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 all decreased. 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 Mr 110 000 and Mr 63 000 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 Mr 74 000 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.

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 exposed 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 antimicrobial 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 (F44, F45 and F49) 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°C.

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°C in 5% CO₂-95% 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°C for 2-3 days under microaerophilic conditions (5% O₂; 10% CO₂; 85% N₂). 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°C 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* (10¹⁰ cfu/mL) was added into the testing well in volume of 5 µL, 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* (10⁶ 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°C in 5% CO₂, 95% air. Cultures were washed and stained with Wright-Giemsa stain, and both the amount of cells adhered by bacteria and bacteria adhering to cells were counted among one hundred cells under the light microscope. In addition, the culture with strain F₄₄ 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 × 10⁴ cells/well at the time of infection), the suspension of bacteria were swang, centrifuged, and the cell-free supernatants were concentrated 20-fold using polyethylene glycol M, 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 × 10¹⁰ 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°C until use. According to Sambrook et al[19], the proteins 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 F₄₄ (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 (10⁶ cfu/mL), heated in a boiling water bath for 1 min 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°C for 0.5 min); annealing (52°C for 1 min); and extension (72°C for 1 min), and final extension at 72°C 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          | HPU5'-GCCAATGTTAAATAGTT3' | 411bp               |
| ureB          | HPU5'-CTCITAATTTGTTTTTAC3' | 115bp               |
| hpaA          | HPYLO3'-GTAACCTTGACAAAACCGGC3' | 375bp               |
| vacA          | VA5'-ATGGAATACAAACACAC-3' | 259bp               |
| cagA          | F5'-GATAACCCCGCAAGCTTGGAGG3 | 349bp               |

ureA: urease gene A; ureB: urease gene B; hpaA: H.pylori adhesin gene A; vacA: vacuolating cytotoxin gene A; 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°C 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   |
| F44    | ++++            | +              |
| F45    | ++++            | ++             |
| F49    | ++++            | +              |

Adherence to Hep-2 cells
According to the following formula, the rate and the index of adherence were calculated:

\[
\text{Rate of adherence} = \frac{\text{amount of cell adherenced by bacteria}}{100 \times 100}\%
\]

\[
\text{Index of adherence} = \frac{\text{amount of bacteria adhering to cells}}{100}
\]

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

| Strain | 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 |

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.
**PCR and PCR-SSCP**

The result of PCR for strain F44 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 F44 and F45, as well as in hpaA gene of strain F49 in coccoid forms. The pattern of SSCP for strain F44 is illustrated in Figure 4.

**DISCUSSION**

*H. pylori* can convert into coccoid forms after induced by antibiotics *in vitro* or *in vivo*. It has been reported that coccoid *H. pylori* is able to colonize and to produce gastric alterations in the suitable animal model. Costas M et al. 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 reinjected 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: 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. 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 is one of the important virulence factors. Infection with cytotoxin-producing *H. pylori* strains was more prevalent among patients with peptic ulcer disease and gastric carcinoma 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*, 87 000 VacA and *M*, 125 000 CagA proteins determining vacuolating cytotoxicity 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 conversion to coccoid form. As shown in the assay for adherence, a few coccoid *H. pylori* still adhered to Hep-2 cells, even invaded 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 recrudescence 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. In this study, the results of Western blot showed that the antigenic fraction with molecular weight in *M*, 110 000 and *M*, 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 ones 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 QX, Liu Y. The value of _Helicobacter pylori_ in gastro-duodenal diseases. *Xin Xiuhaobingzhe 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 Xiang Zazhi*, 1996;4:153-155
4. Blaser MJ, Perez-Perera GI, Kleanthous H, Cover TL, Peck RM, Chyow 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. Mauda S, Yoshida H, Ogura K, Yamaji Y, Inoue T, Mitsuhashi T, Tagawa H, Kawaguchi R, Mori K, Mafune K, Kawabe T, Shigata Y, Ooma T. Assessment of gastric carcinoma risk associated with _Helicobacter pylori_ infection 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 gastric carcinoma. *World J Gastroenterol*, 1998;4:246-248
7. Wang XY, Zhang WD, Zhang YL, Zeng JZ, Sun Y. Relationship between Hp infection and oncogene and tumor suppressor gene expressions in gastric cancer and precancerous. *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 vacuolizing cytotoxin activity. *Gastroenterol Z*, 1999;17:111-113
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 coccyoidal 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 bacillary form. *Microb Immunol*, 1994;38:843-850
12. Bode G, Mauch F, Malfertheiner P. The coccoid forms of _Helicobacter pylori_. *Cancer Res*, 1993;53:1483-1489
13. Costa K, Ruchat B, Allmaier G, Dominguez-Bello MG, Engstrand L, Fank 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, Campoli ED, Dainelli B. _Helicobacter pylori_. A fickle germ. *Microb Immunol*, 1994;38:25-30
16. Benissa M, Babin P, Queralt N, Pezennec L, Ceniayo M, Faucher JL. Changes in _Helicobacter pylori_ ultrastructure and antigens during the transition from the bacillary to the coccoid form. *Infec Immun*, 1994;62:2331-2335
17. Shahamat M, Mai U, Paszko-Kolva C, Kessel M, Colwell RR. Use of autoradiography to assess viability of _Helicobacter pylori_. *Infect Immun*, 1995;63:1282-1289
18. Clayton CL, Kleanthous H, Coates PJ, Morgan DD, Tabacchi S. Sensitivity of detection of _Helicobacter pylori_ by using polymerase chain reaction. *J Clin Microbiol*, 1992;30:192-200
19. Atherton JC, Cao P, Peak RM, Tummuru MKR, Blaser MJ, Cover TL. Mosaicism in vacuolating cytotoxin alleles of _Helicobacter pylori_ association of specific vacA types with cytotoxic production and peptic ulceration. *J Biol Chem*, 1995;270:17771-17777
20. Berry V, Jennings K, Woodnutt G. Bacterial and morphological effects of amoxicillin on _Helicobacter pylori_. AntiMicrob Agents Chemother., 1995;39:1859-1861
21. Costas M, Morgan DR, 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. *Epidemol Infect*, 1997;117:67-71
22. Dunn BE, Cohen H, Blaser MJ. _Helicobacter pylori_. Clin Microbiol Rev., 1997;10:720-741
23. Eaton KA, Brooks CL, Morgan DR, Krakowska S. Essential role of ureA in pathogenesis of gastritis induced by _Helicobacter pylori_ in gnotobiotic piglets. *Infect Immun*, 1991;59:2470-2475
24. Smoot DT, Mohley HT, Chippsdale GR, Lewison JF, Resau JH. _Helicobacter pylori_ urease activity is toxic to human gastric epithelial cells. *Infect Immun*, 1990:58:1999-1994
25. Miao ZH, Shen HF, Chen HJ. Adherent properties of _Helicobacter pylori_ to human epithelial cells. China Natl J New Gastroenterol, 1997;3:35-37
26. Cover TL, Blaser MJ. Purification and characterization of the vacuolating toxin from _Helicobacter pylori_. *J Biol Chem*, 1992;267:10570-10575
27. Pan FK, Liu WD, Ma JL, Zhou T, Zhang Y, 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
28. Yu CZ, Qiao ZM, Xie QH, Guo XQ, Luo P. The relationship between infection of Vaca Hp Helicobacter pylori and gastric system diseases. *Shijie Huaren Xiaohua Zazhi*, 1999;7:439
29. Phadnis SH, Ilver D, Janzon L, Normark S, Westblom UT. Pathological significance and molecular characterization of the vacuolating toxin gene of _Helicobacter pylori_. *Infect Immun*, 1994;62:1557-1565
30. Figure N, Guglielmetti P, Rossolini A, Barberi A, Cusi G, Musmanno RA, Russi M, Quarin M. Cytotoxin production of _Helicobacter pylori_ strains isolated from patients with peptic ulcers and from patients with chronic gastritis only. *J Clin Microbiol*, 1989:27:225-226
31. Ren JP, Wang Y, Xie XJ, Di Q, Qian DM, Men ZR, Li M. The relation between CagA+ _Helicobacter pylori_ and gastric duodenal ulcer. *Shijie Huaren Xiaohua Zazhi*, 1999;7:119
32. Zhang L, Zhang L, Zhang NX, Liu YG, Yan XJ, Han FC, Hou Y. Contrast study on the relation between CagA+ _Helicobacter pylori_ and development of gastro-duodenal ulcer. Shijie Huaren Xiaohua Zazhi*, 1999;7:700-701
33. 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
34. 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
35. 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
36. Zhang L, Jiang J, Pan KF, Liu WD, Ma JL, Zhou T, Perez GI, Blaser MJ, Chang YS, You WC. Infection of _Helicobacter pylori_ with cagA strain in a high-risk area of gastric cancer. *Huaren Xiaohua Zazhi*, 1998;6:40-41
37. Zhang L, Yan XJ, Zhang LX, Han FC, Zhang NX, Hou Y, Liu Y. Serological study of Hp and CagA+ Hp infection. *Shijie Huaren Xiaohua Zazhi*, 2000;8:389-392
38. Tummura 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
39. Nilsson T, Utt M, Nilsson O, Ljungh A, Wadstrom T. Two dimensional electro phoretic and immunoblot analysis of cell surface proteins of spiral-shaped and coccyoidal forms of _Helicobacter pylori_. *Electrophoresis*, 2000;21:2670-2677
40. Mizoguchi H, Fujitsuka T, Kishi K, Nishizono A, Kodama R, Nama M, Diversity in peptid synthesis and viability of _Helicobacter pylori_ coccyoidal forms in response to various stimuli. *Infect Immun*, 1998:66:5555-5560