autotransporter secretion: varying on a theme. *Res Microbiol* 164:562–582. <http://dx.doi.org/10.1016/j.resmic.2013.03.010>.
- Cover T, Blanke S. 2005. *Helicobacter pylori* VacA, a paradigm for toxin multifunctionality. *Nat Rev Microbiol* 3:320–332. <http://dx.doi.org/10.1038/nrmicro1095>.
- Varon C, Mocan I, Mihi B, Péré-Védrenne C, Aboubacar A, Moraté C, Oleastro M, Doignon F, Laharie D, Mégraud F, Ménard A. 2014. *Helicobacter pullorum* cytolethal distending toxin targets vinculin and cactin and triggers formation of lamellipodia in intestinal epithelial cells. *J Infect Dis* 209:588–599. <http://dx.doi.org/10.1093/infdis/jit539>.
- Fox J, Chien C, Dewhirst F, Paster B, Shen Z, Melito P, Woodward D, Rodgers F. 2000. *Helicobacter canadensis* sp. nov. isolated from humans

- with diarrhea as an example of an emerging pathogen. *J Clin Microbiol* 38:2546–2549.
38. Melito P, Munro C, Chipman P, Woodward D, Booth T, Rodgers F. 2001. *Helicobacter winghamensis* sp. nov., a novel *Helicobacter* sp. isolated from patients with gastroenteritis. *J Clin Microbiol* 39:2412–2417. <http://dx.doi.org/10.1128/JCM.39.7.2412-2417.2001>.
 39. Ho B, Dong T, Mekalanos J. 2014. A view to a kill: the bacterial type 6 secretion system. *Cell Host Microbe* 15:9–21. <http://dx.doi.org/10.1016/j.chom.2013.11.008>.
 40. Bleumink-Pluym N, van Alphen L, Bouwman L, Wösten M, van Putten J. 2013. Identification of a functional type VI secretion system in *Campylobacter jejuni* conferring capsule polysaccharide sensitive cytotoxicity. *PLoS Pathog* 9:e1003393. <http://dx.doi.org/10.1371/journal.ppat.1003393>.
 41. Sirianni A, Kaakoush N, Raftery M, Mitchell H. 2013. The pathogenic potential of *Helicobacter pullorum*: possible role for the type VI secretion system. *Helicobacter* 18:102–111. <http://dx.doi.org/10.1111/hel.12009>.
 42. Harrison JW, Dung TTN, Siddiqui F, Korbristrate S, Bukhari H, Tra MPV, Hoang NVM, Carrique-Mas J, Bryant J, Campbell JJ, Studholme DJ, Wren BW, Baker S, Titball RW, Champion OL. 2014. Identification of possible virulence marker from *Campylobacter jejuni* isolates. *Emerg Infect Dis* 20:1026–1029. <http://dx.doi.org/10.3201/eid2006.130635>.
 43. Bondy-Denomy J, Davidson A. 2014. When a virus is not a parasite: the beneficial effects of prophages on bacterial fitness. *J Microbiol* 52:235–242. <http://dx.doi.org/10.1007/s12275-014-4083-3>.
 44. Clark C, Chong P, McCorrister S, Mabon P, Walker M, Westmacott G. 2014. DNA sequence heterogeneity of *Campylobacter jejuni* CJIE4 prophages and expression of prophage genes. *PLoS One* 9:e95349. <http://dx.doi.org/10.1371/journal.pone.0095349>.
 45. Lehours P, Vale F, Bjursell M, Meleforts O, Advani R, Glavas S, Guegueniat J, Gontier E, Lacomme S, Alves Matos A, Menard A, Mégraud F, Engstrand L, Andersson AF. 2011. Genome sequencing reveals a phage in *Helicobacter pylori*. *mBio* 2:e00239-11. <http://dx.doi.org/10.1128/mBio.00239-11>.