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Virulence and potential pathogenicity of coccoid *Helicobacter pylori* induced by antibiotics

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Abstract

AIM **To explore the virulence and the potential pathogenicity of coccoid *Helicobacter pylori* (*H. pylori*) transformed from spiral form by exposure to antibiotic.**

METHODS **Three strains of *H. pylori*, isolated from gastric biopsy specimens of confirmed peptic ulcer, were converted from spiral into coccoid from by exposure to metronidazole. Both spiral and coccoid form of *H. pylori* were tested for the urease activity, the adherence to Hep 2 cells and the vacuolating cytotoxicity to Hela cells, and the differences of the protein were analysed by SDS-PAGE and Western blot. The mutation of the genes including ureA, ureB, hpaA, vacA and cagA, related with virulence, was detected by means of PCR and PCR-SSCP.**

RESULTS **In the coccoid *H. pylori*, the urease activity, the adherence to Hep 2 cells and the vacuolating cytotoxicity to Hela cells alldecreased. In strain F44, the rate and index of adherence reduced from 70.0% ± 5.3% to 33% ± 5.1% and from 2.6 ± 0.4 to 0.96 ± 0.3 (*P*<0. 01), respectively. The invasion of coccoid *H. pylori* into Hep 2 cell could be seen under electronmicroscope. SDS-PAGE showed that the content of the protein with the molecular weight over Mr 74 000 decreased, and the hybriditional signal in band Mr 125 000 weakened, while the band *M*, 110 000 and Mr63 000 strengthened in coccoid *H.pylori* as shown in Western blot. The results of PCR were all positive, and PCR-SSCP indicated that there may exist the point mutation in gene hpaA or vacA.**

CONCLUSION **The virulence and the proteins with molecular weight over Mr74 000 in coccoid *H.pylori* decrease, but no deletion exists in amplification fragments from ureA, ureB, hpaA, vacA and cagA genes, suggesting that coccoid *H.pylori* may have potential pathogenicity.**

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INTRODUCTION

Helicobacter pylori is an important pathogen that causes chronic gastritis and peptic ulcer and may be a risk factor for gastric carcinoma[1-9]. The organism exists in two forms, a spiral form and a coccoid form. The coccoid *H.pylori*, which can be induced by increased oxygen tension, extended incubation and exposured to antibiotics[10-14], is nonculturable but alive [12,15-17], and could be induced to revert to a virulent spiral form *in vivo*[11]. Therefore, the coccoid *H. pylori* has been suspected to play a role in the transmission of the bacteria and to be partly responsible for recrudescence of infection after antimicrobiol treatment. However, the pathogenesis of coccoid *H.pylori* remains unclear. In this study, three strains of coccoid *H.pylori* transformed from spiral form by exposure to metronidazole, were tested for the urease activity, the adherence to Hep-2 cells and the vacuolating cytotoxin activities to Hela cells, the differences of the protein were analysed by SDS-PAGE and Western blot, and mutation of the genes involving ureA, ureB, hpaA, vacA and cagA was detected by means of PCR and PCR-SSCP, in order to explore the virulence and the potential pathogenicity of coccoid *H.pylori*.

MATERIALS AND METHODS

Bacterial strains

Three strains (F_{44}, F_{45} and F_{49}) of *H.pylori* were isolated from gastric biopsy specimens of confirmed peptic ulcer patients. The isolates were spiral shape, positive for catalase, oxidase, urease, and cagA and vacA gene. Stock cultures were maintained in defatted milk at -80℃.

Cells

The Hep-2 cells and Hela cells were maintained in

1640 medium supplemented with 10% fetal calf serum, 200 IU/mL penicillin and 50 μg/mL streptomycin at 37℃ in 5% CO_2-95% air, and recultivated once or twice a week.

Cultivation of H.pylori and induction of coccoid forms

The stored strains of *H.pylori* were cultivated on Brucella agar with 5% sheep blood at 37℃ for 2-3 days under microaerophilic conditions (5% O_2; 10% CO_2; 85% N_2). After subculturing, the bacteria were harvested and suspended in Brucella broth with 10% fetal calf serum, and the suspension was divided into two parts, one as spiral *H.pylori*, the other added with metronidazole at a concentration of 1/2 of MIC values (MIC = 32 mg/L-64 mg/L) at 37℃ under the microaerophilic conditions for a few days until coccoid forms reaching 100%(about 2-3 days).

Examination of urease activity

According to the manufacturer's instructions of the kit detecting fastly urease activity (Sanqiang Company), the suspension of *H.pylori* (10^{10} cfu/mL) was added into the testing well in volume of 5 uL, and on the basis of reacting colors, the urease activity was divided into five grades ("+++ +" "+++", "++","+"and "-").

Assay for adherence to Hep-2 cells

To assay bacterial adherence, Hep-2 cells were grown to confluence on coverslips in culture flask, and the suspension of *H.pylori* (10^8 cfu/mL) were added in a total volume of 0.5mL for Hep-2 cultures and allowed to adhere for 3.5 h at 37℃ in 5% CO_2 95% air. Cultures were washed and stained with Wright-Giemsa stain, and both the amount of cells adhereed by bacteria and bacteria adhereing to cells were counted among one hundred cells under the light microscope. In addition, the culture with strain F_{44} was scraped using a glass club, centrifuged, and the pellet was embedded in Epoxy 618, then the ultrathin sections were cut and examined under a Hu-12A transmission electron microscope.

Assay for cytotoxicity to Hela cells

To assay vacuolating cytotoxicity of *H.pylori*, Hela cells were grown to confluence in 96-well plates (2×10^4 cells/well at the time of infection), the suspension of bacteria were swung, centrifuged, and the cell-free supernatants were concentrated 20-fold using polyethylene glycol M_r 20 000, then sterilized by passage through a 0.25 μm pore-size filter. The concentrated supernatants at the consistency shown in Table 4 were added in a total volume of 0.1 mL to Hela cells, and Brucella broth served as a negative control. After incubation for 48 h, cells were stained and observed under phase microscope. Wells, in which 50% or more cells were vacuolated, were defined as showing a cytotoxic effect[18].

SDS-PAGE of whole cell proteins

The suspension of *H.pylori* (6×10^{10} cfu/mL) were centrifuged, washed once with 0.5 mol/L Tris (pH 7.4), and the pellets were suspended in extraction buffer consisting of 0.75% Tris, 2% sodium dodecyl sulfate, 5% dithiothreitol, 10% glycerol and 0.1% bromophenol blue. The homogenate was heated for 5 min in a boiling water bath and frozen at -20℃ until use. According to Sambrook J *et al*[19], the protein solutions (10 μL) were electrophoresed on SDS-polyacrylamide gels with the stacking and the separating gels containing 5% and 10% acrylamide, respectively, and the gels were stained with Coomassie blue.

Western blot

According to Sambrook J *et al*[19], after SDS-PAGE, the proteins were blotted onto a nitrocellulose membrane under a constant current of 100 mA for 7 h, and the antigenic profiles were studied by Western blotting using serum from the patients infected with strain F_{44} (diluted at 1/50).

PCR and PCR-single-strand conformational polymorphism (PCR-SSCP)

PCR primers and the size of the corresponding PCR products are shown in Table 1[20-22]. The suspension of bacteria were centrifuged, and the pellets were resuspended in distilled water(10^5 cfu/mL), heated in a boiling water bath for 1min to obtain the DNA template, which was added into PCR reaction system in volume of 1/5. PCR was performed as follows: denaturation at 95 for 5 min, followed by 30 cycles of denaturation (94℃ for 0.5 min);annealing (52℃ for 1 min); and extension (72℃ for 1 min), and final extension at 72℃ for 7 min. The PCR products were electrophoretically separated on 0.2% agarose gel, and stained with ethidium bromide.

Table 1 Oligonucleotide primers used for PCR reactions

Gene amplified	Primer sequence	Size of PCR product
ureA	HPU_1:5'-GCCAATGGTAAATTAGTT-3'	411bp
	HPU2:5'-CTCCTTAATTGTTTTTAC-3'	
ureB	HPU_{55}:5'-AATTGCAGAAATATCAC-3'	115bp
	HPU_{17}:5'-ACTTTATTGGCTGGTTT-3'	
hpaA	$HPYLO_1$:5'-GAATTACCATCCAGCTAGCG-3'	375bp
	$HPYLO_2$:5'-GTAACCTTGACAAAACCGGC-3'	
vacA	VA_1F:5'-ATGGAAATACAACAAACACAC-3'	259bp
	VA_1R:5'-CTGCTTGAATGCGCCAAAC-3'	
cagA	F_1:5'-GATAACAGGCAAGCTTTTGAGG-3	349bp
	B_1:5'-TCTGCCAAACAATCTTTTGCAG-3'	

ureA: urease gene A; ureB: urease gene B; hpaA: *H.pylori* adhesin gene A; vacA: vacuolating cytotoxin geneA; cagA: cytotoxin-associated gene A.

SSCP was performed as follows: the mixture consisting of 5 μL of PCR product and 3 μL of loading buffer (95% formamide, 200 mmol/L EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) was heated at 100℃ for 5 min to denature double-stranded DNA and then plunged into ice for 5 min, and electrophoresed on 50% polyacrylamide gel under a constant voltage of 300v for 7 h, the gel was stained with 0.2% silve nitrate.

Statistical analysis

Analysis of data was performed using the Student *t* test. A value of $P < 0.05$ was regarded as statistically significant.

RESULTS

Urease activity

As shown in Table 2, the urease activity of coccoid *H. pylori,* which was transformed from spiral form by exposure to antibiotic in subinhibitory concentrations, decreased.

Table 2 Urease activity of*H.pylori*

Strain	Urease activity	
	Spiral form	Coccoid form
F_{44}	++++	+
F_{45}	++++	++
F_{49}	++++	+

Adherence to Hep-2 cells

According to the following formula, the rate and the index of adherence were calculated:

The rate of adherence = the amount of cell adherenced by bacteria/100×100%

The index of adherence = the amount of bactria adhereing to cells/100

For each coverslip five-fields (one field containing of one hundred cells) were counted, and the mean of all fields is shown in Table 3. As compared with the spiral forms ($P < 0.01$), the adherence of coccoid forms to Hep-2 cells decreased. In addition, invasion of coccoid *H. pylori* into cell could be seen under electron microscope (Figure 1).

Vacuolating activity to Hela cells

As shown in Table 4, vacuolating activity of coccoid *H. pylori* to Hela cell impaired after Hela cells were incubated with different concentrations of supernatants of coccoid *H.pylori* for 48 h.

Table 3 Adherence test of *H.pylori* to Hep-2 cells

	Rate of adherence			Index of adherence		
	F_{44}	F_{45}	F_{49}	F_{44}	F_{45}	F_{49}
Spiral form	70.0 ± 5.3	73.0 ± 5.1	72.6 ± 4.5	2.60 ± 0.4	3.1 ± 0.5	2.90 ± 0.4
Coccoid form	33.0 ± 4.3	40.1 ± 3.7	35.5 ± 4.1	0.96 ± 0.3	1.0 ± 0.3	0.98 ± 0.4
t	12.1	11.7	13.6	7.5	8.1	7.6
P	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Table 4 Vacuolating activity of *H.pylori* to Hela cells

Dilution of concentrated supernatants	Spiral form			Coccoid form		
	F_{44}	F_{45}	F_{49}	F_{44}	F_{45}	F_{49}
No dilution	+	+	+	-	-	-
1:10	+	+	+	-	-	-
1:20	+	+	+	-	-	-
1:40	+	-	+	-	-	
1:80	-	-	-	-		-

Figure 1 Invasion of coccoid *H.pylori* into Hep-2 cell. Transmission electron microscope, × 15 000

SDS-PAGE and Western blot

SDS-PAGE patterns are illustrated in Figure 2A. The content of the proteins with molecular weight over Mr 74 000 decreased, especially the band at Mr 125 000 was presented with deletion in coccoid *H. pylori*. The protein patterns of the three strains of coccoid *H. pylori* were similar. Western blot patterns showed that the hybriditional signal in band M_r 125 000 weakens, meanwhile, strengthens in band M_r 110 000 and M_r 63 000 in all coccoid *H. pylori* as illustrated in Figure 2B.

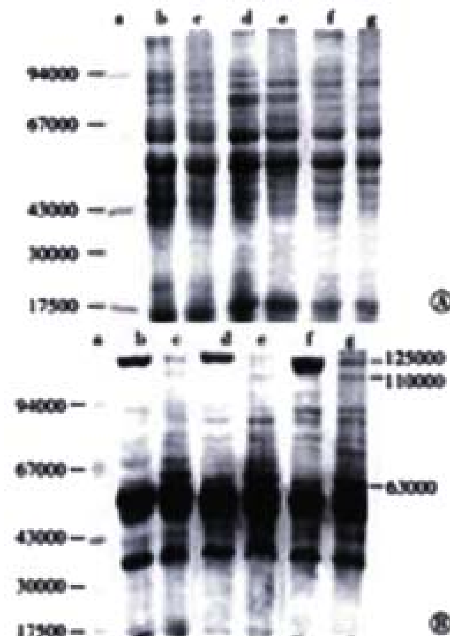

Figure 2 A. SDS-PAGE pattern of whole cell proteins of *H. pylori*, B. Western blot pattern of the proteins of *H. pylori*.
a. marker; b.d.f. spiral forms of *H.pylori*F_{44}, F_{45} and F_{49}, respectively; c.e.g. coccoid forms of *H.pylori* F_{44}, F_{45} and F_{49}, respectively.

PCR and PCR-SSCP

The result of PCR for strain F_{44} is illustrated in Figure 3. The genes detected by PCR, which included ureA, ureB, hpaA, vacA and cagA, were all positive in both spiral and coccoid *H.pylori*. The patterns of SSCP showed that there could exist point mutation in vacA gene of strain F_{44} and F_{45}, as well as in hpaA gene of strain F_{49} in coccoid forms. The pattern of SSCP for strain F_{44} is illustrated in Figure 4.

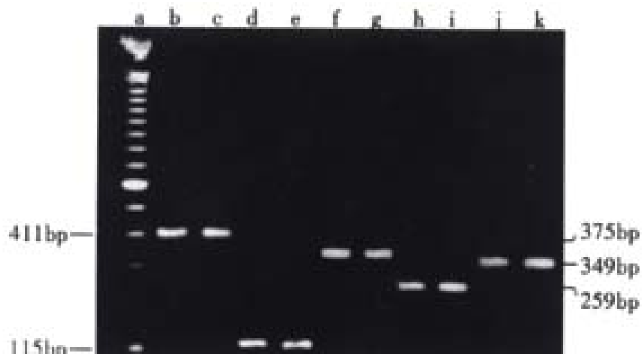

Figure 3 The results of PCR of *H.pylori* F_{44}.
a. PCR marker 100bp lader; b.c. ureA gene; d.e. ureB gene; f.g. hpaA gene; h.i. vacA gene; j.k. cagA gene; b.d.f.h.j. spiral forms of *H.pylori*; c.e.g.i.k. coccoid forms of *H.pylori.*

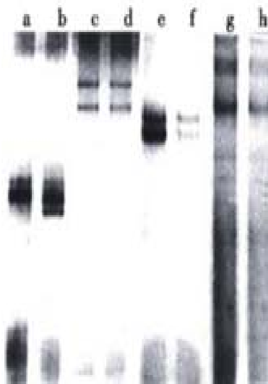

Figure 4 The pattern of PCR-SSCP of *H.pylori* F_{44}.
a.b. vacA gene; c.d. ureA gene; e.f. cagA gene; g.h. hpaA gene; a.c.e.g. spiral forms of *H. pylori*; b.d.f.h. coccoid forms of *H.pylori.*

DISCUSSION

H.pylori can convert into coccoid forms after induced by antibiotics *in vitro* or *in viro*[12,23,24]. It has been reported that coccoid *H.pylori* is able to colonize and to produce gastric alterations in the suitable animal model[11]. Costas M *et al*[25]compared the pre-with post-treatment isolates of *H.pylori* from the same patients by using 1-D SDS PAGE of proteins and considered that recurrent patients were not reinfected with a different strain but that there was recrudescence of the pre-treatment strain. Thus, it is reasonable to suppose that the viability of the coccoid form may account for the wide number of relapses in patients. However, as yet, the pathogenesis of the coccoid form remains unclear.

The putative pathogenic determinants of *H.pylori* can be divided into two major groups[26]: maintenance factors, which allow the bacterium to colonize and remain within the host, and virulence factors, which contribute to the pathogenetic effects of the bacterium. Both urease activity and adherence to epithelia cells of *H.pylori* are important maintenance factors[27-30]. In this study, It is shown that both urease activity and adherence to Hep-2 cell of coccoid *H.pylori* decreased, suggesting that virulence related to colonization in coccoid *H.pylori* reduced. Vacuolating cytotoxin produced by about 50%-60% of *H.pylori* strains[18,31-33] is one of the important virulence factors[34]. Infection with cytotoxin-producing *H.pylori* strains was more prevalent among patients with peptic ulcer disease[35-37]and gastric carcinoma[18,38-42] than among patients with gastritis alone. In our study, the decrease of vacuolating cytotoxicity in coccoid *H. pylori* was found, which may be related with the reduction of the volume of M_r 87 000 VacA and M_r 125 000 CagA proteins determining vacuolating cytotoxicity [34,43] by SDS-PAGE. These findings indicate that the coccoid *H.pylori* is less efficient in the colonizational virulence and vacuolating cytotoxicity,which may make it unlikely to induce an inflammatory response. Thus the alleviation of clinical symptom of the patients after antimicrobiol treatment does not necessarily mean eradication of *H.pylori,* it may also result from the convertion to coccoid form. As shown in the assay for adherence, a few coccoid *H.pylori* still adhered to Hep-2 cells, even invated into them, which indicates that coccoid *H.pylori* is likely to sustain in the host, thus making the recrudescence of infection possible.

In order to display the hereditary background of coccoid *H.pylori*, the genes related with virulence mentioned above (involving ureA, ureB, hpaA, vacA and cagA) were detected by PCR and PCR-SSCP. No deletion was in these genes, but there only existed the point mutation in genes hpaA or vacA. These data demonstrate that the coccoid *H.pylori* may revert into an infectious spiral form under the appropriate conditions and result in recudescence of infection, suggesting that coccoid *H.pylori* may have potential pathogenicity.

According to the features of coccoid *H.pylori*, it may escape the techniques usually applied for their detection such as cultivation and assay for urease activity. Because of no deletion in genes mentioned above in coccoid *H.pylori,* we can use PCR to detect these genes instead of conventional methods to determine whether the bacteria have been completely eliminated after treatment.

Some studies showed that the synthesis of some proteins of coccoid *H.pylori* increased such as 62KDa and >94KDa proteins[16,44,45]. In this study, the results of Western blot showed that the antigenic fraction with molecular weight in M_r 110 000 and M_r 63 000 was detected more intensively in all three strains of coccoid *H.pylori*, as compared with

spiral *H.pylori*. The fractions may be the one degraded from a high-molecular-mass antigen. Further studies are required to determine whether these antigenic proteins have special functions.

REFERENCES

1 Yang SM, Lin BZ, Fang Y, Zheng Y. Ultrastructural observation on relation of *H. pylori* to gastric epithelia in chronic gastritis and peptic ulcer. *China Natl J New Gastroenterol*, 1996;2:152-154
2 Gao GL, Pan BR, Yang SF, Song G, Xu XQ, Liu Y. The value of *Helicobacter pylori* in gastro-duodenal diseases. *Xin Xiaohuabingxue Zazhi*, 1994;2:232-233
3 Li ZX, Zhang WD, Zhou DY, Zhang YL, Guo XP, Yang HT. Relationship between *Helicobacter pylori* and duodenal ulcer. *Xin Xiaohuabingxue Zazhi*, 1996;4:153-155
4 Blaser MJ, Perez-Perez GI, Kleanthous H, Cover TL, Peek RM, Chyou PH, Stemmermann GN, Nomura A. Infection with *Helicobacter pylori* strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. *Cancer Res*, 1995;55:2111-2115
5 Maeda S, Yoshida H, Ogura K, Yamaji Y, Ikenoue T, Mitsushima T, Tagawa H, Kawaguchi R, Mori K, Mafune KI, Kawabe T, Shiratori Y, Omata M. Assessment of gastric carcinoma risk associated with *Helicobacter pylori* may vary depending on the antigen used. *Cancer*, 2000;88:1530-1535
6 Liu WZ, Zheng X, Shi Y, Dong QJ, Xiao SD. Effect of *Helicobacter pylori* infection on gastric epithelial proliferation in progression from normal mucosa to gastriccarcinoma. *World J Gastroenterol*, 1998;4:246-248
7 Wang XH, Zhang WD, Zhang YL, Zeng JZ, Sun Y. Relationship between Hp infection and oncogene and tumor suppressor gene expressions in gastric cancer and precancerosis. *Huaren Xiaohua Zazhi*, 1998;6:516-518
8 Lu W, Chen LY, Gong HS. PCNA and c-erbB-2 expression in gastric mucosal intestinal metaplasia with *Helicobacter pylori* infection. *Shijie Huaren Xiaohua Zazhi*, 1999;7:111-113
9 Cai L, Yu SZ, Zhang ZF. *Helicobacter pylori* infection and risk of gastric cancer in Changle County, Fujian Province, China. *World J Gastroenterol*, 2000;6:374-376
10 Catrenich CE, Makin KM. Characterization of the morphologic conversion of *Helicobacter pylori* from bacillary to coccoid forms. *Scand J Gastroenterol*, 1991;26(Suppl 181):58-64
11 Cellini L, Allocati N, Angelucci D, lezzi T, Campli ED, Marzio L, Dainelli B. Coccoid *Helicobacter pylori* not culturable in vitro reverts in mice. *Microbiol Immunol*, 1994;38:843-850
12 Bode G, Mauch F, Malfertheiner P. The coccoid forms of *Helicobacter pylori*. Criteria for their viability. *Epidemiol Infect*, 1993;111:483-490
13 Costa K, Bacher G, Allmaier G, Dominguez-Bello MG, Engstrand L, Falk P, de Pedro MA, Portillo FG. The morphological transition of *Helicobacter pylori* cells from spiral to coccoid is preceded by a substantial modification of the cell wall. *J Bacteriol*, 1999; 181:3710-3715
14 Xu ZM, Zhou DY, Pan LJ, Song S. Transformation and reversion of *Helicobacter pylori in vitro*. *Shijie Huaren Xiaohua Zazhi*, 1999;7:215-217
15 Cellini L, Allocati N, Carnpli ED, Dainelli B. *Helicobacter pylori*: A fickle germ. *Microbiol Immunol*, 1994;38:25-30
16 Benaissa M, Babin P, Quellard N, Pezennec L, Cenatiempo Y, Fauchere JL. Changes in *Helicobacter pylori* ultrastructure and antigens during conversion from the bacillary to the coccoid form. *Infect Immun*, 1996;64:2331-2335
17 Shahamat M, Mai U, Paszko-Kolva C, Kessel M, Colwell RR. Use of autoradiography to assess viability of *Helicobacter pylori* in water. *Appl Environ Microbiol*, 1993;59:1231-1235
18 Cover TL, Dooley CP, Blaser MJ. Characterization of and human serologic response to proteins in *Helicobacter pylori* broth culture supernatants with vacuolizing cytotoxin activity. *Infect Immun*, 1990;58:603-610
19 Sambrook J, Fritsch EF, Maniatis T. editor, Jin DY, Li MF. Translation. Molecular cloning a laboratory manual. 2nd ed. *Beijing: Kexue Chubanshe*, 1992:880-888
20 Evans DG, Evans DJ, Lampert HC, Graham DY.Restriction fragment length polymorphism in the adhesin gene hpaA of *Helicobacter pylori*. *Am J Gastroenterol*, 1995;90:1282-1289
21 Clayton CL, Kleanthous H, Coates PJ, Morgan DD, Tabaqchali S. Sensitive detection of *Helicobacter pylori* by using polymerase chain reaction. *J Clin Microbiol*, 1992;30:192-200
22 Atherton JC, Cao P, Peek RM, Tummuru MKR, Blaser MJ, Cover TL. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori* association of specific vacA types with cytotoxin production and peptic ulceration. *J Biol Chem*, 1995;270:17771-17777
23 Berry V, Jennings K, Woodnutt G. Bactericidal and morphological effects of amoxicillin on *Helicobacter pylori*. *Antimicrob Agents Chemother*, 1995;39:1859-1861
24 Zuo JS, Xu KC. Two cases of conversion of *Helicobacter pylori* from the bacillary to the coccoid form after antibiotic treatment. *Zhonghua Xiaohua Zazhi*, 1991;11:250
25 Costas M, Morgan DD, Owen RJ, Morgan DR. Differentiation of strains of *Helicobacter pylori* by numerical analysis of 1 D SDS-PAGE protein patterns:Evidence for post treatment recrudescence. *Epidemiol Infect*, 1991;107:607-617
26 Dunn BE, Cohen H, Blaser MJ. *Helicobacter pylori*. *Clin Microbiol Rev*, 1997;10:720-741
27 Eaton KA, Brooks CL, Morgan DR, Krakowka S. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. *Infect Immun*, 1991;59:2470-2475
28 Smoot DT, Mobley HT, Chippendale GR, Lewison JF, Resau JH. *Helicobacter pylori* urease activity is toxic to human gastric epithelial cells. *Infect Immun*, 1990;58:1992-1994
29 Borén T, Falk P, Roth KA, Larson G, Normark S. Attachment of *Helicobacter pylori* to human gastric epithelium mediated by blood group antigens. *Science*, 1993;262:1892-1895
30 Wang ZX, Shen HF, Chen HJ. Adherent properties of *Helicobacter pylori* to human epithelial cells. *China Natl J New Gastroenterol*, 1997;3:35-37
31 Cover TL, Blaser MJ. Purification and characterization of the vacuolating toxin from *Helicobacter pylori*. *J Biol Chem*, 1992; 267:10570-10575
32 Pan KF, Liu WD, Ma JL, Zhou T, Zhang L, Chang YS, You WC. Infection of *Helicobacter pylori* in children and mode of transmission in a high risk area of gastric cancer. *Huaren Xiaohua Zazhi*, 1998;6:42-44
33 Yu CQ, Zou QM, Xie QH, Guo XQ, Luo P. The relation between infection of $VacA^+$ *Helicobacter pylori* and digestive system diseases. *Shijie Huaren Xiaohua Zazhi*, 1999;7:439
34 Phadnis SH, Ilver D, Janzon L, Normark S, Westblom TU. Pathological significance and molecular characterization of the vacuolating toxin gene of *Helicobacter pylori*. *Infect Immun*, 1994; 62:1557-1565
35 Figura N, Guglielmetti P, Rossolini A, Barberi A, Cusi G, Musmanno RA, Russi M, Quaranta S. Cytotoxin production by Campylobacter pylori strains isolated from patients with peptic ulcers and from patients with chronic gastritis only. *J Clin Microbiol*, 1989;27: 225-226
36 Ren JP, Wang Y, Xie XJ, Di X, Qian DM, Men ZR, Li M. The relation between $CagA^+$ *Helicobacter pylori* and gastro duodenal diseases. *Shijie Huaren Xiaohua Zazhi*, 1999;7:119
37 Zhang LX, Zhang L, Zhang NX, Liu YG, Yan XJ, Han FC, Hou Y. Contrast study on the relation between CagA of *Helicobacter pylori* and development of gastroduodenal ulcer. *Shijie Huaren Xiaohua Zazhi*, 1999;7:700-701
38 Hu FL, Guo F, Jia BQ. Study on the relationship between the toxin of *Helicobacter pylori* and the gastric cancer. *Zhonghua Neike Zazhi*, 1998;37:620-621
39 Brenner H, Arndt V, Stürmer T, Stegmaier C, Ziegler H, Dhom G. Individual and joint contribution of family history and *Helicobacter pylori* infection to the risk of gastric carcinoma. *Cancer*, 2000; 88:274-279
40 Rugge M, Busatto G, Cassaro M, Shiao YH, Russo V, Leandro G, Avellini C, Fabiano A, Sidoni A, Covacci A. Patients younger than 40 years with gastric carcinoma. *Cancer*, 1999;85:2506-2511
41 Zhang L, Jiang J, Pan KF, Liu WD, Ma JL, Zhou T, Perez Perez GI, Blaser MJ, Chang YS, You WC. Infection of *H.pylori* with $cagA^+$ strain in a high-risk area of gastric cancer. *Huaren Xiaohua Zazhi*, 1998;6:40-41
42 Zhang L, Yan XJ, Zhang LX, Han FC, Zhang NX, Hou Y, Liu YG. Seroepidemiological study of Hp and $CagA^+$ Hp infection. *Shijie Huaren Xiaohua Zazhi*, 2000;8:389-392
43 Tummuru MKR, Cover TL, Blaser MJ. Cloning and expression of a high-molecular mass major antigen of *Helicobacter pylori*: evidence of linka ge to cytotoxin production. *Infect Immun*, 1993; 61:1799-1809
44 Nilsson T, Utt M, Nilsson HO, Ljungh A, Wadstrom T. Two dimensional electro phoretic and immunoblot analysis of cell surface proteins of spiral-shaped and coccoid forms of *Helicobacter pylori*. *Electrophoresis*, 2000;21:2670-2677
45 Mizoguchi H, Fujioka T, Kishi K, Nishizono A,Kodama R, Nasu M. Diversity in protein synthesis and viability of *Helicobacter pylori* coccoid forms in response to various stimuli. *Infect Immun*, 1998;66: 5555-5560

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