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RESEARCH ARTICLE

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Study of the relationships among known virulence genes, coccoid transformation and cytotoxicity of *Helicobacter pylori* in different clinical diseases

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ABSTRACT

Background: *Helicobacter pylori (H. pylori)* has infected approximately 4.4 billion individuals worldwide. The known virulence genes and the existing *H. pylori* typing methods have not been shown to have a recognized correlation with its infectivity. The aim of this study was to elucidate the relationships among known important virulence genes, coccoid transformation, and cytotoxicity of *H. pylori* isolated from individuals with different clinical diseases to provide guidance for the development of new virulence typing methods for *H. pylori*.

Methods: The known important virulence genes of 35 *H. pylori* strains were identified by whole-gene next-generation sequencing (WGS) and polymerase chain reaction (PCR). The chronological changes in the proportion of coccoid forms of *H. pylori* and their ultramicroscopic structures were observed chronologically using transmission electron microscopy. Human gastric mucosal epithelial cells (GES-1) were infected with *H. pylori* strains in vitro to evaluate cytotoxicity of *H. pylori*.

Results: There were no significant correlations among the known important virulence genes, coccoid transformation and cytotoxicity of *H. pylori* isolated from patients with different clinical diseases. We developed a new virulence classification based on the defensive and offensive abilities of *H. pylori*.

Conclusions: Coccoid transformation and virulence are two independent characteristics of *H. pylori* that reflect its defensive and offensive abilities, respectively. These two abilities work synergistically, warranting the construction of a new virulence typing method for *H. pylori*. However, the correlation between the new virulence classification and pathogenic ability still needs to be further verified.

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Introduction

Helicobacter pylori (*H. pylori*) has been recognized as a cause of various gastrointestinal diseases, such as gastritis, gastric ulcers, gastric cancer and gastric mucosa-associated lymphoma [1]. Approximately 4.4 billion people are infected with *H. pylori* worldwide [2]. *H. pylori* infection can lead to different types and severities of gastric diseases. However, the specific factors involved are still unclear. Virulence genes, the host, the gastric microenvironment and their interactions may all affect the degree of *H. pylori* infection [3,4].

Studies on *H. pylori* virulence genes have mostly focused on cytotoxin-associated gene A (*cagA*), vacuolating cytotoxin gene A (*vacA*), duodenal ulcer promotion gene A (*dupA*), induced by contact with epithelium gene A (*iceA*), blood group antigen – binding adhesin gene A (*babA*), outer inflammatory protein-encoding gene A (*oipA*), and sialic acid-binding adhesin gene A/B (*sabA/B*) [5–11], among which the *cagA* and *vacA* genes are the most studied. The 3'-end of the *cagA* gene has a polymorphism. The C-terminus of the CagA protein from different types of strains has different types of EPIYA (glutamate-proline -isoleucine-tyrosine-alanine) motifs. The flanking sequences on both sides of the EPIYA-D/C motif has been used to classify *H. pylori* into an East Asian-type strains(EPIYA-D) and a Western-type strains (EPIYA-C) [12]. The EPIYA-D form, i.e. the eastern-type strain, has been shown to more effectively activate signalling pathways [13] and is significantly associated with the development of gastric cancer [14]. The *vacA* gene is present in all *H. pylori* strains, and the alleles mainly include s1/s2 in the *vacA* signalling region and m1/m2 and i1/i2 in the intermediate region [15,16]. The *vacA*-

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containing s1, m1, and i1 subtypes are more toxic than are the s2, m2, and i2 subtypes [16–18] and are more closely related to gastric diseases [19–22]. Xiang et al. divided *H. pylori* clinical strains into two types, type I (CagA+/VacA+) and type II (CagA−/VacA−), based on the expression of the CagA and VacA proteins [23]. Moreover, type I strains were more closely associated with gastric ulcers and duodenal ulcers [24]. On this basis, *Krzyżek* et al. divided *H. pylori* into highly virulent strains (type I, *cagA*+/*vacA* s1), low-virulent strains (type II, *cagA*−/*vacA* s2) and intermediate-virulent strains (type III, *cagA*−/*vacA* s1 or *cagA*+/*vacA* s2) according to the *vacA* genotype subtype [25].

Compared with other bacteria, *H. pylori* shows surprising adaptability under stress conditions [26]. *H. pylori* can undergo morphological transformation to adapt to environmental changes. Under suboptimal environmental conditions (changes in oxygen concentration, temperature or pH in the growth environment, prolonged culture, exposure to antibiotics or proton pump inhibitors, etc.) [27,28], transformations to coccoid occur. *H. pylori* in the coccoid state is usually viable but not culturable, i.e. in the viable but nonculturable (VBNC) state, and *H. pylori* in this state retain metabolic activity and toxicity [29–31] and have the ability to return to the helical rod morphology [32]. The coccoid *H. pylori* strain has increased tolerance to drugs [33] and evasion of the human immune response [34], leading to treatment failure in related diseases [35]. Krzyżek, who studied the relationship between virulence genes and the coccoid transformation of 13 *H. pylori* strains, proposed that the coccoid transformation of *H. pylori* was positively correlated with its virulence [25]. That result was based on the unverified actual virulence of the strains used in the experiment and remains to be confirmed.

Studies on *H. pylori* virulence, virulence-related genes, and coccoid transformation have been published recently, but these three factors have not been comprehensive analyzed with regard to their relationships among each other. In this study, the whole-genome sequence of *H. pylori*, coccoid transformation morphology and in vitro cell infection data were combined to investigate the relationships among known virulence genes, coccoid transformation and cytotoxicity. This study provides a reference for the development of new typing methods related to *H. pylori* virulence.

Materials and methods

Bacteria and cells

Thirty-five clinical *H. pylori* strains used in this study were sourced from the *Helicobacter pylori* strain library of the Chinese Center for Disease Control and Prevention (China CDC) (Table 1). Out of the 35 strains, 34 strains were isolated from patients with various stages of gastric diseases (including gastritis, gastric ulcer, gastric cancer and gastric MALT lymphoma) in eight regions of China, and the *H. pylori* Sydney strain 1 (SS1) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) (Table 1). This study was approved by the ethics committee of China CDC and follows the tenants of the Declaration of Helsinki. *H. pylori* was cultured on Columbia agar base supplemented with 5% sheep blood at 37°C under microaerobic conditions [36]. Microscopy, urease, oxidase, and catalase activity tests and mass spectrometry were used to identify *H. pylori* strains. A human gastric mucosa epithelial cell line (GES-1) was purchased from BeNa Culture Collection (Beijing, China) and cultured in RPMI-1640 medium supplemented with 10% FBS at 37°C in a 5% CO_2 humidified incubator. When reaching a confluence of 80–90%, a trypsin solution was used to passage the GES-1 cells after washing them with RPMI-1640.

Analysis of the vacA and cagA EPIYA motifs by PCR

Total DNA from *H. pylori* strains was extracted using a genomic DNA extraction kit (FastPure® Bacteria DNA Isolation Mini Kit, Vazyme). Polymerase chain reaction (PCR) for the *vacA* s1, *vacA* s2, *vacA* m1, *vacA* m2, *vacA* i1 and *vacA* i2 alleles and the EPIYA motifs was performed according to methods described previously [16,37,38]. The primers were synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China) and Beijing DIA-UP Biotechnology Co., Ltd. (Beijing, China), and the primer sequences are presented in Table 2.

Whole genome sequencing

The genome sequencing of 35 *H. pylori* isolates was performed by Shanghai Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). A genome library was constructed after DNA fragmentation with a Covaris M220 (Thermo, Waltham, USA). The prepared libraries then were used for paired-end Illumina sequencing (2 × 150 bp) on an Illumina Novaseq 6000 (Illumina Inc., San Diego, CA, USA). The data generated from the Illumina platform were used for bioinformatics analysis. Raw reads obtained after sequencing were filtered using fastp software (version 0.20.0) [39] followed by assembly with SOAPdenovo [40]. Glimmer [41] was used for predicting the CDS, tRNAscan-SE [42] was used to predict tRNA, and Barrnap was used to predict rRNA. The virulence factors of *H. pylori*

Table 1. The isolation origin, virulence gene profile, and coccoid forms proportion of 35 *H. pylori* isolates were determined via 5-day continuous culture, and the cytotoxicity of these isolates was assessed.

H.pylori strains	Clinical Origin	Regional Origin	Profile of virulence genes							Coccoid forms proportion (%) Culture time					Cytotoxicity (%)
			cagA	*vacA*	*babA*	*dupA*	*iceA*	*sabA*	*sabB*	24 h	48 h	72 h	96 h	120 h	
A1	GC	Beijing	East	s1m2i1	+		+	+	+	26.67	49.68	87.06	81.99	86.44	51.42
A2	GC	Zhoushan	East	s1m2i1	+	+	+	+	+	25.44	71.2	97.03	95.75	88.82	66.26
A3	GC	Haerbing	East	s1m2i1	+	+	+	+		27.43	45.73	66.06	77.37	81.83	43.39
A4	GC	Yantai	East	s1m1i1	+		+	+		29.56	49.02	94.54	95.1	92.05	38.63
A5	GC	Haerbing	West	s1m2i1	+		+	+		57.22	71.45	80.52	89.01	74.98	38.07
A6	GC	Beijing	East	s1m2i1	+		+	+	+	26.54	47.71	55.64	73.55	82.37	44.1
A7	GC	Hangzhou	East	s1m1i1	+		+	+	+	25.04	77.24	92.56	91.86	68.67	33.84
A8	GC	Haerbing	East	s1m1i1	+		+	+		65.52	94.16	94.21	87.99	92.92	67.44
A9	GC	Haerbing	East	s1m2i1	+		+	+		76.28	80.19	92.38	97.13	96.96	76.93
A10	GC	Yantai	East	s1m1i1	+		+	+		66.27	55.74	83.23	82.29	95.89	35.02
A11	GC	Yantai	East	s1m1i1	+		+	+		78.95	89.48	59.98	90.09	97.16	28.91
K1	GU	Xian	East	s1m1i1	+	+	+	+	+	39.02	91.22	94.88	97.05	95.68	45.05
K2	GU	Haerbing	East	s1m2i1	+		+	+		45.85	92.51	90.34	97.85	98.04	77.55
K3	GU	Xian	East	s1m2i1	+		+	+		32.28	71.61	90.35	96.75	97.26	40.11
K4	GU	Dali	East	s1m2i1	+		+	+	+	61.32	35.43	77.63	85.95	93.23	37.25
K5	GU	Haerbing	East	s1m2i1	+		+	+	+	30.55	62.65	90.43	90.2	94.99	49.81
K6	GU	Kunming	East	s1m2i1	+	+		+		49.24	60.92	77.35	85.91	93.12	39.73
K7	GU	Beijing	East	s1m1i1	+	+	+	+		17.35	18.81	45.29	76.98	84.73	42.53
K8	GU	Hangzhou	East	s1m2i1	+		+	+		50	80	80	90	95	39.81
K9	GU	Beijing	East	s1m2i1	+		+	+	+	38.88	63.45	88.3	83.96	83.93	46.88
K10	GU	Hangzhou	East	s1m1i1	+		+	+		84.22	54.62	64.67	56.98	90.75	52.63
M1	GML	Beijing	East	s1m1i1	+		+	+	+	39.64	47.78	36.46	36.73	38.41	42.52
M2	GML	Hangzhou	East	s1m2i1	+		+	+	+	71.37	67.19	64.67	87.74	87.01	33.51
Y1	Gastritis	Dali	East	s1m2i1	+	+	+	+	+	12.2	76.42	86.65	82.94	88.6	39.31
Y2	Gastritis	Xian	East	s1m1i1	+	+	+	+		24.38	60.15	94.98	93.94	94.37	56.01
Y3	Gastritis	Haerbing	East	s1m1i1	+		+	+	+	47.42	94.78	96.1	95.26	94.69	44.38
Y4	Gastritis	Xian	East	s1m2i1	+			+	+	56.27	70.63	88.6	96.75	92.32	62.81
Y5	Gastritis	Kunming	East	s1m2i1	+		+	+	+	47.26	60.32	74.71	86.58	79.19	22.28
Y6	Gastritis	Kunming	East	s1m1i1	+		+	+	+	17.9	31.22	46.94	44.04	42.96	65.39
Y7	Gastritis	Beijing	East	s1m2i1	+		+	+	+	40.58	67.97	87.93	94.6	87.52	28.67
Y8	Gastritis	Beijing	East	s1m1i1	+		+	+	+	68.81	60.49	64.34	69.94	69.08	40.7
Y9	Gastritis	Haerbing	West	s1m2i1	+	+	+	+		91.39	45.19	66.66	79.16	87.41	39.31
Y11	Gastritis	Hangzhou	East	s1m2i1	+			+		66.2	53.44	70.75	62.02	80.87	36.74
Y12	Gastritis	Hangzhou	East	s1m1i1	+		+	+	+	46.52	65.4	61.97	69.01	61.5	40.6
SS1	Gastritis	Sydney	West	s2m2i2	+			+		41.3	25.08	73.08	88.03	93.88	27.85

GU: gastric ulcer; GC: gastric cancer; GML: gastric MALT lymphoma; SS1: standard strains.

Table 2. PCR primers used in this study for virulence gene analysis.

DNA region(s) amplified	Primer name	Primer sequence	Amplicon Size(s) (bp)	Reference
vacA s1/*vacA* s2	VAI-F	5'-ATGGAAATACAACAAACACAC-3'	259/286	37
	VAI-R	5'-CTGCTTGAATGCGCCAAAC-3'		
vacA m1/*vacA* m2	VAG-F	5'-CAATCTGTCCAATCAAGCGAG-3'	267/642	37
	VAG-R	5'-GCGTCAAAATAATTCCAAGG-3'		
vacA i1	VacF1	5'-GTTGGGATTGGGGGAATGCCG-3'	495	16
	C1R	5'-TTAATTTAACGCTGTTTGAAG-3'		
vacA i2	VacF1	5'-GTTGGGATTGGGGGAATGCCG-3'	495	16
	C2R	5'-GATCAACGCTCTGATTTGA-3'		
EPYIA-C	Cag2	5'-GGAACCCTAGTCGGTAATG-3'	501	38
	CagAWest	5'-TTTCAAAGGGAAAGGTCCGCC-3'		
EPYIA-D	Cag2	5'-GGAACCCTAGTCGGTAATG-3'	495	38
	CagAEast	5'-AGAGGGAAGCCTGCTTGATT-3'		

isolates were predicted by the Virulence Factors Database (VFDB) [43]. The draft genome information in NCBI GenBank database of 35 *H. pylori* strain are presented in Table 3.

Coccoid transformation assay

H. pylori was cultured under normal conditions, and morphological coccoid transformation was promoted by prolonged culture. Samples were collected at culture times of 24, 48, 72, 96, and 120 hours. The samples were processed using negative staining and ultrathin sectioning and observed with a Hitachi H-7700 transmission electron microscope; four images were acquired at each time point. Normal *H. pylori* are 2 ~ 4 μm in length and 0.5 ~ 1 μm in width. Most of the strains had 2 ~ 6 flagella, which were 2 ~ 3 μm long and 45-nm thick (Figure 1a). As the culture time increased, the morphology of the bacteria gradually changed from rod-shaped to

Table 3. The genome information of 35 *H. pylori* strains.

H. pylori strains	BioProject	BioSample	Genome accession
A1	PRJNA1122956	SAMN41805561	JBELOO0000000000
A2	PRJNA1122956	SAMN41805562	JBELOP000000000
A3	PRJNA1122956	SAMN41805563	JBELOQ000000000
A4	PRJNA1122956	SAMN41805564	JBELOR000000000
A5	PRJNA1122956	SAMN41805565	JBELOS000000000
A6	PRJNA1122956	SAMN41805566	JBELOT000000000
A7	PRJNA1122956	SAMN41805567	JBELOU000000000
A8	PRJNA1122956	SAMN41805568	JBELOV000000000
A9	PRJNA1122956	SAMN41805569	JBELOW000000000
A10	PRJNA1122956	SAMN41805570	JBELOX000000000
A11	PRJNA1122956	SAMN41805571	JBELOY000000000
K1	PRJNA1122956	SAMN41805572	JBELOZ000000000
K2	PRJNA1122956	SAMN41805573	JBELPA000000000
K3	PRJNA1122956	SAMN41805574	JBELPB000000000
K4	PRJNA1122956	SAMN41805575	JBELPC000000000
K5	PRJNA1122956	SAMN41805576	JBELPD000000000
K6	PRJNA1122956	SAMN41805577	JBELPE000000000
K7	PRJNA1122956	SAMN41805578	JBELPF000000000
K8	PRJNA1122956	SAMN41805579	JBELPG000000000
K9	PRJNA1122956	SAMN41805580	JBELPH000000000
K10	PRJNA1122956	SAMN41805581	JBELPI000000000
M1	PRJNA1122956	SAMN41805582	JBELPJ000000000
M2	PRJNA1122956	SAMN41805583	JBELPK000000000
Y1	PRJNA1122956	SAMN41805584	JBELPL000000000
Y2	PRJNA1122956	SAMN41805585	JBELPM000000000
Y3	PRJNA1122956	SAMN41805586	JBELPN000000000
Y4	PRJNA1122956	SAMN41805587	JBELPO000000000
Y5	PRJNA1122956	SAMN41805588	JBELPP000000000
Y6	PRJNA1122956	SAMN41805589	JBELPQ000000000
Y7	PRJNA1122956	SAMN41805590	JBELPR000000000
Y8	PRJNA1122956	SAMN41805591	JBELPS000000000
Y9	PRJNA1122956	SAMN41805592	JBELPT000000000
Y11	PRJNA1122956	SAMN41805593	JBELPU000000000
Y12	PRJNA1122956	SAMN41805594	JBELPV000000000
SS1	PRJNA1122956	SAMN41805595	JBELPW000000000

SS1: standard strains.

"U-shaped," and finally, a coccoid with a compact membrane structure and a diameter of 0.8–1.5 μm was formed (Figure 1b,c). The proportion of coccoid cells for each strain at each time point was determined based on the average of the proportions coccoid cells in the four SEM images.

In vitro cell infection experiment

GES-1 cells (1×10^4 per well) were cultured in 96-well plates (Costar #3599) and adhered to the walls after 8 h. The cells were divided into three groups: blank group, control group (cells only), experimental group (cells and *H. pylori* co-cultured at MOI = 1800:1 for 24 h). At least 4 wells were prepared for each *H. pylori* strain, and at least five independent experiments were performed for each infection procedure.

LDH cytotoxicity assay

For the LDH cytotoxicity assay, the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, USA) was used according to the technical bulletin with slight modifications. Following treatment, the Lysis Solution (10×) provided with the kit was used to lyse all the cells in the control group and the residual cells in the experimental group. The percent cytotoxicity was calculated using the following formula: percent cytotoxicity = (LDH in control group cells – LDH in experimental group cells)/(LDH in control group cells – LDH in blank group) ×100. The final result was taken as the average of five experiments.

Statistical analysis

Statistical analysis was performed using the unpaired student's t-test (GraphPad Prism, CA) or Fisher's test (IBM SPSS Statistics 25.0). Differences were considered statistically significant at $p < 0.05$.

Results

Virulence genes of H. pylori isolates

Genome-wide sequencing revealed that 35 *H. pylori* strains all contained the *cagA*, *vacA*, *babA*, and *sabA* genes, and strains with the *dupA*+, *iceA*+ and *sabB*+ genotypes accounted for 22.56% (8/35), 88.57% (31/35), and 51.43% (18/35) of the *H. pylori* strains, respectively (Table 1). The electrophoresis results for the PCR

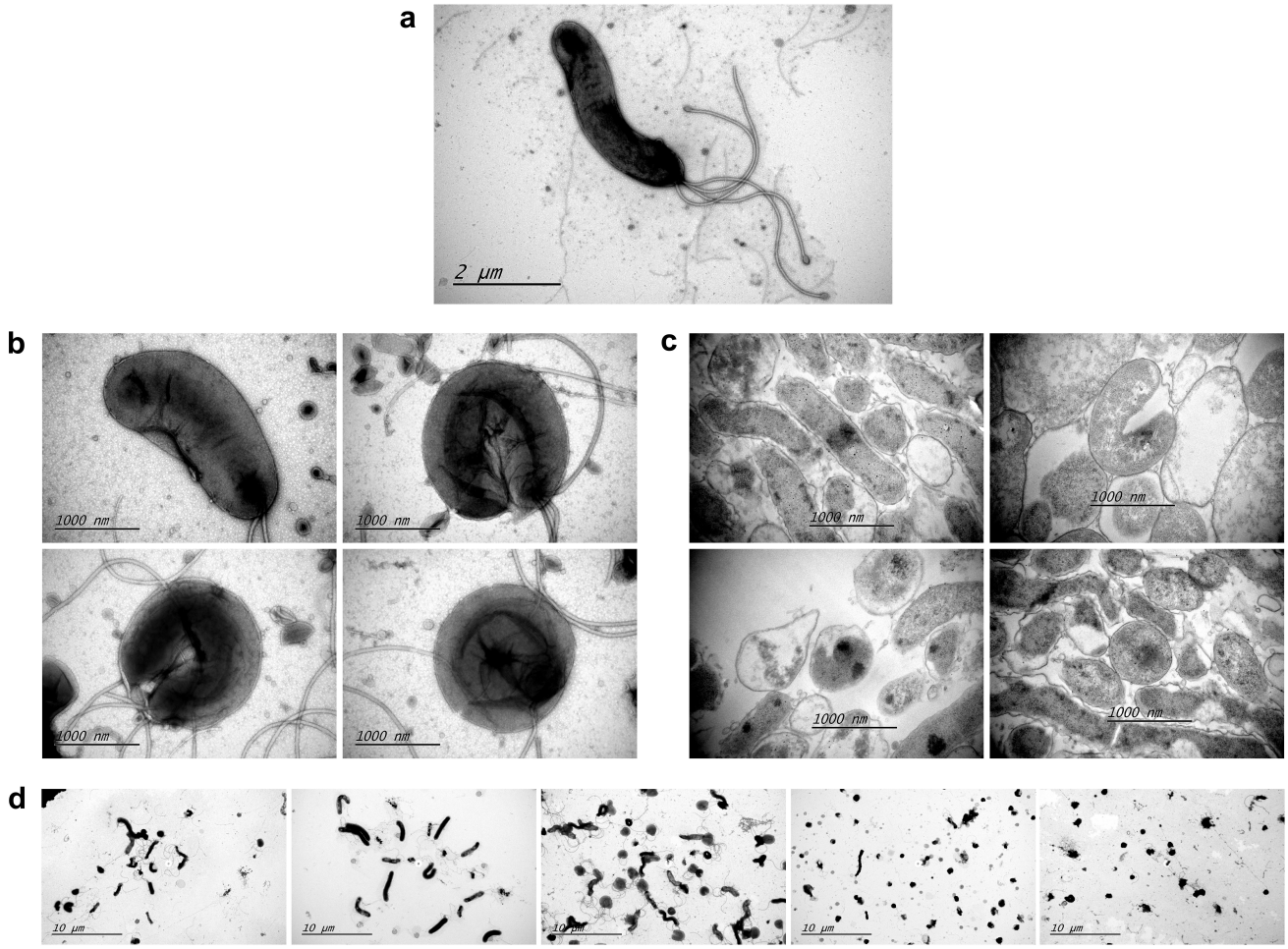

Figure 1. Coccoid transformation of *H. pylori*. (a) Morphology of strain SS1 (negative staining). (b) *H. pylori* ultrastructure during coccoid transformation (negative staining). (C) *H. pylori* ultrastructure during coccoid transformation (ultrathin sectioning). (D) Coccoid transformation of SS1 in 5 days of continuous culture.

amplification products of each genotype of *cagA* and *vacA* are shown in Figure 2. Among the 35 strains, only 3 strains, including SS1, were Western-type strains (8.6%), and the rest were East Asian-type strains (91.4%). The genotypes of 35 *H. pylori vacA* strains were dominated by s1, m2 and i1, accounting for 97.1% (34/35), 60.0% (21/35) and 97.1% (34/35), respectively. Only the SS1 strain had the s2i2 subtype,

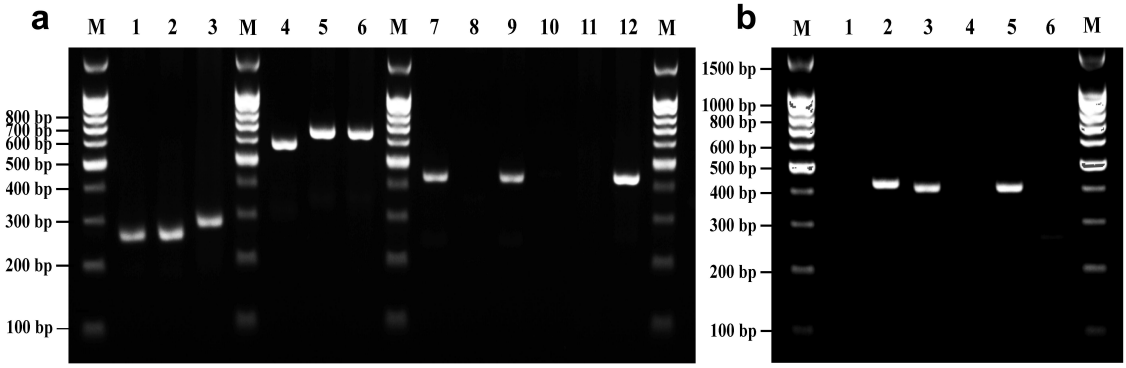

Figure 2. PCR amplification results of the typing related genes of *H. pylori*. (a) Lane M, 1-12: 100bp marker (biosharp), strain Y8 (s1), Y9 (s1), SS1 (s2), Y8 (m1), Y9 (m2), SS1 (m2), Y8 (i1), Y8 (i2), Y9 (i1), Y9 (i2), SS1 (i1), SS1 (i2). (b) Lane M, 1-6: 100bp marker (biosharp), Y8 (EPYIA-C), Y8 (EPYIA-D), Y9 (EPYIA-C), Y9 (EPYIA-D), SS1 (EPYIA-C), SS1 (EPYIA-D).

with the remaining 34 strains all having the s1i1 subtype; that is, all the s1-type *vacA* alleles were of the i1 type, and the s2-type alleles were all of the i2 type, findings that are consistent with the results reported by Rhead et al. [16]. S and m were included in three combination types, i.e. s1m1, s1m2 and s2m2; the slm2 type was the main type, with a total of 20 strains (57.1%), followed by the s1m1 type, with 14 strains (40.0%), and the s2m2 type, with only 1 strain (2.9%; SS1).

Coccoid transformation assay

After 5 days of culture, the morphology of most bacteria was coccoid, but some bacteria were partially coccoid. When the bacteria died, the bacterial cells ruptured. Additionally, a large number of coccoid bacteria had irregular morphologies, and their flagella fell off (Figure 1d).

The coccoid transformation speeds of the strains were quite different. For the SS1 strain (Figure 3a), the proportion of coccoid cells showed a decreasing trend during the first two days of culture, with the proportion of coccoid cells on the second day being the lowest. The coccoid transformation rate of the K1 strain was significantly greater than that of the SS1 strain, and the optimal conditions were reached after 1 day of culture; the proportion of coccoid cells was greater than 90% on the 2nd day, far greater than that observed for the SS1 strain. The cells were cultured for 5 days. Among the 35 strains, those for which the proportion of coccoid cells increased by more than 30% between the 1st and 2nd days and for which the coccoid transformation rate was greater than 50% within 5 days were classified as high-coccoid transformation strains, and those for which the proportion of coccoid cells increased by less than 30% were classified as strains with low coccoid transformation. The proportions of coccoid and helical rod bodies at different time points were counted for the 35 *H. pylori* strains (Table 1), and a line graph was drawn. The line graphs for the two groups of strains with high coccoid transformation and low coccoid transformation are shown in Figure 3b,c. The coccoid transformation data of the two groups of strains were analyzed according to the number of days of culture (Figure 4). After culturing for 1 day, there was no significant difference in the proportions of coccoid cells between the two groups of strains; however, on the 2nd day, there was a significant difference ($p < 0.001$), which was maintained until the 3rd day ($p < 0.01$). On the 4th and 5th days, both groups of strains basically completed coccoid transformation, and there was no significant difference in the proportion of coccoid cells.

A comparison of the genotype data of the two groups of bacteria revealed that the degree of coccoid transformation of *H. pylori* was not significantly associated with the Eastern or Western types of the *cagA* ($p = 0.58$), *vacA* m1/m2 ($p = 0.49$), *dupA* ($p = 1.00$), *iceA* ($p = 1.00$), and *sabB* ($p = 0.74$) (Figure 5a). Like the other 33 strains, M1 and Y6 had the lowest coccoid transformation, and both contained the *babA* and *sabA* genes. According to the virulence typing criteria of Krzyżek et al., M1 and Y6 are type I highly virulent strains, and SS1 is a type III intermediate – virulent strain, which is consistent with our results indicating that compared with M1 and Y6, SS1 has a significantly stronger coccoid transformation ability. The results for the other strains were the opposite.

In vitro cell infection experiment

Percent cytotoxicity refers to the cell lethality rate of *H. pylori* attacking GES-1 cells in vitro for 24 hours, which reflects the offensive abilitiy of *H. pylori*. In in vitro-infected cells, the K2 strain was the most virulent, with an average lethality rate of 77.55% at 24 h; the Y5 strain was the least virulent, with a lethality rate of 22.28%, and the lethality rate of the SS1 standard strain was 27.85% (Table 1). Joint analysis of the cytotoxicity and genotype data of 35 *H. pylori* strains revealed that the cytotoxicity of *H. pylori* was not significantly correlated with the Eastern-Western types of *cagA* ($p = 0.18$) or with the *vacA* m1/m2 ($p = 0.93$), *dupA* ($p = 0.74$), *iceA* ($p = 0.62$), and *sabB* ($p = 0.70$) (Figure 5b). The results showed that the type III intermediate virulent strain SS1 was more cytotoxic than the type I highly virulent strain Y5 [25]. Thirty-five strains were analyzed for their relationship with the cytotoxicity and coccoid transformation, and the results ($p = 0.08$) showed that there was no statistical association between the virulence of the strains and coccoid transformation (Figure 5c).

Discussion

H. pylori has coexisted with humans for more than 80,000 years [44]. During long-term coevolution, *H. pylori* has acquired the ability to withstand harsh external environments, and coccoid transformation is a form of expression of this ability. As one of the most important characteristics of *H. pylori*, coccoid transformation is helpful for *H. pylori* tolerance to drug treatment, immune escape, and survival in harsh

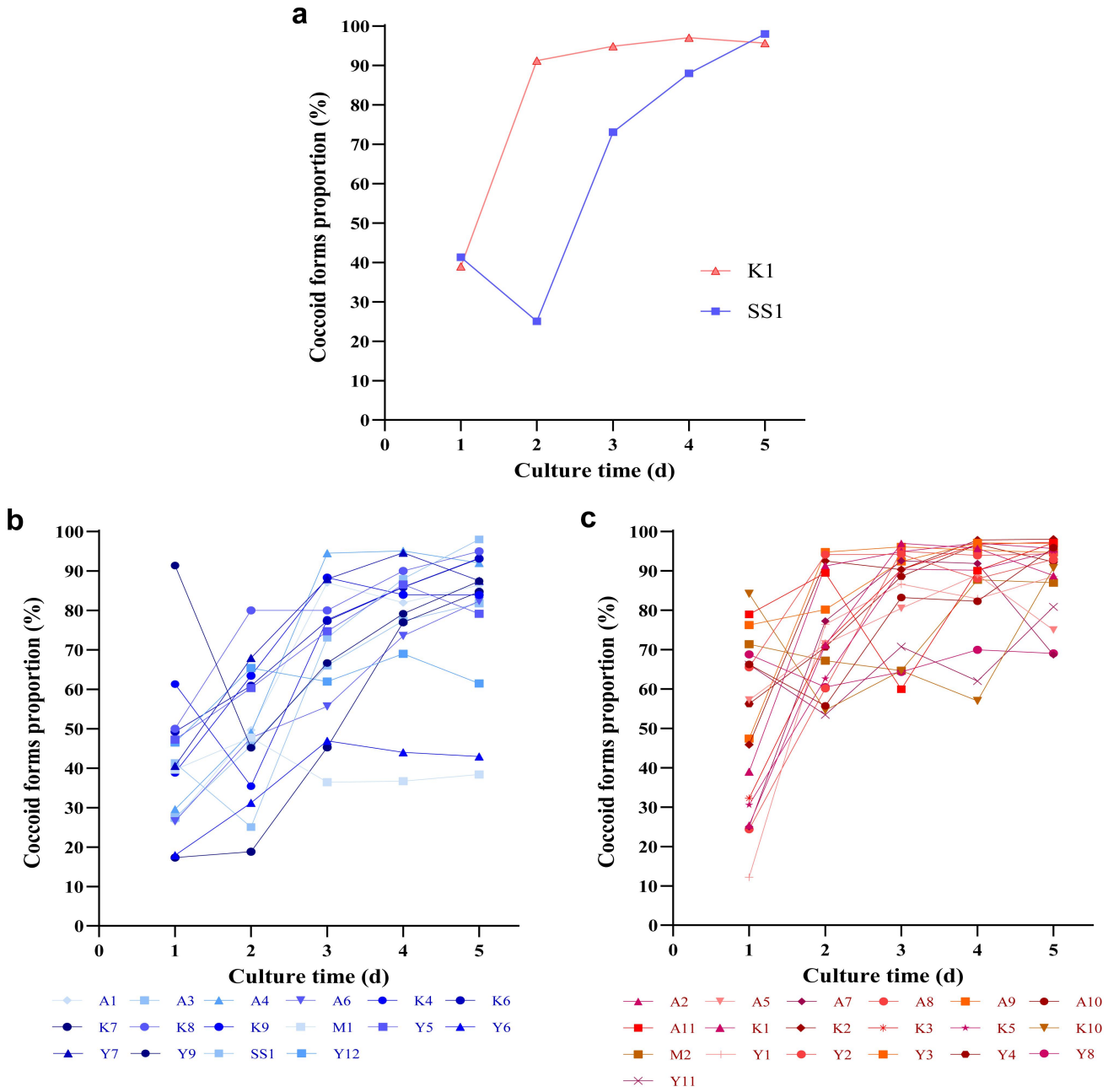

Figure 3. Coccoid transformation rate. (a) Coccoid transformation rate in SS1 and K1 cultures. (b) Coccoid transformation rate in strains with a low coccoid transformation ability in 5 days of continuous culture. (c) Coccoid transformation rate in strains with a high coccoid transformation ability in 5 days of continuous culture.

environments [33,34,45,46], reflecting the defence ability of *H. pylori*. This study explored the relationships among the coccoid transformation, cytotoxicity and known related virulence genes of *H. pylori*.

The results of this study showed that the coccoid transformation of *H. pylori* was not significantly associated with currently known important virulence genes. In this study, transmission electron microscopy was used to calculate the proportion of coccoid forms of *H. pylori*. In addition, scanning electron microscopy, Autoradiography technique, Fluorescent in situ hybridization, PCR and real time (RT)-PCR can also be used to detect coccoid forms [47]. During the transformation process of *H. pylori*, the cell wall structure changes, and the flagella are shed. This change may lead to a decrease in the ability of the host immune system to recognize the bacteria. The reduction in volume and surface area caused by coccoid transformation also reduces the nutrient consumption needed for *H. pylori* to survive and enables *H. pylori* to reduce its

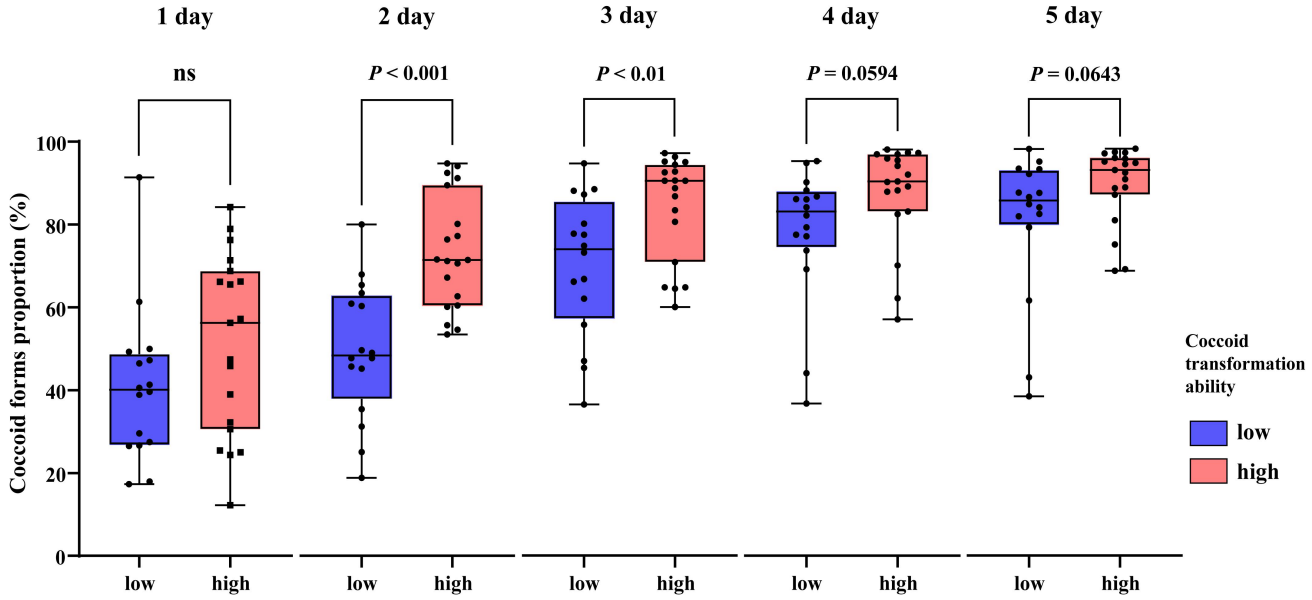

Figure 4. Analysis of coccoid form proportion of strains with low and high coccoid transformation ability during continuous culture for 5 days. ns: $p > 0.1$.

contact with unfavorable factors in the environment as much as possible, reducing the risk of exposure to the immune system. In the face of unfavorable growth conditions, *H. pylori* undergoes coccoid transformation sooner and faster to reduce nutrient demand, reduce metabolic activity, and increase resistance to harsh environments, all of which are desirable for survival. In our study, we found that for some strains, the proportion of coccoid cells was maintained at 50% or greater for 5 consecutive days of culture, indicating that these strains are sensitive to unfavorable conditions and that a long-term high proportion of coccoid cells is conducive to continued survival. We classified this type of strain as "strains with high coccoid transformation." These results are inconsistent with those reported by Krzyżek et al. [25] There are three main reasons for this inconsistency. First, the dimensions used when collecting coccoid transformation data are different. Krzyżek et al. focused on the change in the length of *H. pylori* within 1 day under suboptimal culture conditions; however, in the present study, we investigated the change in the proportion of coccoid cells during culture for 5 consecutive days. Second, the 35 *H. pylori* strains used in this study were isolated from patients with gastritis (11 isolates), gastric ulcers (11 isolates), gastric cancer (11 isolates), or gastric MALT lymphoma (2 isolates), and Krzyżek et al. used 13 *H. pylori* strains from unknown disease sources; there, the strains used in the present study are more representative. Third, Krzyżek et al. predicted the virulence of *H. pylori* through the virulence genotype, but virulence was not positively correlated with the true virulence of the strain (as evidenced in the present study). After *H. pylori* transforms from a helical to coccoid form, the content of unsaturated fatty acids significantly increases, and the efflux ability of antibiotics increases [45]. Moreover, the genome does not undergo significant changes [48], and *H. pylori* can still express *cagA*, *vacA*, *babA* and other related virulence genes [49]. Notably, the expression of the *spoT* gene in coccoid cells was 30-fold greater than that in helical bodies [50]; however, the regulatory mechanism of the *spoT* gene is still unclear. Catherine et al., through gene complementation experiments with *amiA* gene deletion strains, reported that the *amiA* gene was needed for *H. pylori* coccoid transformation [34]. However, 35 *H. pylori* strains in this study all had this gene (data not shown), and the genes related to the regulation of coccoid transformation rate are still unknown. The ability of *H. pylori* to deform is important for *H. pylori* infection. In the future, we will conduct an in-depth study on the related defence genes involved in coccoid changes.

We used an in vitro cell assay to determine the virulence of 35 *H. pylori* strains. The GES-1 cell line was established by Ke et al. in 1994 [51]. In 1996, Ning et al. applied sonicated *H. pylori* suspensions to GES-1 cells to observe the formation of micronuclei [52]. Since then, *H. pylori* infection of GES-1 cells has been widely used in the study of relevant pathogenic mechanisms and signalling pathways [53–57]. Wang et al. also used in vitro cell experiments to determine

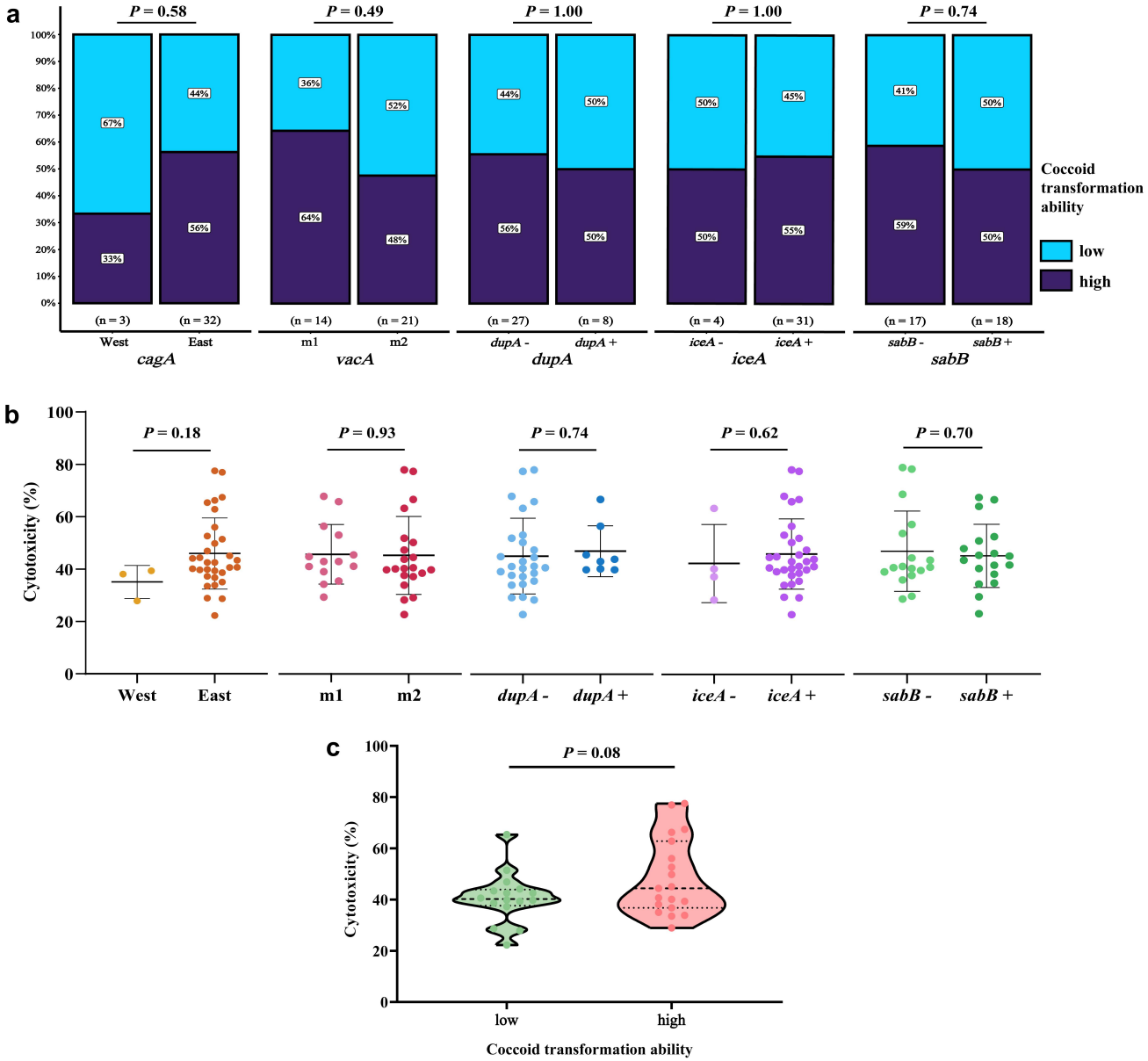

Figure 5. Relationships among *H. pylori* virulence genes, coccoid transformation ability and cytotoxicity. (a) Relationships between coccoid transformation ability and *H. pylori* virulence genes. (b) Relationship between the cytotoxicity of *H. pylori* and virulence genes. (c) The cytotoxicity and coccoid transformation ability of *H. pylori* were also assessed.

the virulence of 20 *H. pylori* clinical isolates [58]. In contrast, in the present study, as an infection method, we used a high bacterial load (MOI 1800:1). In pilot experiments, we initially infected GES-1 cells at an MOI of 300:1. We found that cell death decreased after 24 h of infection and that the difference among the different strains was not significant. When the bacterial concentration was gradually increased until the MOI reached 1800:1, there was a significant difference in cell death among the strains. The coculture conditions used for bacteria and cells were not ideal for the growth of *H. pylori*. *H. pylori* cells undergo a large amount of coccoid transformation, which reduces their offensive and adhesion abilities [59]. This finding is also in line with the view that coccoid transformation reflects the defensive ability of *H. pylori*. The use of high loads of *H. pylori* to infect host cells is not novel. Cole et al. infected AGS cells and Kato III cells with *H. pylori* at an MOI of 1000:1, and the cells still produced large amounts of IL-8 [60].

The results of this study showed that the cytotoxicity of *H. pylori* was not significantly associated with the important virulence genes that have been extensively studied. There is considerable controversy about the many virulence factors associated with the pathogenicity of *H. pylori*, with CagA and VacA being the most

studied. Currently, no CagA virulence-related domains have been found to be similar to those of any known bacterial protein toxin, nor does CagA exhibit all the toxic effects of typical toxins [61]. Because CagA is highly dependent on the type IV secretion system, CagA cannot be freely transported on the host cell membrane in the absence of bacteria [62,63]. In addition, VacA and CagA can inhibit each other via many functions: VacA can reduce the hummingbird phenotype in host cells induced by the CagA protein [64,65] and VacA also has an inhibitory effect on the CagA-activated transcription factor NFAT in cultured gastric epithelial cells [66]. CagA can reduce VacA-induced vacuolation, counteract the apoptotic activity of VacA, impair VacA internalization and intracellular trafficking, and stimulate the expression of antiapoptotic genes [67]. This mutual antagonism, called "friendly fire," is more conducive to preventing the exacerbation of gastric diseases caused by *H. pylori* and the ability to cause persistent infection in the stomach [68,69]. In highly virulent strains, are there other unknown factors that guide *H. pylori* to disrupt gastric homeostasis, exacerbate gastric diseases, and induce the development of gastric cancer? The discovery and validation of novel *H. pylori* virulence-related genes are highly important and may also constitute a breakthrough point in unravelling the pathogenicity of *H. pylori*.

The results of our study also showed that there was no significant association between the coccoid transformation and cytotoxicity of *H. pylori*. The virulence of *H. pylori* is the ability to damage cells and tissues, which is a reflection of offensive ability. The ability of *H. pylori* to undergo coccoid transformation is an important part of its defence. Offensive ability and defensive ability are important independent characteristics of *H. pylori* infection, rather than being positively correlated, as described in the literature [25]. The virulence of *H. pylori* plays a critical role in its pathogenicity, but the important role of the coccoid transformation of *H. pylori* should also receive attention. There is abundant evidence that *H. pylori* is a facultative intracellular parasite [70,71] and can form coccoid cells [72], which undoubtedly greatly increases *H. pylori* immune evasion. In contrast to the native form of *H. pylori*, the coccoid form of *H. pylori* has no metabolites that can stimulate the production of immune factors such as NF-κB and IL-8 [34], and during the process of coccoid transformation, the expression of many surface antigens on the bacteria changes [73]. This means that in the face of recognition and attack by the immune system, *H. pylori* rapidly undergoes coccoid transformation, a process that is conducive to its survival; when the body's immunity declines, *H. pylori* then transforms into a helical morphology, causing repeated infections. Therefore, the actual virulence, that is, the infection ability, of strains with high cytotoxicity and low coccoid transformation will be affected.

The actual cause of *H. pylori* infection is a high infection rate among the population; however, the prevalence rate is far lower than the infection rate, and most infected people are asymptomatic carriers [74]. Currently, there is a lack of *H. pylori* virulence typing data that can effectively guide clinical diagnosis. Therefore, the need to construct a novel virulence typing criterion that can screen highly pathogenic strains is particularly urgent. Given that the existing typing criteria only reference the relevant virulence genes of *H. pylori* and that the pathogenicity of *H. pylori* does not depend solely on its virulence, we considered the ability of *H. pylori* to undergo coccoid transformation. The classification criteria for the novel virulence of *H. pylori* were used. Based on results obtained through the present study, we divided the 35 *H. pylori* strains used in the study into low, intermediate, and high virulent categories based on their coccoid transformation and cytotoxicity and analyzed the relationships between their virulence and the sources of the strains (Figure 6). According to the novel virulence typing criteria of this study, among the strains isolated from gastric ulcers, gastric cancer and gastric MLAT lymphoma, 78% were strains had intermediate virulence and above ($p < 0.01$). *H. pylori* infection is not the only factor involved in gastric diseases. Dietary habits, genetic inheritance, and the environment are all non-negligible influencing factors. In addition, the duration of infection is also an important cause of disease severity. Therefore, the symptoms of the patient at the time of strain isolation are not the final clinical outcome after infection. Therefore, strains isolated from patients with gastritis are highly virulent, and strains isolated from patients with gastric cancer have low virulence.

In conclusion, this study explored the relationships among the coccoid transformation, cytotoxicity and known related virulence genes of *H. pylori*, and the results proved that there were no significant correlations among them. Coccoid transformation and virulence are two independent characteristics of *H. pylori* that reflect its defensive and offensive abilities, respectively. These two abilities work synergistically, warranting the construction of a new virulence typing method for *H. pylori*. However, the correlation between the new virulence classification and pathogenic ability still needs to be further verified.

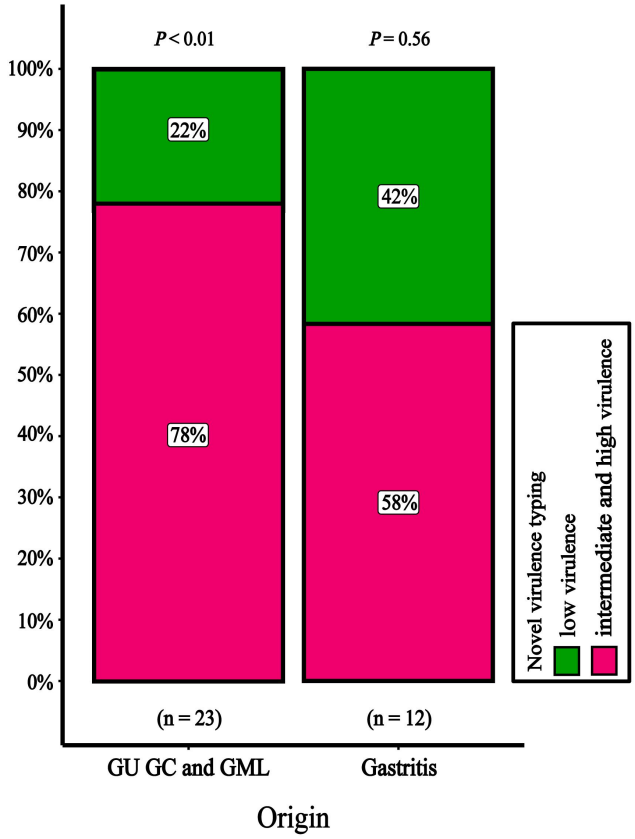

Figure 6. Relationships between the novel virulence typing of *H. pylori* and the origin of the strains. GU: gastric ulcer; GC: gastric cancer; GML: gastric MALT lymphoma.

It is necessary to acknowledge the limitations of this study. First, only 2 gastric MALT lymphoma isolates were involved in this study due to the rarity of clinical cases of gastric MALT lymphoma. In addition, if animal experiments were used in this study, the results would undoubtedly be more convincing. However, a large number of previous mouse infection experiments showed that *H. pylori* strains are actually difficult to colonize in the stomach of mice. Therefore, in vitro cell infection experiments were used to analyze the virulence of *H. pylori* in this study. We are developing methods for stable colonization of *H. pylori* clinical isolates in mice. In another study, we analyzed the protein expression of host cells infected by *H. pylori*, and the results indicated that the virulence grading of the novel virulence typing of *H. pylori* strains was related to their infectivity (unpublished study). Next, we will focus on discovering and identifying molecular markers (genes, proteins and lipid) related to the novel virulence typing, as well as analyzing the correlation between bacterial virulence and infectivity using animal experiments and visual imaging techniques (tissue pathology, electron microscopy and mass spectrometry imaging).

Ethics statement

The studies involving human participants were reviewed and approved by National Institute for Communicable Disease Control and Prevention Chinese Center for Disease Control and Prevention Ethical Committee (ethical approval number: ICDC-2020010). The patients/participants provided their written informed consent to participate in this study.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions

Yao Xiao and Di Xiao designed the study. Jianzhong Zhang provided the *Helicobacter pylori* strains. Yao Xiao, Binghua Zhang, Huifang Zhang, Zehui Zhang, Fanliang Meng and Xin Zhao performed the experiments. Yao Xiao analyzed and interpreted the data. Yao Xiao and Di Xiao drafted the manuscript. Di Xiao performed critical proofreading. All authors approved the final submitted manuscript.

Data availability statement

The data that support the findings of this study are openly available in Science Data Bank (https://www.scidb.cn/en/s/BNBVZz).

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